GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

HPV and associated cancers: from screening, prevention, molecular biomarkers to targeted therapy

- HABILITATION THESIS -

Ramona Gabriela Ursu MD, PhD
Associate Professor
2021

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Abbreviations list:

AIDS: Acquired immunodeficiency syndrome
AJCC: American joint committee on cancer
ASCH: Atypical squamous cells cannot exclude HSIL
ASCUS: Atypical squamous cells of undetermined significance
BOTSCC, base of tongue squamous cell carcinoma;
CIN: Cervical intraepithelial neoplasia
CSF: cerebrospinal fluid
DCIS: ductal carcinoma in situ
ECM: extracellular matrix
FFPE: formalin fixed paraffin embedded
FGFR: fibroblast growth factor receptors
HIV: Human Immunodeficiency Virus
HNSCC: head and neck squamous cell carcinoma
HPV−, human papillomavirus negative;
HPV, human papillomavirus;
HPV: Human papillomavirus
HPV+, human papillomavirus positive;
HR HPV: high risk Human papillomavirus
HSIL: High grade squamous intraepithelial lesion
IARC: International Agency for Research on Cancer
IHC: immunohistochemistry
LCM: laser capture microdissection
LEEP: loop electrosurgical excision procedure
LR HPV types: low risk Human papillomavirus
LSIL: low grade squamous intraepithelial lesion
MALDI TOF: matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
OPSCC: oropharyngeal squamous cell carcinoma
p16, p16INK4a tumor suppressor protein;
PI3K: Phosphoinositide 3-kinases
SPARC: secreted protein acidic and rich in cysteine;
TIL: tumor infiltrating lymphocyte
TSCC: tonsillar squamous cell carcinoma
VHB: Hepatitis B virus
WHO: World Health Organization

Abstract of the thesis

The habilitation thesis is a summary of the most important academic, professional and, especially, scientific accomplishments realized after finishing the doctoral studies (2011-2021). Starting from these achievements and, in accordance with the latest trends in scientific research, I have included, in the latter part of the habilitation thesis, several future research projects on which I will focus in the future. According to the guidelines approved by CNATDCU, the thesis is structured in three sections, as follows:

Section I, which presents the professional, academic and scientific achievements, realized in the postdoctoral period.

Section II is referring at the scientific accomplishments.

Chapter 1: HPV & cervical cancer

Chapter 1 details one of the main research activities undertaken immediately after obtaining the PhD title in medical sciences: HPV and cervical cancer. The doctoral studies, conducted under the guidance of Professor Iancu, have given me the opportunity to combine the most modern molecular biology testing methods with careful monitoring of the patients, in collaboration with gynecologists (Dr. Anton, Dr. Onofriescu, Dr. Nemescu). Thus, I have evaluated the distribution of HPV genotypes through PCR genotyping, detecting 37 viral genotypes, in over 500 patients with different results of the Papanicolau stain diagnosis (NILM, ASCUS, ASCH, LSIL, HSIL). Besides the epidemiological relevance of detecting these HPV genotypes, which has emphasized the need for anti-HPV vaccination in our region, this study has detected highly oncogenic genotypes even in patients who had a normal cytological exam, which suggests the necessity for a more sensitive screening test, in order to detect which patients are at risk of developing cervical cancer.

Another line of research has been the risk assessment of developing cervical cancer, of a group of HIV-positive female patients. In this group of patients, the detection of infections with multiple HPV genotypes has been considered significant. The prevalence of HPV in this group has increased from 33% to 66%, paralleling the decrease in immunity. All women, regardless of HIV status, should take part in national screening programs for cervical cancer, through clinically validated tests and at an affordable price.

Another postdoctoral study has evaluated the efficiency of excisional surgery (loop electrosurgical excision procedure - LEEP) in the removal of HR HPV. Cervical samples from patients with CIN and HR HPV have been genotyped 6 months after undergoing LEEP. We have therefore assessed the influence of various risk factors for the persistence of HR HPV. Persistent infections have been detected in 22% of cases with HPV genotypes 16, 18, 31, 39 51, and 66. The risk factors for the persistence of HR HPV identified were: age over 30, multiparity, the use of oral contraceptives and CIN2-3. In this study we have underlined the importance of implementing cervical cancer screening actions through clinically validated HPV tests.

A final study of this chapter pertains to the efficiency of anti-HPV vaccination. Thus, courtesy of the collaboration with a team of researchers from Karolinska Institute, Stockholm, we have assessed the impact that anti-HPV vaccination has on the prevalence of high-grade lesions in female teenagers, in the period between 2008-2018. The percentage of vaccinated women has risen from 10.7% (2008-2010) up to 82.1% (2017-2018). The prevalence of the 27 types of HPV (HR HPV combined with the LR ones included in the vaccine formula – 16, 18, 6, 11), has been lower in vaccinated women than in non-vaccinated ones. The vaccination with the quadrivalent vaccine has significantly lowered the prevalence of HPV, but it is important to

highlight that the HR HPV types which are not included in the vaccine are still a potential risk factor for the study group. The author's personal contributions to this research has been published in: *Virol J*, (IF=2.343), *Plos One* (IF=3.057), *Front Cell Infect Microbiol* (IF=4.123).

Chapter 2: HPV & head and neck cancers

The participation to the postdoctoral project „Parteneriat strategic pentru creşterea calităţii cercetării ştiinţifice din universităţile medicale prin acordarea de burse doctorale şi postdoctorale - DocMed.net_2.0", Contract nr. POSDRU/ 159/ 1.5/ S/ 136893, Universitatea de Medicină și Farmacie "Grigore T. Popa" Iași with the subject "HPV infections – risk factor for special population groups" has been another important moment for my research activity. During the annual HPV conference in Seattle, I had the opportunity to be evaluated for my poster presentation by a team of experts in the field. Therefore, I had the opportunity to initiate a research project with Dr. Massimo Tommasino, chief of the department for Infections and Cancer Biology Group, International Agency for Research on Cancer, Lyon, France. Four years of collaboration with researchers from Lyon, Germany, Italy, USA have followed, and I have learned what international interdisciplinary collaboration means, in which the centerpiece is scientifically valid information. I have tested a considerable number (200) of tumoral samples of HNSCC, selected by Dr. Mihai Danciu and Dr. Irene Spiridon from "Sf. Spiridon" Hospital in Iasi. I have participated directly in the processing of these samples, during a Visiting Scientist mobility program at IARC, where I purified the DNA for the samples, I genotyped HPV by multiplex PCR, I stained for $p16^{INK4a}$, and I prepared the sections for RNA testing which were sent to the German Cancer Research Center (DKFZ), Division of Molecular Diagnostics of Oncogenic Infections, Heidelberg, Germany. In this study I have used a very strict evaluation algorithm for the HNSCC samples> triple testing DNA/HPV, RNA/HPV and evaluating $p16^{INK4a}$, according to the IARC criteria of implying a virus as the etiological agent of a certain cancer.

The presentation of the intermediary results of this study, as an oral presentation at the IARC Conference in Manduria, Italy (*Emerging Issues in Oncogenic Virus Research*) has facilitated my collaboration with Professor Tina Dalianis, Chief of the Department for Oncology and Pathology at Karolinska Institute, Stockholm, Sweden. From that moment, another four years of collaboration and further training have started, as part of Professor Dalianis' Lab, during the students' summer vacation. During this period I have been a part of different teams, utilizing the most modern diagnostic methods available at the Institute. I had the joy of two internal research grants from UMF Iasi to obtain consecutive financing, which has facilitated the publication of 9 ISI articles in collaboration with the team from Karolinska Institute. Here, I have continued testing HNSCC tumor samples with similar methods to those at IARC regarding multiplex genotyping, but I have extended the spectrum of the tests especially for BOTSCC, TSCC, for salivary tumors and hypopharyngeal tumors, in association with new tumoral markers.

The author's personal contributions to this research has been published in: *Plos One* (IF=2.776), *Anticancer Res* (IF=1.935), *Diagn Pathol* (IF=2.528), *Cancer Med-US* (IF=3.491), *Acta Otolaryngol* (IF=1.157).

Chapter 3: Other oncogenic viruses

I have genotyped HBV by PCR assay followed by strip-hybridization (INNO LiPA HBV Genotyping assay) for patients with chronic hepatitis B, but also for pregnant patients who were detected positive for HBsAgs during pregnancy. The HBV genotypes detected were A and D, in accordance with the WHO data regarding their geographic distribution. Genotype F, detected

in the pregnant patients group has been a novelty for our area. Another study in which I assessed the presence of oncogenic viruses was that in which I tested the presence of 10 different polyomaviruses in salivary tumors, by multiplex PCR. For the malignant tumors that were tested, the analysis of the 10 genotypes did not implicate any of them as an etiological agent in neither of the tumoral subtypes. The most important results were published in *Rev Romana Med Lab* (IF=0.945) and in *Anticancer Res* (IF=1.935).

Chapter 4: Targeted cell lines therapy

This chapter includes studies regarding the sensitivity testing of several cell lines to different cytostatics in association with FGFR and PI3K inhibitors. The tests were undertaken in Karolinska Institute's new labs, where together with my fellow colleagues, I have worked with cell lines originating from BOTSCC and TSCC malignant tumors, which were positive or not for HPV. Similarly, I have tested medulloblastoma cell lines to similar therapeutic associations. After being treated with specific inhibitors, the studied cell lines were analyzed by methods such as viability, proliferation, cytotoxicity and apoptosis evaluation, using the Incucyte System. The purpose of this research was to identify the most efficient therapeutic association for the tumors that were tested, with minimal adverse effects for the patients.

The most important results were published in *Anticancer Res* (IF=1.994) și Front Oncol. 2021 May 11;11:640490. (IF=6.244).

In **section III**, future professional, academic and research projects are presented. The first two are strongly interlinked, with some similar aspects being: dedication, excellence and professionalism. The future scientific projects will include research that will, naturally, continue to evaluate the carcinogenic role of HPV in cutaneous tumours, but also of other oncogenic viruses (BK, EBV, HCV) in renal cancers. I will try to implement methods of testing the sensitivity of cell lines in our University as well. Thanks to the interdisciplinary collaboration, I intend to implement antibiotic sensitivity testing of *Helicobacter pylori* by molecular biology methods.

Section IV includes a selection of bibliographical references cited in this habilitation thesis.

Rezumat

Teza de abilitare prezintă pe scurt, cele mai importante realizări profesionale, academice și, în special, pe cele științifice obținute după finalizarea studiilor doctorale (2011-2021). Pornind de la aceste realizări și în concordanță cu actualele tendințe în cercetarea științifică, am inclus în finalul tezei de abilitare, câteva direcții de cercetare pe care mă voi axa în viitor.
Conform criteriilor recomandate și aprobate de CNATDCU, teze de abilitare este structurată în 3 secțiuni, după cum urmează:
Secțiunea I prezintă realizările profesionale, academice obținute postdoctoral.
Secțiunea II se referă la realizările științifice.

Chapter 1: HPV & cervical cancer

Capitolul 1 detaliază una dintre activitățile constante de cercetare avute în perioada imediat următoare obținerii titlului de doctor în științe medicale: HPV și cancerul cervical. Doctoratul realizat sub îndrumarea D-nei Profesor Iancu mi-a oferit oportunitarea de a îmbina testările moderne de biologie moleculară cu monitorizarea atentă a pacientelor, în colaborare cu medicii ginecologi (Dr. Anton, Dr. Onofriescu, Dr. Nemscu). Am evaluat astfel distribuția genotipurilor HPV prin genotipare PCR, care a detectat 37 de genotipuri virale, în rândul a peste 500 de paciente cu diferite diagnostice Papanicolau (NILM, ASCUS, ASCH, LSIL HSIL). Pe lângă importanța epidemiologică a detectării acestor genotipuri HPV care au susținut necesitatea implementării vaccinării anti HPV și în zona noastră, acest studiu a detectat genotipuri înalt oncogene și în rândul pacientelor cu citologie normală, ceea ce sugerează necesitatea unui test de screening mai sensibil pentru detectarea pacientelor la risc de a dezvolta cancer cervical.

O altă direcție de cercetare a fost evaluarea riscului unui lot de paciente cunoscute HIV pozitive, de a evolua către cancerul cervical. Semnificativă a fost detecția de infecții cu multiple genotipuri HPV pentru această categorie de paciente. Prevalența HPV în rândul acestor paciente a crescut de la 33% la 66%, în paralel cu scăderea imunității. Toate femeile, indiferent de statusul infecției HIV, ar trebui să participe la un screening organizat național al cancerului cervical, prin metode validate clinic, la prețuri accesibile.

Un alt studiu postdoctoral a evaluat eficiența intervenției chirugicale excizionale (*loop electrosurgical excision procedure* - LEEP) în îndepărtatea HR HPV. Probe cervicale de la paciente cu CIN și HR HPV au fost genotipate la 6 luni după LEEP. Am evaluat astfel influiența a variați factori de risc pentru persistența HR HPV. Infecțiile persitente au fost detectate în 22% din cazuri cu genotipurile HPV 16, 18, 31, 39, 51 și 66. Factorii de risc pentru persistența HR HPV după LEEP detectați au fost vârsta peste 30 ani, multiparitatea, utilizarea contraceptivelor orale și CIN2-3. Și în acest studiu am subliniat importanța implementării în țara noastră a unui screening cervical organizat cu ajutorul testelor HPV validate clinic.

Un ultim studiu în acest capitol se referă la eficiența vaccinării anti HPV. Astfel, grație colaborării cu echipa de cercetători de la Institutul Karolinska, Stockholm, am evaluat impactul vaccinării anti HPV asupra prevalenței leziunilor cervicale de grad înalt în rândul adolescentelor, în perioada 2008 – 2018. Proporția femeilor vaccinate a crescut de la 10.7% (2008 – 2010) până la 82,1% (2017 – 2018). Prevalența celor 27 de tipuri HPV (HR HPV combinate cu cele LR incluse în formula vaccinală - 16, 18, 6, 11), a fost mai scăzută în rândul femeilor vaccinate, în comparație cu cele nevaccinate. Vaccinarea cu vaccinul tetravalent a scăzut semnificativ prevalența HPV, dar important de subliniat însă este că tipurile HR HPV neincluse în vaccin, rămân un potențial factor de risc pentru lotul de studiu analizat.
Contribuțiile personale în urma acestor cercetări au fost publicate în: *Virol J,* (IF=2.343), *Plos One* (IF=3.057), *Front Cell Infect Microbiol* (IF=4.123).

Chapter 2: HPV & head and neck cancers

Participarea la proiectul postdoctoral „Parteneriat strategic pentru creşterea calităţii cercetării ştiinţifice din universităţile medicale prin acordarea de burse doctorale şi postdoctorale - DocMed.net_2.0”, Contract nr. POSDRU/ 159/ 1.5/ S/ 136893, Universitatea de Medicină și Farmacie “Grigore T. Popa” Iași cu tema ”Infecțiile HPV - factor de risc pentru grupuri populaționale speciale”, a fost un alt moment important în activitatea mea de cercetare. În cadrul conferinței HPV anuale din Seattle, am avut oportunitatea de a fi evaluată la prezentarea posterului, de către o echipă de specialiști în domeniu. Astfel, am avut șansa de a iniția un proiect de cercetare cu Dr. Massimo Tommasino, șeful departamentului Infections and Cancer Biology Group, International Agency for Research on Cancer, Lyon, Franța. Au urmat 4 ani de colaborare cu cercetători din Lyon, Germania, Italia, SUA și am învațat ce înseamnă colaborarea interdisciplinară internațională, în care informația științifică corectă, validată este elementul primordial. Am testat astfel un număr reprezentativ (200) de probe tumorale de HNSCC, selectate de Dr. Mihai Danciu și Dr. Irene Spiridon de la Spitalul Sf. Spiridon din Iași. La prelucrarea acestor probe am participat efectiv în cadrul unei mobilități de tip *Visiting Scientist* la IARC, unde am purificat ADN-ul din probele selectate, am genotipat HPV prin multiplex PCR, am colorat pentru $p16^{INK4a}$ și am pregătit secțiunile pentru testarea ARN ce au fost trimise pentru analiză la German Cancer Research Center (DKFZ), Division of Molecular Diagnostics of Oncogenic Infections, Heidelberg, Germany. În acest studiu am utilizat un algoritm strict de evaluare ale acestor probe tumorale HNSCC: tripla testare ADN/HPV, ARN/HPV și evaluarea $p16^{INK4a}$, conform criteriilor IARC, de a implica un virus ca agent etiologic al unui cancer.

Prezentarea rezultatelor intermediare, ca prezentare orală, ale acestui studiu la conferința IARC din Manduria, Italia (*Emerging Issues in Oncogenic Virus Research*), a facilitat inițierea colaborării cu Dna Profesor Tina Dalianis, Șefa Departamentului de Oncologie Patologie de la Institutul Karolinska, Stockholm, Suedia. Din acel moment au urmat alți 4 ani de stagii de perfecționare în laboratorul Domniei Sale, în vacanțele de vară ale studenților. În timpul acestor stagii am lucrat în diverse echipe, utilizând cele mai moderne metode de diagnostic disponibile în acest institut de cercetare. Am avut bucuria ca două din granturile interne de cercetare ale UMF Iasi să primească finanțare consecutivă, ceea ce a facilitat publicarea a 9 articole ISI în colaborarea cu echipa de la Institutul Karolinska. Am continuat aici testările tumorilor HNSCC cu metode similare celor de la IARC în ceea ce privește genotiparea multiplex, dar am extins testarea în special pentru BOTSCC, TSCC, pentru tumori salivare si cancere hipofaringiene, în asociere cu noi markeri tumorali.

Contribuțiile personale în urma acestor cercetări au fost publicate în: *Plos One* (IF=2.776), *Anticancer Res* (IF=1.935), *Diagn Pathol* (IF=2.528), *Cancer Med-US* (IF=3.491), *Acta Otolaryngol* (IF=1.157).

Chapter 3: Other oncogenic viruses

Am realizat genotipări ale VHB prin PCR urmat de hibridizarea pe strip (INNO LiPA HBV Genotyping assay) pentru pacienți cu hepatice cronice VHB, dar și pentru paciente însărcinate, detectate pozitive pentru AgHBs în cursul sarcinii. Genotipurile VHB detectate au fost A și D, conform datelor OMS în ceea ce privește distribuția geografică a acestora. Genotipul F detectat în lotul pacientelor însărcinate a fost un element de noutate pentru zona noastră. Un alt studiu în care am evaluat prezența de virusuri oncogene a fost cel în care am testat prezența a 10 polyomavirusuri diferite în tumori salivare, prin testare multiplex PCR. Pentru tumorile

maligne testate, analiza acestor 10 tipuri de poliomavirusuri nu a implicat nici unul ca factor etiologic important în nici unul din subtipurile tumorale.
Cele mai importante rezultate au fost publicate în *Rev Romana Med Lab* (IF=0.945) și în *Anticancer Res* (IF=1.935).

Chapter 4: Targeted cell lines therapy

Acest capitol include studii de testare a sensibilității unor linii celulare față de diferite citostatice în asociere cu inhibitori FGFR și PI3K. Testările au fost realizate în laboratoarele noi ale Institutului Karolinska, unde împreună cu ai mei colegi de proiecte, am lucrat cu linii celulare provenite de la tumori maligne BOTSCC și TSCC, pozitive sau nu pentru HPV. De asemenea, am testat linii celulare de tip meduloblastom la asocieri terapeutice similare. După tratare cu inhibitorii specifici, liniile celulare au fost analizate prin metode cum ar fi evaluarea viabilității, a proliferării, citotoxicității și apoptozei, cu ajutorul sistemului Incucyte. Scopul acestor testări este de a identifica cea mai eficientă asociere cu efect terapeutic pentru tumorile testate, cu efecte adverse minime pentru pacienți.
Cele mai importante rezultate au fost publicate în *Anticancer Res* (IF=1.994) și Front Oncol. 2021 May 11;11:640490. (IF=6.244).

În **secțiunea III** sunt prezentate proiectele de viitor în plan profesional, academic și de cercetare.
Primele două sunt puternic interconectate, câteva trăsături fiind comune: dedicarea, excelența și profesionalismul. Viitoarele proiecte științifice vor include cercetări care vor continua bineînțeles evaluarea rolului carcinogen al HPV în tumori cutanate, dar și a altor virusuri oncogene (BK, EBV, HCV) în cancere renale. Voi încerca să implementez metode de testare a sensibilității liniilor celulare și în Universitatea noastră. Grație colaborării colegiale interdisciplinare, intenționez testarea sensibilității la antibiotice a *Helicobacter pylori* prin metode de biologie moleculară.

Secțiunea IV include o selecție a referințelor bibliografice citate în această teză de abilitare.

SECTION I. Summary of personal professional, academic activities

I. 1. TEACHING ACTIVITY

I started my academic career with the residency in Laboratory Medicine, at the University of Medicine and Pharmacy "Grigore T. Popa" of Iași. In March 2004 I was admitted by contest as a junior assistant at Microbiology Discipline, under the guidance of Prof. Dr. Dumitru Buiuc and Prof. Dr. Luminița Smaranda Iancu. My academic career continued so that in 2008 I was promoted by contest as Assistant Professor. The complexity of the teaching activity stimulated me and made me want to reach a level of performance that would give me comfort and prestige as a young educator in front of a group of students. As a junior assistant I start teaching to Romanian students, and then, as an assistant, I worked with students from English section. Gradually I went from the status of a person who attends a course as part of the auditorium, to that of a Lecturer. Following the directions of the University of Medicine and Pharmacy "Grigore T. Popa" Iasi, I also tutored at the College of Nurses, Bacău, and Iasi divisions. In 2014, I have been appointed as Senior lecturer, by contest. I continued to teach Microbiology to the second-year medical students, but I also began to teach Clinical Microbiology to sixth year medical students, English seria. My teaching activity is referring also to resident doctors, from Laboratory Medicine, Microbiology, Epidemiology, and Infectious Diseases. In 2019, I was promoted by contest from the position of Senior Lecturer to that of Associate Professor.

I. 2. RESEARCH ACTIVITY

I may say that I start my research activity during my licence thesis, when I tested hepatitis viruses B, C and D, under guidance of Profesor Luminița Smaranda Iancu. Then I had the privilege to receive periodically training mobilities at Virology Institute Ștefan Nicolau, from Bucharest, under the supervision of Professor Dr. Simona Ruță and Researcher Dr. Gabriela Anton, where I first learned about molecular biology techniques. In the academic year 2006 - 2007 I attended the courses of a Master in Clinical Epidemiology. The study methodology in multicausal diseases, under supervision of Professor Dr. Doina Azoicăi. In 2008, I was admitted by a contest to the PhD program with the thesis “Asymptomatic sexually transmitted diseases. Clinical utility of high-risk HPV type viral load quantification by Real Time PCR” carried out under the supervision of Professor Luminița Smaranda Iancu. Once I began my PhD, I started attending numerous medical conferences, both in the country and abroad. During my PhD thesis I implemented for the first time in the Microbiology Laboratory the PCR assay, both Real Time PCR and PCR followed by hybridization on strips.
The viral genotyping was the main research activity starting with my PhD thesis. During doctoral period, the I genotyped by Linear Array HPV Genotyping test over 500 cervical samples from precancerous lesions and in situ cancer. For the main high risk HPV types (16,18, 31, 52) and I quantified the viral load using Real Time PCR assay in correlation with severity of cervical intraepithelial lessions. The results obtained in this PhD thesis were among the first in the North-Eastern Romania region, regarding the prevalence of HR HPV types in association with different cytologic diagnosis, and even in women with normal Pap test.
My doctoral study demonstrated the relationship between HPV and cervical cancer. The major outcomes of my PhD thesis were obtaining the “Proficiency qualifying for HPV DNA Genotyping Proficiency Study 2010”, organized by WHO Global Reference Laboratory for HPV Diagnosis and Control, and the scientific results were published in the paper: *Ursu RG,*

Onofriescu M, Nemescu D, Iancu LS. HPV prevalence and type distribution in women with or without cervical lesions in the Northeast region of Romania. Virol J 2011; 8:558.

During Postdoctoral Fellowship (Leader Professor Dr. Dragoș Pieptu) under the topic: "The HPV infections – risk factors for special populations" (2014 - 2015), I realized a comparative study between the prevalence of HPV types in normal population and in HIV positive women. I detected, as expected, increased number of HPV genotypes, in HIV positive patients, which was correlated with low CD4 number and with increased RNA/HIV viral load. Furthermore, the HPV prevalence in HIV positive women was correlated with the number of sexual partners, which confirm this risk factor, and underlined the utility of organized screening of cervical cancer, especially in this category of women. The results obtained in this study were published in the paper *Ursu RG, Onofriescu M, Luca A, Prisecariu LJ, Sălceanu SO, Nemescu D, Iancu LS. The Need for Cervical Cancer Control in HIV-Positive and HIV-Negative Women from Romania by Primary Prevention and by Early Detection Using Clinically Validated HPV/DNA Tests. PLoS One 2015;10(7):e0132271.*

In another project developed in our University, I genotyped HBV using INNO LiPA assay, detecting 6 genotypes of HBV. Beside the A and D genotypes which are common for our area, I also detected the F genotype, that was not previously mentioned in Romania.

At IARC, I started a study collaboration, part of HPVAHEAD project. Thanks to this project, I tested HPV by a very strict algorithm (triple testing: DNA/HPV, RNA/HPV and p16) in 200 head and neck squamous cell carcinomas (HNSCC) samples from Iasi. As a result of being *Visiting Scientist* at Infections and Cancer Biology Group, Head Dr. Massimo Tommasino, IARC, Lyon, France, 2015, I studied for the first time in Romania the HPV involvement in head and neck cancers and she published the paper: *Ursu RG, Danciu M, Spiridon IA, Ridder R, Rehm S, Maffini F, McKay-Chopin S, Carreira C, Lucas E, Costan VV, Popescu E, Cobzeanu B, Ghetu N, Iancu LS, Tommasino M, Pawlita M, Holzinger D, Gheit T. Role of mucosal high-risk human papillomavirus types in head and neck cancers in Romania. PLoS One 2018;13(6):e0199663.*

From 2017, I had the great opportunity to work each summer at Karolinska Institute, with different colleagues from Oncology – Pathology Department, having the support of Karolinska Institute's team leader. Also, I applied and obtained funding for two consecutive projects from her home University, in collaboration with Karolinska Institute.

During 4 Mobilities Research at Karolinska Institute (The Cancer Society in Stockholm Grant - 2017, PN-III-P1-1.1-MC2018-3106 – 2018, PN-III-P1-1.1-MC2019-1029- 2019, 2020) carried out at Karolinska Institute, Department of Oncology-Pathology, Head Prof. Tina Dalianis, I took part at many experiments and project and I published 9 articles in journals with high visibility. The published papers in collaboration increased visibility of our University at the international level, and opened the path for future research projects and scientific network. One of the latest paper is *Mints M, Landin D, Näsman A, Mirzaie L, Ursu RG, Zupancic M, Marklund L, Dalianis T, Munck-Wikland E, Ramqvist T. Tumour inflammation signature and expression of S100A12 and HLA class I improve survival in HPV-negative hypopharyngeal cancer. Sci Rep 2021;11(1):1782.*

The visibility and the impact of the scientific contribution is given by the total number of citation (without self-citation) according to Web of Science Core Collection (141), Hirsch index, according to Web of Science Core Collection (8), h-index on google scholar (11).

SECTION II. Scientific accomplishments

1. HPV & cervical cancer: scientific context

Epidemiology

Epidemiological research over the last decade has demonstrated that Human Papillomaviruses (HPV) are the most widespread and common sexually transmitted infections worldwide (de Sanjosé et al., 2007). It has been estimated that more than 80% of sexually active women and men will acquire at least one HPV infection by the age of 45 years (Chesson et al., 2014). However, most of them will be transient infections without any clinical impact. In women, 90% of incident HPV genital infections clear within two years (Rodríguez et al., 2008).

Cervical cancer is the fourth most frequent cancer among women and the fourth leading cause of cancer deaths worldwide, with an estimated 528,000 new cases and 266,000 new deaths in 2012 (Ervik et al., 2016). Cervical cancer is particularly observed in women with no use of (or no access to) cervical cancer screening. Out of 100 cases of invasive cervical cancer, 84 will be reported in women living in poor resource settings where access to cervical cancer screening and treatment remain scarce.

Sustained decreases in the incidence of cervical cancer have been recorded in most developed countries during the last part of the 20th century followed by stability of the rates during the 21st century. Although introduction of regular screening followed by adequate treatment are the most obvious triggers of such trends, changes in co-factors such as reproductive and sexual behaviour can also contribute to time variations in risk. For example, delaying age of sexual initiation, reducing the number of sexual partners or having fewer children may indirectly contribute to lower acquisition of HPV infections and ultimately affect incidence of HPV related cancers. Of all the HPV types with oncogenic potential, HPV16 remains in the pre-vaccination era the most predominant type in all locations, and particularly in sites other than the cervix. The contribution of HPV16 exceeds 80% of all HPV-positive cancer cases (Bruni et al., 2017).

HPV infection is involved in the majority of precancerous anogenital lesions, including cervical intraepithelial neoplasia (CIN). Their early detection is strongly dependent on screening practices, as they are generally asymptomatic. Data are, however, limited as these precancerous lesions are rarely reported in cancer registries. Nevertheless, between 263,227 and 503,010 cases of CIN2+ were diagnosed in Europe in 2015 (Hartwig et al., 2017). A variable proportion of these lesions could regress spontaneously, but once they are detected, they require treatment and close follow up. The majority will probably benefit from surgical elimination. Multiple infections and a wider spectrum of different HPV types are more commonly detected in precancerous lesions compared to cancer cases, but HPV16 is still the commonest type detected in all sites (Bruni et al., 2017).

Screening tests for cervical cancer

To quantify and compare the burden of HPV infection across populations, in 2010 Bruni L, et al., combined 194 studies from 59 countries (Bruni et al., 2010) through meta-analysis. This analysis pooled results from close to one million women with normal cytological findings who were tested for HPV with PCR techniques or Hybrid capture 2. The authors found that on average, 12% of women worldwide had a detectable cervical HPV infection varying by geography and age. When including more recent surveys, the same author found that in women with normal cytology, high-risk HPVs account for 70% of HPV infections (Bruni et al., 2017). This data underlines the need of more sensitive screening test to be implemented.

American, Australian and European guidelines recommend implementation of HPV-based cervical cancer screening. Several countries (e.g., Sweden) have recently introduced the HPV test for primary screening or are considering to switch from cytological to viral screening in the near future. The evidence supporting this paradigm shift is derived from randomised trials demonstrating a reduced incidence of cervical precancer and cancer among women with a negative HPV test compared to those with a negative cytology result. Two assays were used in the pivotal trials: Hybrid Capture II (HC2, Qiagen, Gaithersburg, MD, USA) and GP5+6+ PCR-EIA which both detect DNA of 13 or 14 high-risk (hr) HPV types. Based on international consensus, equivalency criteria have been accepted that other hrHPV DNA tests have to fulfil in order to accept them in cervical screening. These criteria include good intra- & inter-reproducibility and non-inferior accuracy to detect CIN2 or worse lesions, compared to the two standard comparator tests (Meijer et al., 2009). In 2015, a systematic review of screening and validation studies was performed which yielded a list of assays fulfilling the international criteria (Arbyn M, Clin Microbiol Infect 2015). The Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Branchburg, NJ, USA) enables type-specific identification of 37 HPV types. The aggregate of 13 hrHPV types identified with this test was found in VALGENT 3 to be as sensitive but more specific for CIN2+ compared to HC2 (Xu L et al., 2018).
A future version of the guidelines should define longitudinal criteria applicable for assays that target other molecules than HPV DNA (RNA, proteins, methylation markers).

Elimination of cervical cancer

Global eradication of cervical cancer is a prioritized goal on the international public health agenda. Existing HPV vaccines are effective for preventing HPV infection (J. Dillner, "IPVS Webinar: Elimination of cervical cancer: Now, later or never?", 2020). Three human papillomavirus (HPV) vaccines are available against up to nine HPV types. In 2006, the first HPV vaccine Gardasil, against HPV16, 18, 6 and 11, was approved by the Food and Drug Administration. This was the case for Cervarix (against HPV16 and 18) and Gardasil-9 (against HPV16, 18, 31, 33, 45, 52, 58, 6 and 11) in 2009 and 2014, respectively (Barra F et al., 2019). In countries that have had HPV vaccination programs for some time, the infection is today only maintained by younger adults (age<30 years of age). If young adult women are vaccinated concomitantly with HPV screening, the HPV reproductive rate will decrease to the level that should lead to rapid elimination of the infection. Combined with ambitious close-out screening to identify the cervical cancer precursors that HPV had caused before circulation was stopped, rapid and permanent elimination of cervical cancer can be reached (de Sanjosé, S., et al. 2007). The HPV vaccination was implemented in some countries in national programmes, in boys and girls, and in countries like Australia, the HPV vaccine implementation like routine to all population, led to decrease of genital infections. Regarding global elimination of HPV is considered to be clearly better conditions than was the case for polio. In 2016 WHO declares global elimination of cervical cancer as a prioritized aim. In 2018 Australia declares aim to eliminate cervical cancer by 2028. This is based on vaccination of all women (up to 26 years) (2006), on gender neutral vaccination (2012) and on switch to HPV-screening (2017) (1).

The major strategies for cervical cancer elimination are: (1) catch-up vaccination (stop the circulation of HPV, as soon as possible) and (2) catch-up screening (offer HPV screening to those who may have been infected before HPV circulation was stopped) (Lehtinen M, et al., 2019).

Eradication of HPV 16 is possible by vaccination of both sexes, vaccine population coverage > 70% up to age 24, gender neutral vaccination like a common strategy, vaccination up to age 26, (e.g. USA). A FASTER eradication of cervical cancer is proposing on top of the routine (HPV screening + vaccination of both sexes) a one-time campaign that invites all women aged 23-25 to both HPV vaccination and HPV screening. Only HPV-positive women need follow-

up (most women can be released from the screening program). Also the eradication procedure involve to invites all high-risk women to HPV screening. Using this strategy there is the choice of eliminating oncogenic HPV types and cervical cancer (Bosch FX et al., 2016).

Clinical trials, observational studies, and modelling analyses are currently being conducted to evaluate the efficacy, immunogenicity, effectiveness, and cost-effectiveness of single-dose HPV vaccination. If demonstrated to be effective, single-dose HPV vaccination could facilitate new options for current national programs by simplifying delivery and lowering program costs. For low- and middle-income countries that have delayed introducing HPV vaccines because of financial, logistical or other barriers, a single-dose HPV vaccination schedule could accelerate introduction of HPV vaccines into national immunization schedules (PATH: Single-Dose HPV Vaccine Evaluation Consortium Evidence Review, 2020).

Given the COVID-19 pandemic, when and where screening is judged to be sufficiently safe, it is important that any screening encounter that we recommend is very economical but also accurate, leading to clinical management of only a small group of women with a high probability of precancer. There is particularly interesting in validating complementary screening approaches concentrating on two areas: 1) Self-sampled, rapid HPV testing that distinguishes clinically distinct type groups useful for management decisions (HPV16; HPV18/45; HPV31/33/35/52/58, and the lower risk HPV39/51/56/59/68); 2) The use of "artificial intelligence" (more accurately, machine-learning) interpretation of smartphone images as a decision aid for health workers performing Visual Inspection with Acetic Acid (VIA) (Zhiyun Xue et al., 2020)

World Health Organization launches cervical cancer elimination strategy

The World Health Assembly recently launched a global strategy to accelerate the elimination of cervical cancer as a public health problem. The first time ever, the world has committed to eliminate a cancer. The strategy focuses on three pillars including prevention through HPV vaccination, screening and treatment of precancerous lesions, and treatment and palliative care for invasive cervical cancer. Eliminating this deadly disease is within reach with a collective effort to meet the targets set by the WHO by 2030. The targets that each country should meet include 90% of girls fully vaccinated with the HPV vaccine by the age of 15; 70% of women screened using a high-performance test by the age of 35, and again by the age of 45; and 90% of women identified with cervical disease receive treatment (Global strategy to accelerate the elimination of cervical cancer as a public health problem. Geneva: World Health Organization, 2020).

This research direction has been realized by publishing the following articles:

- **Ursu RG**, Onofriescu M, Nemescu D, Iancu LS. HPV prevalence and type distribution in women with or without cervical lesions in the Northeast region of Romania, *Virol J*, 2011, 8: art. no 558. IF=2.343
- **Ursu RG**, Onofriescu M, Luca A, Prisecariu LJ, Salceanu SO, Nemescu D, Iancu LS. The need for cervical cancer control in HIV-Positive and HIV-Negative women from Romania by primary prevention and by early detection using clinically validated HPV/DNA tests, *Plos One*, 2015, 10(7): art. no e0132271. IF=3.057
    - *Awarded by the UEFISCDI competition "Premierea rezultatelor 2015" PN-II-RU-PRECISI-2015-9-10591*
- **Ursu RG,** Anton AC, Nemescu D, Iancu LS. Risk factors for high-risk human papilloma virus persistence after loop excision procedure as treatment of cervical dysplasia, GINECO.eu, 2015, 11(4): 176-179 (Scopus, EBSCO)
- Ahrlund-Richter A, Cheng LQ, Hu YOO, Svensson M, Pennhag AAL, **Ursu RG**, Haeggblom L, Grun N, Ramqvist T, Engstrand L, Dalianis T, Du J. Changes in Cervical Human Papillomavirus (HPV) Prevalence at a Youth Clinic in Stockholm, Sweden, a Decade After the Introduction of the HPV Vaccine, *Front Cell Infect Microbiol*, 2019, 9: art. no 59. IF=4.123
    - *Awarded by the UEFISCDI competition "Premierea rezultatelor 2019" PN-III-P1-1.1-PRECISI-2019-35709*

***Ursu RG**, Onofriescu M, Nemescu D, Iancu LS. HPV prevalence and type distribution in women with or without cervical lesions in the Northeast region of Romania, Virol J, 2011, 8: art. no 558. IF=2.343*

Background: Cervical cancer is a major public health problem worldwide. While Romania has the highest incidence of cervical cancer in Europe, the prevalence of HPV has not been evaluated. We report the first data on HPV prevalence and type distribution in Northeast Romania. **Methods:** HPV prevalence and genotype distribution was investigated in 514 consecutively women with or without cervical lesions in Northeast Romania. Genotyping was performed with Linear Array Genotyping/Roche kit. **Results:** In our study group, 192/514 (37.4%) patients were positive for HPV (infected with single and with multiple HPV types). Most frequent types were: 16 (10.5%), 53 (5.44%), 51 (5.05%), 52 (4.08%) 18 (2.91%) and 31 (2.73%). **Conclusions:** Infection with high risk types of HPV is common in Northeast Romania. Enhanced and systematic screening for cervical cancer is needed. Our results call for the implementation of a National HPV vaccine program in Romania.
Keywords: HPV, Screening, Cytology, Prevalence, Persistence, Vaccinatio

Human Papilloma Virus (HPV) is the necessary cause of cervical cancer (de Villiers et al., 2004) and several screening methods have been developed to assess its prevalence in risk groups or patients (Walboomers et al., 1999). Although 15 HPV types are associated with high risk (HR) for cervical cancer, two HPV types, 16 and 18, account for 70% of cervical cancer cases (Muňoz et al., 2003). Each HR HPV type associates different carcinogenetic risks (Bosch et al., 2006). Using HPV/DNA genotyping tests can inform on the natural history of HPV infection by type and collectively, determine incidence, prevalence, and progression of HPV infection. Furthermore, screening for a broad array of HPV types can inform decisions on the

need for HPV vaccine in a given population and enable early detection of women who are infected with HPV and at risk for cervical cancer.

Romania has the highest incidence of cancer in Europe, with an incidence of 23.9/100,000 (Ferlay et al., 2010). In Romania, systematic screening for cervical cancer (i.e., cytology or HPV/DNA testing) is not available. Furthermore, no data on HPV genotype prevalence in Romania is available (WHO/ICO Information Centre on HPV and Cervical Cancer). A government attempt in 2009 to implement HPV vaccination in Romanian young girls failed because of the lack of medical information on the prevalence of HPV genotypes.

The goal of this study was to establish HPV prevalence and genotype distribution in women with and without cervical lesions in Northeast Romania (a population of 4.5 million people) in an attempt to gather sufficient information to support future vaccination program.

Results and discussion

After 2 years of systematic testing the following results were obtained in our study group with regards to classical risk factors for developing cervical cancer: 71/514 (13.8%) of interviewed subjects declared use of oral contraceptives; 146/514 (28.4%) had other gynecologic infections (*Neisseria gonorrhoeae, Candida spp, Trichomonas vaginalis, Treponema pallidum*); 85/514 (16.5%) reported cigarette smoking, while and 53/514 (10.3%) declared having more than three sexual partners. Papanicolau smear test results were as follows: ASCUS - 71/514 (13.8%), ASCH - 49/514 (9.5%), LGSIL - 107/514 (20.8%), HGSIL - 41/514 (8.0%), inflammatory - 31/514(6.0%) while a normal Pat test was reported for 164/514 (31.9%) patients. Note that 51/514 (9.9%) did not had any cytology examination prior to the study, while presenting with macroscopic and colposcopic suspicion for HPV infection. These results are comparable to those reported in other regions of Romania (Bosoteanu et al., 2011). The overall testing of the samples in our study gave the following relative prevalence results: out of the total 514 tested samples, 192 (37.3%) samples were positive for HPV/DNA, 120 of which (23.3%) were single HPV type infections and 72 (14%) tested positive for multiple HPV types (Table 1).

Table 1: The prevalence of HPV genotypes for all infections

HPV TYPE	Frequency	Percent
NEGATIVE	322	62,6
16	54	10,5
53	28	5,44
51	26	5,05
52	21	4,08
18	15	2,91
31	14	2,73
CP6108	13	2,52
6, 45 *	12	2,33
33	11	2,14
42	10	1,94
58	9	1,75
68, 73 *	8	1,55
66	7	1,36
84	6	1,16
62, 70*	5	0,97
82	4	0,77
55	3	0,58
56	2	0,38

11, 35, 39, 40, 61, IS39 *	1	0,19

HR HPV types were detected in 8/108 (7.42%) of women with no Pap test and in different percent for patients with Pap smear scores: 15/108 (13.9%) ASCUS, 16/108 (14.8%) ASC-H, 19/108 (17.6%) LGSI, 21/108 (19.4%) HGSIL and 7/108 (6.5%) having "inflammatory" cytological result. In 22/108 (20.4%) cases positive for HR HPV, the result of conventional smear was reported as "normal" (Figure 1). Since the relative prevalence of different HPV type may inform the composition of the polyvalent vaccine in a given region, we focused on the eight first HPV types detected that were HPV 16, 53, 51, 52, 18, 31, CP6108, 6 and HPV 42. The highest prevalence for multiple HPV type infection was observed in the age group of 21-30 years: 19/40 (47.5%), while women in the age group of 31-40 years had the highest prevalence of HR HPV types: 40/108 (37%). Of all the HR HPV positive women detected, 20/71 (18.5%) declared use of oral contraceptives, 32/146 (29.6%) had associated gynecological pathology, 31/85 (28.7%) reported cigarette smoking and 16/53 (14.8%) reported more than 3 sexual partners. Of all the DNA/HPV negative women, 11.3% declared use of oral contraceptives, 27% had associated gynecological pathology, 12.6% reported cigarette smoking and 5.5% reported more than 3 sexual partners.

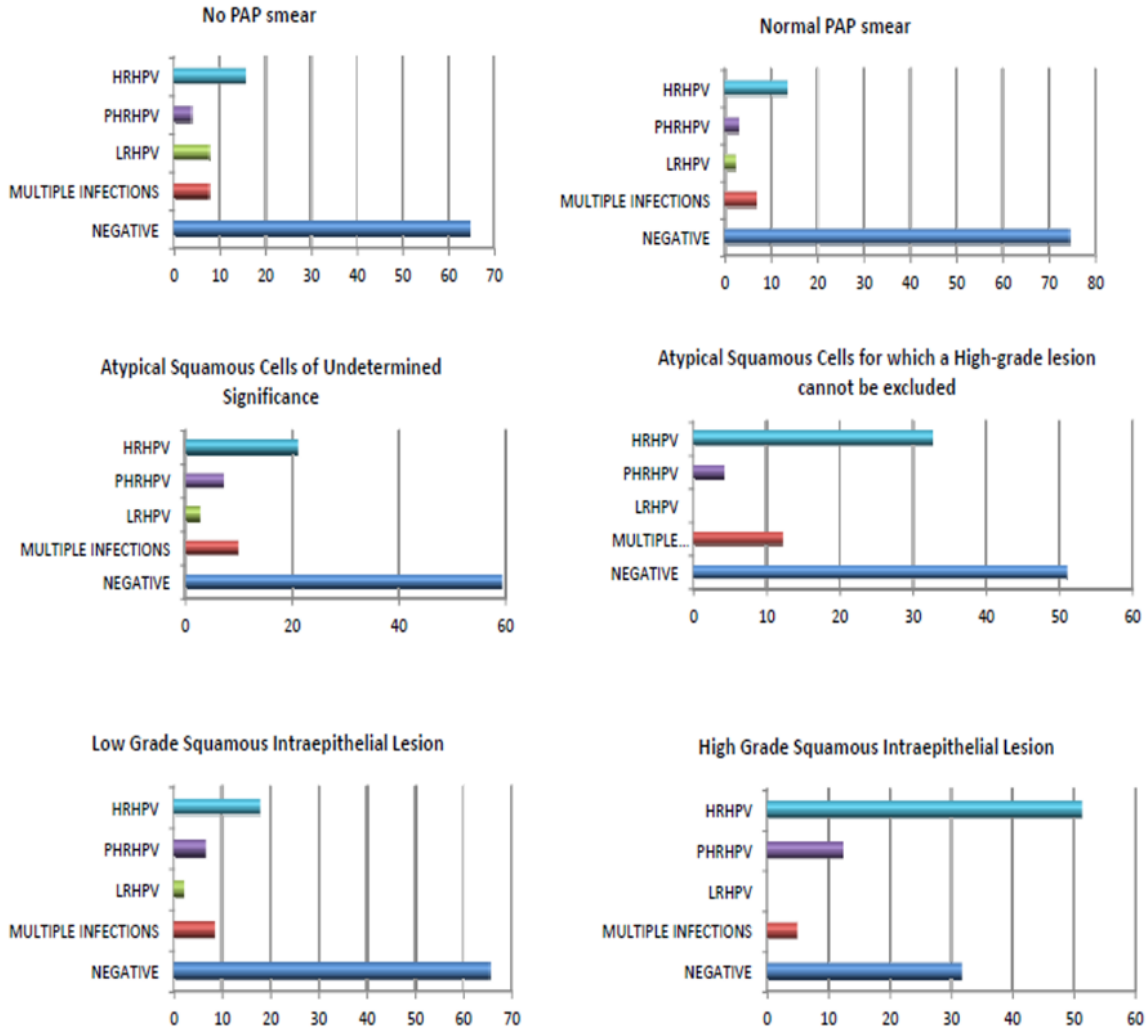


Figure 1: HPV genotype prevalence in 483 women with/and without cervical lesions from Northeast Romania (on the X axis are the percentages for each category according to the

classification IARC for HPV types: HRHPV, PHRHPV, LRHPV; total cases: 483 - except 31/514 cases with inflammatory cytology) (HR HPV types: High Risk HPV types, pHR HPV: probably High Risk HPV types, LR HPV types: Low Risk HPV types).

Multiple HPV types infections were found in 8/72 (11.1%) persons who were never tested for Papanicolau cytology and 20/72 (27.8%) women with normal result. For patients that received other scores, these percents varied between 5.6 and 20.8%. One explanation for detecting DNA/HPV in women with normal cytology is that the majority of HPV infections are transient. In the group of patients that were tested for cytologic examination, we have detected 18/51 (35.3%) positive with HR HPV (high risk oncogenic types), pHR HPV (probable high risk types), LR HPV (low risk oncogenic types) and multiple HPV type infections (de Sanjose et al., 2001). The prevalence of HPV genotypes included in the quadrivalent vaccine was as follows: HPV type 16-10.5% (54/514), HPV type 18-2.91% (15/514), HPV type 6-2.33% (12/514) and HPV type 11-0.19% (1/514). Women identified to carry with HR HPV infection will follow the European Guidelines for quality assurance in cervical cancer screening (2008): if the cytology is normal, they will repeat the HPV DNA test after 6-12 month; if they present with an abnormal Pap smear, they will be monitored by repetition of the smear, and referral for colposcopy. Women with HR HPV and HGSIL usually are candidate for excisional procedures, but the choice of treatment for women with HSIL will depend on the suspected diagnosis, the size and type of transformation zone, age and fertility aspirations (Jordan et al., 2009).

The relative prevalence of HPV types was similar in patients infected with single genotypes and in those with multiple infections. Multiple infections were produced by two to eight genotypes per sample. Note that these results may be due to either a numerous reexposure rate or to a high sensitivity of the assay used in our study in comparison to other HPV genotyping techniques (INNO LiPA, Reverse Line Blot) (Sabol et al., 2008). In order for the typing to meaningful, quality control and standardized panels of samples would need to be implemented for the regional reference laboratories (Eklund et al., 2010).

Thus, our study identified a high prevalence of HPV infection associated with cervical cancer in our area and therefore supports implementation of primary prevention of cervical cancer by vaccination in Northeast Romania, and in all country as well. In our region, screening of cervical cancer is not systematic and the quality control guidelines for conventional smears are not implemented (Anttila et al., 2009). Additionally, general population lack knowledge regarding connection between HPV and cervical cancer and necessity of performing regular screening for detecting precancerous cervical lesions (Badulescu et al., 2011). However, as supported by our study, a systematic testing for HPV both in general population as well as in gynecological patients has to potential to significantly reduce the rates of cervical cancer and therefore a strategy for systematic screening is badly needed. It is considered that full spectrum genotyping is useful for evaluation HPV epidemiology, and to the study of the impact of HPV vaccination and for clinical practice, to assess the persistence of HPV infection: stratification of risk for CIN 2+, ASCUS follow-up or treatment follow up. However, in Romania, similar to numerous other nations, screening for cervical cancer is not systematic and even conventional smear is not done on a regular basis (Anttila et al., 2009). In addition, cytology has a lowest sensitivity in comparison with DNA/HPV tests (53% versus 96%) (Cuzick et al., 2006). This calls for an alternative strategy consisting of improving the infrastructure for molecular diagnostic to enable systematic HPV detection using generic tests (Jordan et al., 2009, Cuzick J et al., 2008, Meijer CJ et al., 2009) followed by genotyping of the positive samples in regional reference centers. Also, implementation of a systematic screening program in Romania using generic cost effective DNA/HPV detection tests, would further imply an improvement of clinical sensitivity (cross hybridization with HR genotypes) and specificity (i.e., increase the detection cut-off of the assays) of screening tests. Also, DNA/HPV tests should be validated analytically and clinically to be adequate for diagnostic use (Meijer et al., 2009).

There is currently an open national cervical cancer screening pilot study undergoing in Romania. However, this study is still regional and aims to a 10% coverage only. These numbers are clearly different from those observed in other EU countries (i.e., in Netherland the coverage is 77%). This demonstrate that in order to significantly improve the efficacy of cervical cancer screening there is an important need for education of women as well as of medical professionals too (Anttila et al., 2009). At the same conclusion reached Apostol et al, regarding implementing in Romania of one organized cervical cancer screening (Apostol et al, 2010). Arbyn M. et all identified in 2007 the burden of cervical cancer in Romania requiring implementation of one well-organized cervical cancer program; the same authors published in 2010, by joinpoint regression method that the incidence and mortality through cervical cancer in Romania is increasing (Arbyn et al., 2007; Arbyn et al., 2010).

Being a new member of EU, in Romania there are still challenges regarding starting of one organised programmes for cervical cancer: the main reason is the lack of the infrastructure necessary to implement it (Nicula et al., 2009). Even though our pilot study was carried out on a limited number of patients, it covered a large number of patients and generated results that may permit the policy makers to initiate cervical cancer screening and vaccination programs for teenagers.

Conclusions

In developing countries as Romania, HPV testing can be used in primary cervical cancer screening. This will be beneficial for patients who will thus avoid excessive referrals to colposcopy and overtreatment (Jordan et al., 2009; Cuzick et al.; 2006, Wright et al., 2007). In the future, screening via HPV/DNA tests and vaccination must become coordinated, with programs providing the most cost-effective cervical cancer prevention strategies. Our results regarding epidemiological issues are comparable with WHO data at global and European level (WHO/ICO Information Centre on HPV and Cervical Cancer). Also, they are similar with the overwhelming epidemiological evidence for the role of sexual activity in the transmission of anogenital HPV infections (de Sanjosé et al., 2010; Cuschieri et al, 2004). The efficiency of prevention programs (screening, vaccination) is highest if the aim of these programs is focused on community needs. In this respect, the high prevalence of HR HPV documented by our study justifies entirely the necessity of cervical cancer prevention programs in Romania.

Methods

The study was carried out between September 2009 and November 2011, and included 514/1896 consecutive women (aged: 17-84 years, median: 36.5) from the "Cuza-Vodă" Gynecology Clinical Hospital. All patients were monitored by clinical gynecological examination, cytology exam and some of them, by colposcopic examination. Women with abnormal Pap smears (ASCUS–Atypical Squamous Cells of Undetermined Significance, ASC-H–Atypical Squamous Cells for which a High-grade lesion cannot be excluded, LGSIL–Low Grade Squamous Intraepithelial Lesion, HGSIL–High Grade Squamous Intraepithelial Lesion or inflammatory result) or with only suggestive colposcopy exam were genotyped for HPV infection. Each participant signed an informed consent and the study was approved by the Bioethical Committee of the "Gr. T. Popa" University of Medicine and Pharmacy of Iaşi, and each woman completed a questionnaire concerning possible cofactors for cervical cancer (e.g., smoking, genital coinfections, oral contraceptive use, number of sex partners), and provided cytological results of previous Papanicolau tests. Cervical cells were sampled with a cervical brush and they were transported in Cobas PCR Cell Collection Media (Roche). DNA purification was done using the High Pure PCR Template (Roche); HPV detection was performed using the Linear Array HPV Detection kit (Roche), and then DNA was genotyped with the Linear Array HPV Genotyping Test (Roche, Iaşi, Romania).

Genotyping via this technique involves one step of PCR amplification (ABI 9700): the final volume contained 50 µl of purified DNA and 50 µl of master mix (Roche). The thermal profile

included the next steps: HOLD program: 2 min/50°C; an initial denaturation of 9 min at 95°C was followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with a final extension for 7 min at 72°C. The amplicons have been denatured at the end of PCR amplification. HPV types were detected by hybridizations of the amplicons. Hybridization was performed in a TwinCubator (HAIN, Life Science) using Working Hybridization Buffer, Working Ambient Wash Buffer, Working Stringent Wash Buffer, Working Conjugate, Working Citrate Buffer and Working Substrate (Roche). The technique was validated through the use of positive and negative controls at each shift; the strips contain two bands for beta globin, which checks if the sample was correctly processed.
Linear Array HPV Genotyping test (Roche) is detecting 14 HR HPV (high risk) types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and 24 pHR HPV (probably high risk) and LR HPV (low risk) types (6, 11, 26, 39, 40, 42, 45, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 81, 82, 83, IS39, CP6108). We validated our HPV genotyping technique by obtaining proficiency for all the HPV types included in WHO HPV Proficiency Study, 2010. For the main HR HPV types (16, 18, 33, 52) we confirmed the results of LA genotyping result by qPCR/MX3005P (Stratagene manufacturers) detection using quantitative evaluation.

***Ursu RG**, Onofriescu M, Luca A, Prisecariu LJ, Salceanu SO, Nemescu D, Iancu LS. The need for cervical cancer control in HIV-Positive and HIV-Negative women from Romania by primary prevention and by early detection using clinically validated HPV/DNA tests, Plos One, 2015, 10(7): art. no e0132271. IF=3.057*

Abstract

Background

In Romania, a country with no organized national surveillance program regarding cervical cancer, the early diagnosis of HPV (Human Papilloma Virus) infections is a major requirement, especially in HIV-infected women. The objective of this study was to determine the HPV prevalence and type distribution in young HIV-positive women and to assess the difference in the risk factors for developing cervical cancer compared to those of HIV-negative women.

Method

We conducted one cross-sectional cohort study from June 2013–September 2014, including 1,032 women: 992 HIV- women who were 36.5 years old (limits: 17 84) and 40 HIV + women who were 22.9 years old (limits: 17 30) with iatrogenic HIV infected. We detected
HPV types with the Linear Array HPV Genotyping test (Roche, Romania).

Results

DNA/HPV was detected in 18/40 (45%) of the HIV+ patients and in 350/992 (35.2%) of the HIV- patients (OR = 1.5, 95%CI 0.762.96). After age adjustment, the overall HPV prevalence was 51.6%in HIV+ versus 63.2%in HIV- women aged under 25, and 22.2% in HPV+ versus 47.2% in HIV- women aged 25–34. We detect HIV being a risk factor for acquiring multiple HPV type infections (OR = 2.30, 95% CI 0.885.97). The eight most common HPV types (high-risk, and low-risk) for women below age 30, HIV+ / - were: HPV 16, 18, 31, 51, 58, 68, and 6 and 82 respectively. To assess the risk factors of HIV-positive women for acquiring HPV infection, we analyzed the CD4/μL, ARN/HIV copies/μL, the age group, the number of sexual partners, smoking, and the type of HPV infection (single versus multiple
infections). We found that the number of sexual partners and smoking are statistically significant risk factors.

Conclusion

Even though there are no significant differences regarding the prevalence of HPV infection in HIV + versus HIV – patients, multiple infections were more frequent in the first group. In

our study group young HIV-infected patients under HAART therapy, high number of sexual partners (more than 3) and smoking were detected to be risk factors. Future organized screening for HPV infection using sensitive and specific methods are necessary at the national level in Romania.

Introduction

The primary cause of cervical cancer is persistent infection with high-risk human papillomavirus (HR HPV) types (Walboomers et al., 1999). HPV is a common virus that is sexually transmitted, and most cervical HPV infections resolve spontaneously within 2 years. Cervical cancer is one of the main causes of cancer incidence and death in women, most notably in low- and middle-income countries (Castellsagué et al., 2008). According to GLOBOCAN 2012, Romania is the leading country in Europe regarding the incidence (28.6/100.000) of cervical cancer (Ferlay et al., 2013). Special populations, like immunosuppressed individuals, pregnant women, and homosexuals are high risk groups for acquiring HPV infections (Ezechi et al., 2014). The therapy of HPV-related diseases in case of HIV-positive persons can be difficult and HIV status accelerates evolution to HPV-associated cancers (Denny et al., 2012).
In 1989, in Romania it was an important nosocomial HIV epidemic, when many institutionalized children contracted HIV through blood transfusions and/or infected needles (World Health Organization 2013. Key facts on HIV epidemic in Romania and progress in 2011). On 30 June 2014, the total number of HIV/AIDS cases (cumulative for the periodfrom1985-2014) in Romania was 19,696 (The evolution of HIV/AIDS infection in Romania 31 December 2014, http://www.cnlas.ro/images/doc/31122014_eng.pdf). The ECDC 2013 Report reveals that the percentage of HIV diagnoses in young people was the highest for Romania, reporting that > 35% of the HIV diagnoses are among young people (European Centre for Disease Prevention and Control. Annual Epidemiological Report 2013).
In Romania 2012, the national government attempted to implement organized and free cervical screening by cytology but there was no centralized quality control or specific training and audits. Even under these conditions, it was possible to detect approximately 120 new cases of cervical cancer annually (data not published). The program has been stopped because of the lack of financial and political support, and screening by Pap test is now possible by request. The HPV vaccination program also failed, even though Cervical Cancer Action states that in Romania, there is a national program for HPV vaccination (HPV vaccine map—Cervical Cancer Action).
In settings where HIV is endemic, screening for cervical cancer is particularly important. Although Romania is not included in the endemic area, HIV-positive patients represent a high-risk group for many infections, including HPV. The aim of this study was to evaluate the HPV type prevalence and distribution in HIV-positive women and to assess the difference in the risk factors for developing cervical cancer compared to those of HIV-negative women.

Material and Methods

Study population

We conducted one cross-sectional cohort study from June 2013- September 2014, including 1032 HIV-negative women from “Cuza-Vodă” Gynecology Clinical University Hospital Iaşi and 40 HIV-positive/AIDS women from Infectious Disease “St. Parascheva” Clinical University Hospital Iaşi. The average age for 992 HIV- women was 36.5 years old (limits: 17 - 84) and 22.8 year old (limits: 17 - 30) for40 HIV +patients. Half of the first study group (514 HIV-negative women respectively) was included in a previous study that was published in 2011 (Ursu et al., 2011). The HIV-positive women were iatrogenically infected, and the medium period of treatment for the last therapy regimen was 5 years. The HAART therapy used the classic regim with 2–4 drugs: an association between two inhibitors of protease, nucleosidic inhibitors of reverse transcriptase, protease inhibitor and non-nucleosidic inhibitor of reverse

transcriptase (kaletra, abacavir + lamivudine, invirase, zidovudine + lamivudine, ritonavir, didanosine, darunavir, andetravirine). All of the patients were monitored by clinical gynecological, cytology and colposcopic examinations. All HIV positive women were sexually active, and majority of those from HIV negative group (97.3%, bellow 70 years old, according with their answers from questionnaires); we also included in our study pregnant women (13 / 32.5% HIV-positive women and 16 / 1.6% HIV-negative women).

Ethics statement

Thestudy was approved by the Bioethical Research Committee of the "Gr. T. Popa" University of Medicine and Pharmacy Iaşi. All participants in the study signed informed consent form after they were shown/presented the benefits of inclusion in this study and filled out a questionnaire that was administered by an interviewer.

Data collection and laboratory procedures

The study participants completed a questionnaire concerning the known cofactors for cervical cancer (e.g., smoking, genital co-infections, oral contraceptive use, and number of sex partners) and provided cytological results of previous Pap tests. HPV genotyping was performed in the Virology Laboratory of "Gr. T. Popa" University of Medicine and Pharmacy, Iași, using the Linear Array (LA) HPV genotyping method as previously described (Ursu et al., 2011). The LA method identified 37 HPV types: 13 high risk (HR) (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and 24 low or intermediate risk (LR) (6, 11, 26, 39, 40, 42, 45, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108).

Statistical analysis

The age was categorized into the following groups: <25; 25–34; 35–44; 45–54; and 55+.years. Descriptive statistics were prepared for demographic variables: age, births, pregnancies, abortions, DNA/HPV presence, multiple HPV infections, and HR and LR HPV types. According to the presence or absence of HIV infection, after age adjustment, we used chi-2 test for OR, CI 95%, to determine statistically significant differences between the proportions for each variable. If OR was > 1, we considered the tested variable being a risk factors for acquiring HPV infections. The CD4+ cell count and ARN/HIV viral load were both divided into three groups (CD4/μL: < 200, 200–499, > 500), respectively ARN/HIV copies/mL: < 10,000, 10,000– 1,000,000, >100,000), based on the median value for each respective variable. To assess the risk factors for HIV-positive women for developing cervical cancer, we used the Kruskal-Wallis test. Statistical analysis was performed using SPSS version 20.0 software. A p value of <0.05 was considered statistically significant.

Results

For HIV-positive women, we detected 18/40 (45%) positive for DNA/HPV; the HPV prevalence for HIV-negative women was 35.2% (350/992).Among the HIV+ women, the most prevalent HR HPV were HPV 52 (12.5%), HPV 31 and 68 (each 10%), HPV 16 and 51 (7.5%) and HPV 18 (5%). For HIV-negative women, the descending order of HR HPV types was HPV 16 (11%), HPV 52 (4%), HPV 18 (3.9%), HPV 31 and 51 (3.7% each), and HPV 58 (2.75%). (S1 Table). There was a difference regarding the LR HPV types that were included in the vaccine formula: 10% HPV 6 and5% HPV 11 in HIV-positive women versus 2.3% HPV 6 and 0.19% HPV 11 in HIV-negative women. We also detected that some LR HPV types (54, 81 and 83) were found only in HIV + women. The prevalence of multiple HPV infections was 32.5% (13/ 40) for the HIV + women versus 20% (119 / 992) in HIV-negative women. Multiple HPV infections in HIV-positive women included the following types:16, 18, 31, 39, 51, 52, 58, 59, and 68; in one case, infection involved the following types:53, 59, 61, 68 and 84.

Discussion

This is a pilot study assessing for the first time in Romania the HPV types distribution in HIVpositive women, and their risk for developing precancerous lesions and cervical cancer,

was compared to that of HIV-negative women. The HPV type distribution in HIV-negative women in the present study does not bring any new significant information than those previous published [10]: the same high prevalence for HPV types 16, 53, 51, 52, 18 and 31 was observed in a study group that was twice as large (992 versus 514 women genotyped). Therefore, we will focus in this discussion on the results of HPV genotyping in HIV-positive women. In Fig 1 we compared theHPV prevalence in HIV-positive women and HIV-negative women by age group and can be observed that the HPV prevalence in HIV-positive women is higher in the age group < 25 years old, for multiple HPV types infections (OR = 2.30 for multiple HPV type infections). Higher HPV genotype prevalence found for the HIV-positive, can be directly linked to generation-related differences in sexual behavior. In HIV-negative women, the HPV prevalence between women of different generations decreased with age increasing. We acknowledge that the group of HIV+ women who were infected iatrogenically is very small mainly because in Romania all HIV positive patients are treated and monitorized in 8 regional centers; in Iaşi Center from "Sf Parascheva" Hospital there are around 1000 patients treated with HAART therapy since 2010; therefore, many differences compared to HIV-uninfected women will not be statistically significant even if they are rather strong.

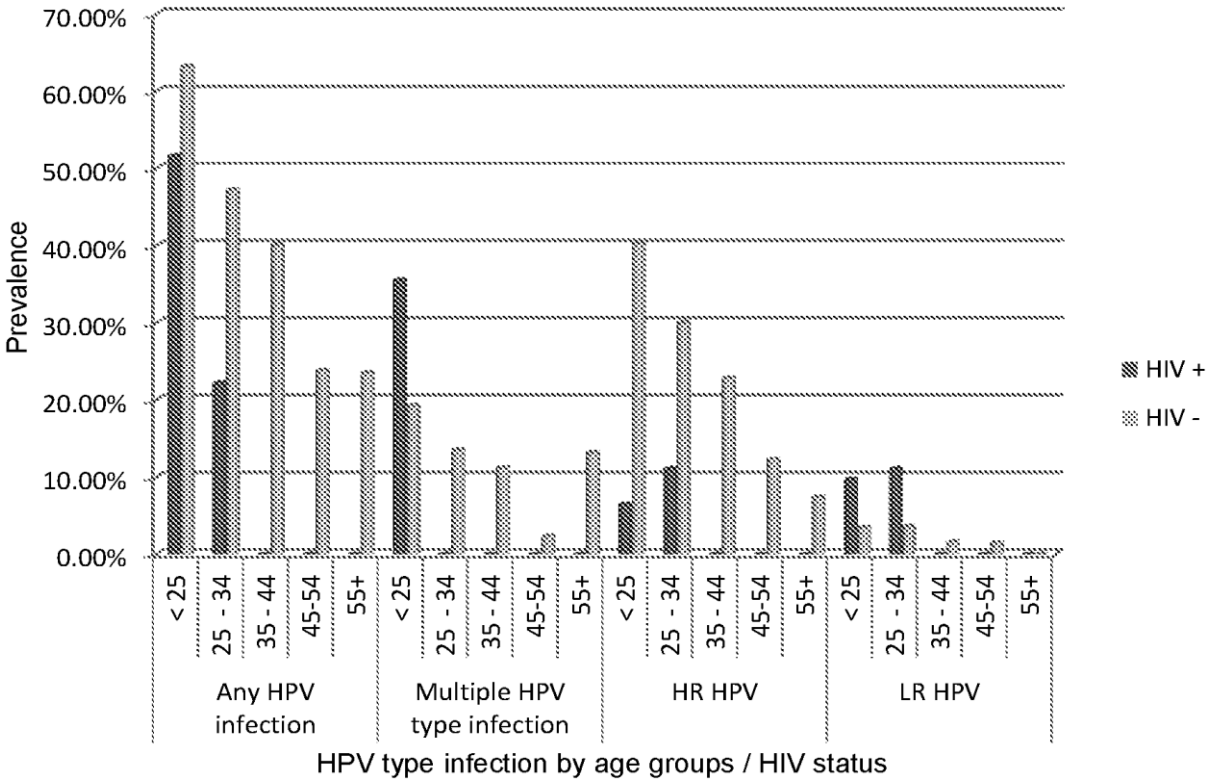


Fig 1. The HPV prevalence in HIV-positive women and HIV-negative women, compared by age group.

The demographic data regarding the tested women are shown in Table 1.We evaluated if HIV represent a risk factor in acquiring HPV infection and we have detected significant association for multiple HPV type infections (OR = 2.30) and LR HPV types (OR = 2.95). After age adjustment of the two study groups, we found like risk factor age < 25 years old, (OR = 5.83), number of birth (OR = 2.94 for 1 and 3.90 for 2), abortion (OR = 2.34 for 2 and 27.29 for three), pregnancy (OR = 17.62 for the group < 25 years old and 47.25 for 25–34), inflammatory Pap test (OR = 2.38). Also, after age adjustment, the overall HPV prevalence was 51.6% in HIV+ versus 63.2% in HIV- women aged under 25, and 22.2% in HPV+ versus 47.2% in HIV- women

aged 25–34. We conclude that HPV prevalence is lower in HIV+ than in HIV- women when adjusted for age. HIV-positive women are more susceptible to HPV infection associated with multiple types and with loss of detection (Heard et al., 2013; De Vuyst et al., 2012). These HIV-positive women belong to a special cohort of children who were HIV infected iatrogenically at birth or in the first years of life; the detected prevalence of HPV infection (45%) in HIV-positive women agrees with the results of other studies from other countries that reported HPV prevalence rates ranging from 36.5% to 72.2% (Clifford et al., 2006; Desruisseau et al., 2009; Firnhaber et al., 2013; Stuardo et al, 2012; Dames et al, 2014; Vaccarella et al., 2013).

The high HPV prevalence in this study group HIV-positive women can be explained by at least two main factors: the level of immunity, as demonstrated by a low medium level of CD4 cells (onaverage369 cells/mm3), and by a relatively high ARN/HIV median value (8575 copies / mL). For the patients with CD4 > 200 cells/mm3 and ARN/HIV < 20,000, the HPV prevalence was 33.33%, while for the patients having CD4 < 200 and ARN/HIV > 20,000, the HPV prevalence was doubled to 66.66%. A similar tendency was detected by Dames DN et al (in 2014), who reported that HR HPV infection increased 7 times in the case of CD4+ ≤ 200 cells/mm3 versus> 200 in a study group of 167 non-pregnant HIV-positive women [17]. As HPV type distribution is affected by age, we compared in Fig 2 the HPV types distribution to women below age 30 and to the eight most common HPV types (regardless of whether they are high-risk or low-risk), and we observed that the most frequent HPV types, both for HIV+/- women included the 3 types contained in the actually vaccine formula (HPV 16, 18, 6). HPV 16 seems to be relatively under-represented in HIV-positive women, as expected. We are confident in our HPV genotyping results as we have obtained proficiency in HPV genotyping with the LA method in WHO 2010 HPV proficiency testing; even this is a time consuming method, LA does not have the risk of cross-hybridization that can appear in other methods that are used for HPV genotyping, such as enzyme immunoassay (Kojic et al., 2014).

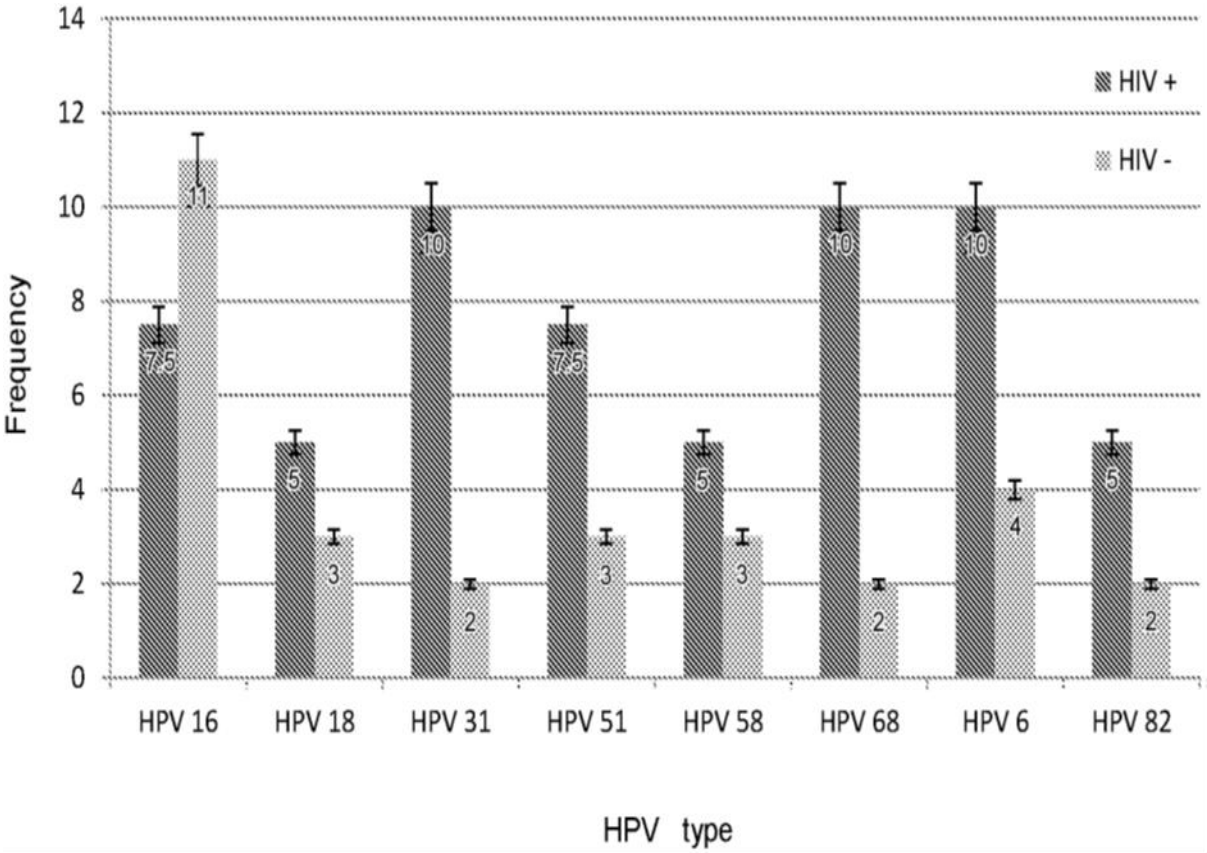


Fig 2. The eight most common HPV types (high-risk or low-risk) for

women below age 30, HIV+ /-

Table 1: The demographic characteristics of the two study groups

CHARACTERISTIC			HIV POSITIVE (n=40)	HIV NEGATIVE (n=307) ≤ 30 YEARS	OR	95 % CI	P values for chi-2 test
AGE							
	< 25		31 (77,5%)	114 (37,1%)	5.83	2.55÷13.71	0.001
	25 - 34		9 (22,5%)	193 (62,9%)	0.17	0.07÷0.39	0.001
BIRTH							
	< 25						
		1	5 (16,1%)	7 (6,1%)	2.94	0.74÷11.49	0.155
		2	0 (0,0%)	0 (0,0%)	-	-	-
		3	0 (0,0%)	0 (0,0%)	-	-	-
	25 - 34						
		1	1 (11,1%)	36 (18,7%)	0.55	0.02÷4.53	0.896
		2	1 (11,1%)	6 (3,1%)	3.90	0.48÷26.64	0.726
		3	1 (11,1%)	10 (5,2%)	2.29	0.31÷14.98	0.988
ABORTION							
	< 25						
		1	3 (9,7%)	15 (13,2%)	0.71	0.15÷2.88	0.831
		2	3 (9,7%)	5 (4,4%)	2.34	0.41÷12.21	0.484
		3	1 (3,2%)	0 (0,0%)	-	-	0.483
	25 - 34						
		1	3 (33,3%)	34 (17,6%)	2.34	0.44÷11.28	0.453
		2	0 (0,0%)	9 (4,7%)	0.00	0.00÷14.34	0.870
		3	2 (22,2%)	2 (1,0%)	27.29	2.29÷337.9	0.001
PREGNANCY							
	< 25		10 (32.3%)	3 (2,6%)	17.62	3.98÷89.21	0.001
	25 - 34		3 (33.3%)	2 (1,0%)	47.25	5.08÷531.9	0.001
PAP TEST							
No PAP test							
	< 25		5 (16,1%)	28 (24,6%)	0.59	0.18÷1.83	0.452
	25 - 34		2 (22,2%)	23 (11,9%)	2.11	0.28÷12.19	0.068
NORMAL							
	< 25		20 (64,5%)	46 (40,4%)	2.69	1.10÷6.67	0.028
	25 - 34		5 (55,6%)	74 (38,3%)	2.01	0.45÷9.26	0.493
ASCUS							
	< 25		1 (3,2%)	6 (5,3%)	0.60	0.03÷5.39	0.997
	25 - 34		0 (0,0%)	25 (13,0%)	0.00	0.00÷4.26	0.525
ASC-H							
	< 25		1 (3,2%)	6 (5,3%)	0.60	0.03÷5.39	0.997
	25 - 34		0 (0,0%)	25 (13,0%)	0.00	0.00÷4.26	0.525
LGSIL							
	< 25		0 (0,0%)	12 (10,5%)	0.00	0.00÷1.51	0.049
	25 - 34		1 (11,1%)	43 (22,3%)	0.44	0.02÷3.60	0.704
HGSIL							
	< 25		0 (0,0%)	4 (3,5%)	0.00	0.00÷5.79	0.660
	25 - 34		0 (0,0%)	12 (6,2%)	0.00	0.00÷10.19	0.960
INFLAMMATORY							
	< 25		4 (12,9%)	9 (7,9%)	1.73	0.41÷6.08	0.609
	25 - 34		1 (11,1%)	9 (4,7%)	2.38	0.34÷16.83	0.932
HPV PREVALENCE							
Any HPV infection							
	< 25		16 (51.6%)	72 (63,2%)	0.62	0.26÷1.49	0.337
	25 - 34		2 (22.2%)	91 (47,2%)	0.32	0.04÷1.74	0.261
Multiple HPV type infection							
	< 25		11 (35,5%)	22 (19,3%)	2.30	0.88÷5.97	0.096
	25 - 34		0 (0,0%)	26 (13,5%)	0.00	0.00÷4.07	0.503
HR HPV							
	< 25		2 (6,5%)	46 (40,3%)	0.10	0.02÷0.47	0.001
	25 - 34		1 (11,1%)	58 (30%)	0.29	0.01÷2.38	0.397
LR HPV							
	< 25		3 (9,7%)	4 (3,5%)	2.95	0.49÷16.88	0.344
	25 - 34		1 (11,1%)	7 (3,6%)	3.32	0.42÷22.32	0.802
CD4/µl							
			369 (14 – 774)				

ARN/HIV copies / mL							
			8575 (0 –343450)				

The Linear Array HPV Genotyping Strip contains a cross reactive probe (probe line 14) that hybridizes with HPV genotypes 33, 35, 52 and 58. HPV 52 can be interpreted correctly according with the manufacturer's instructions. We confirmed the HPV 52 type presence with real-time PCR by testing for the E6 oncogene of HPV 52.

One weakness of our study is that 13 of the pregnant HIV-positive women (32.5%) refused second sampling for the cytological examination because these women had a bloody cervix. We respected the patient's decision in accordance with their informed consent; this possibility could have been avoided if LBC (liquid-based cytology) were available. Regarding the Pap smear examination, only two smears were detected with cytological abnormalities: one had LSIL (low-grade squamous intraepithelial lesion) and infection with HPV 55 and 73, and one had ASC-US (atypical squamous cells of undetermined significance)and AGC (atypical glandular cells) with HPV 31, 61, and 84; the rest of the tested HIV-positive women had a negative Pap smear for intraepithelial lesions or malignity or an inflammatory result despite the high frequency of HPV type infection. Other studies reported an increased prevalence of HPV in association with high-grade lesions in HIV-positive women [12, 13]; in our study, the low number of high-grade lesions can be explained by the median age of the study group (22.9 years), requiring further testing to be able to conclude that the HPV infection was transitory or incident infection instead of persistent. Self-reported numbers of current STD symptoms (4/40) were not connected with any HPV infection. To assess the risk factors of HIV-positive women for acquiring HPV infections, we analyzed the CD4/μL (< 200, 200–499, > 500), ARN/HIV copies/mL (< 10,000, 10,000–1,000,000, >100,000), age group, number of sexual partners and smoking in relation to the type of HPV infection (single versus multiple infections). The Kruskal Wallis score was significant for the number of sexual partners (> 3) and smoking condition (Table 2: The risk factors of HIV-positive women for acquiring HPV infections).

Table 2: The risk factors of HIV-positive women for acquiring HPV infections

		HPV TYPE INFECTION			
		Negative	**Multiple infections**	**Single HPV type infection**	**P value Kruskal-Wallis test**
CD4/μL					
	< 200	3 (50%)	3 (50%)	0	0.625 a)
	200 – 499	13 (56.5%)	8 (34.8%)	2 (8.7%)	0.323 b)
	≥ 500	6 (54.5%)	2 (18.2%)	3 (27.3%)	0.959 *)
ARN/HIV copies/mL					
	< 10,000	12 (60%)	5 (25%)	3 (15%)	0.234 a)
	10,000 – 100,000	9 (56.2%)	5 (31.3%)	0	0.321 b)
	>10,000	1 (25%)	3 (75%)	0	0.359 *)
Sexual partners					
	< 3	21 (75%)	4 (14.3%)	3 (10.7%)	0.001 a) 0.025 b)
	≥ 3	1 (8.3%)	9 (75%)	2 (.7 %)	0.001 *)
Smoking					
	No	19 (65.5%)	6 (20.7%)	4 (13.8%)	0.031 a) 0.737 b)
	Yes	3 (27.3%)	7 (63.6%)	1 (9.1%)	0.034 *)

a) Multiple *vs* negative infections
b) Single HPV type *vs* negative infections

*) HPV positive *vs* negative infections

For the HIV- negative women, the risk factor for multiple infections was only the number of sexual partners. With the exception of one single case (who refused the therapy), all of the HIV + cases received HAART therapy, which was given for the last 5 years. Antiretroviral therapy was associated with lower prevalence of HPV infections, similar to other authors [14]. Recent studies have reported that vaccines against cervical cancer are efficient even in sexually active women HIV positive; our data regarding the HPV type distribution in HIV + women, even without cervical intraepithelial lesion, supports this vaccine implementation in our country [19]. The most important strength of our study is the characteristics of HIV-positive women who were infected at birth or soon after, and this is the first study, to our knowledge, to assess the HPV type distribution in this category of patients from Romania. Additionally, the genotyping method has good strength and was demonstrated to be proficient by aWHO panel. The weaknesses of this study include the low number of HIV-positive women, explained by the real number of HIV—positive registered in our geographic area, and the lack of biopsies for them, due to the low medium age (22.9 years), which did not allow the gynecologist to see visible lesions in the colposcopic examination. Additionally, the cross-sectional feature of our pilot study did not enabled for investigating the effects of the CD4 count and HAART on HPV infection longitudinally. To study the natural history of HPV infection, it would be necessary to perform one longitudinal study to assess the type of HPV infection, transitory versus persistent, considering that persistent HR HPV infections is a major risks for cervical cancer development. It is well known that only a small proportion of women infected with pathogenic HPV will develop a cancer. Therefore detecting such infections will unnecessarily alarm women and may lead to aggressive intervention from physicians, with a decrease in quality of life and fertility. Screening for pathogenic HPVs is costly. In addition, cervical cancer will develop (although rarely) in HPV- women. The method of choice for a national surveillance program could remain the cytological examination of Pap smears, in the countries in which Pap smears is organized and HPV infection prevalence is low. For our country, in which in 2015 aproximatively 40% of family physicians did not registered themselves in the national cervical screening program (data not published), we can conclude that women registered to those doctors will not be not included in the future screening. In countries where cervical screening is well organized (e.g. Netherlands) it was observed that even with a very well organized cytology based cervical screening programm, the incidence and mortality did not decreased in the last 10 years. For this reason Pap test will be replaced with clinically validated test, more sensitive and specific, being also known that a HR HPV negative woman is having low risk for developing precancerous lesions.

Conclusions

The HAART therapy and young age seems to be protective against developing precancerous cervical lesions in our study group, even the HPV prevalence and the multiple HPV type distribution were higher than those in the HIV-negative study group. The HPV prevalence in HIVpositive women increased (from 33.3% to 66.6%) in parallel with decreasing immunity. In particular, we noticed that serotypes 54, 81 and 83 were isolated only from HIV-positive patients. All women, regardless of their HIV status, should participate in an organized cervical cancer screening using clinical validated methods at affordable prices. In our region, it is necessary to improve medical knowledge of general population for a better understanding of the impact of HPV vaccination on the future cervical cancer incidence. Additionally, it would be cost efficient to use an HR HPV clinically validated test for the primary screening of cervical cancer.

Ahrlund-Richter A, Cheng LQ, Hu YOO, Svensson M, Pennhag AAL, ***Ursu RG****, Haeggblom L, Grun N, Ramqvist T, Engstrand L, Dalianis T, Du J. Changes in Cervical Human Papillomavirus (HPV) Prevalence at a Youth Clinic in Stockholm, Sweden, a Decade After the Introduction of the HPV Vaccine, Front Cell Infect Microbiol, 2019, 9: art. no 59. IF=4.123*

Abstract

Aim. This study aimed to follow the impact of human papillomavirus (HPV) catch-up and vaccination the past decade on the very high cervical HPV-prevalence in women at a youth clinic in central Stockholm.

Background. 2008-2010, cervical HPV-prevalence (70%) and HPV16 prevalence (34%) were high in non-vaccinated women at a youth clinic in Stockholm. 2013-2015, after the introduction of the quadrivalent-Gardasil® HPV-vaccine, HPV16 and HPV6 prevalence had decreased. Here, cervical HPV-prevalence was investigated 10 years after primary sampling.

Material and methods. 2017-2018, 178 cervical swabs, from women aged 15-23 years old, were tested for 27 HPV types by a bead-based multiplex method. HPV-prevalence data were then related to vaccination status and age and compared to HPV-prevalence in 615 samples from 2008-2010 and 338 samples from 2013-2015 from the same clinic, and to HPV types in 143 cervical cancer cases during 2003-2008 in Stockholm.

Results. The proportion of vaccinated women increased from 10.7% in 2008-2010 to 82.1% in 2017-2018. The prevalence of all 27 HPVs, all high-risk HPVs (HR-HPVs) and the combined presence of the quadrivalent-Gardasil® types HPV16, 18, 6 and 11, was lower in vaccinated compared to unvaccinated women (67.4% vs. 93.3%, p=0.0031, 60.1% vs. 86.7%, p=0.0057 and 5.8% vs. 26.7%, p=0.002 respectively). Furthermore, HPV16 prevalence in non-vaccinated women 2017-2018 was lower than that in 2008-2010 (16.7% and 34.7% respectively, p=0.0471) and similar trends were observed for HPV18 and 11. In both vaccinated and non-vaccinated women, the most common non-quadrivalent-Gardasil® vaccine HR-HPV types were HPV39, 51, 52, 56 and 59. Together they accounted for around 9.8% of cervical cancer cases in Stockholm during 2003-2008, and their prevalence tended to have increased during 2017-2018 compared to 2008-2010.

Conclusion. Quadrivalent-Gardasil® vaccination has decreased HPV-vaccine type prevalence significantly. However, non-vaccine HR-HPV types still remain high in potentially high-risk women at a youth clinic in Stockholm.

Key words. HPV, cervical HPV-prevalence, HPV-vaccine, women, youth clinic, Quadrivalent-Gardasil® vaccination, school-based vaccine program, catch-up vaccination

Introduction

Human papillomavirus (HPV) infections represent the most common sexually transmitted diseases, affecting millions of people worldwide. About 90% of HPV infections are cleared within 2 years without any need for medical intervention (Ho et al., 1998). However, persistent infections with high-risk HPV (HR-HPV) types can progress to cancer (Ho et al., 1998; Tommasino, 2014). Low-risk HPV (LR-HPV) types do not usually cause cancers but are associated with the occurrence of genital warts and respiratory papillomatosis (Ho et al., 1998; Tommasino, 2014). There is also evidence that links HPV with other types of cancer, e.g. vaginal, vulvar, penile, anal, as well as oropharyngeal cancer, especially tonsillar and base of tongue cancer (Forte et al., 2012; Dunne and Park, 2013; Tommasino, 2014; Nasman et al., 2017).

The US Food and Drug Administration (FDA) has approved three HPV-vaccines: quadrivalent-Gardasil®, Cervarix® and Gardasil®9, consisting of type-specific HPV L1 virus-like particles (VLPs) that induce type-restricted protection (Schiller and Lowy, 2018). All three vaccines prevent HPV16 and HPV18 infection, which account for about 70% of cervical cancer and

precancerous cervical lesions (Koutsky et al., 2002; Schiller and Lowy, 2018). Quadrivalent-Gardasil® also protects against HPV6 and HPV11, which cause 90% of genital warts (Schiller and Lowy, 2018). Gardasil®9 targets an additional five cancer-causing HPV types (HPV31, 33, 45, 52, and 58) (Schiller and Lowy, 2018). These vaccines may also have some cross-protection against other less common HR-HPV types (Kavanagh et al., 2017). Since the first licensure of HPV-vaccination in 2006, many countries have implemented publicly funded HPV-vaccination programs, with a variety of observational studies documenting vaccine efficacy and impact (Gallagher et al., 2018). HPV-vaccination is documented to be safe, immunogenic, and associated with decreased HPV infection rates and lowered risk of HPV related diseases (Drolet et al., 2015; Sankaranarayanan et al., 2016; Niccolai et al., 2017). Seven years (2007–2014) follow-up of the female three-dose HPV-vaccine series in nine high-income countries demonstrated a 68% decrease in the incidence of HPV16 and HPV18 (Drolet et al., 2015). In Sweden, HPV-vaccination was gradually introduced and subsidized between 2006 and 2011 for 13-17 years old girls, but initially with a fairly low coverage (Vanska et al., 2018). In 2012, the quadrivalent-Gardasil® vaccine was introduced into the school-based vaccination program for 10-12 years old girls. Meanwhile a catch-up vaccination included young women up to 26 years of age in Stockholm, Sweden, which increased the total vaccine coverage (Vanska et al., 2018).

In 2008-2010, when catch-up vaccination was still low (10.7%), we reported a cervical HPV-prevalence of 74% (with 34.7% infected by HPV16, and 10.1% infected by HPV18 respectively) in HPV non-vaccinated individuals at a youth clinic in Stockholm, Sweden (Ramqvist et al., 2011). In a follow-up study performed 2013-2015 at the same youth clinic, we showed that HPV-vaccination and catch-up vaccination coverage had increased to 71.0% and correlated with a significant decrease in cervical prevalence of HPV16 and HPV6 both covered by the quadrivalent-Gardasil® vaccine (Grun et al., 2016). However, very few studies focus on HPV types not targeted by the present HPV-vaccines. Given the very high pre-vaccine prevalence of HR-HPV infection in young women at the Stockholm youth clinic, it was of special interest to further assess the current coverage of the vaccination program and to evaluate the impact of HPV-vaccination on cervical HPV-prevalence one decade after our first survey (Ramqvist et al., 2011).

We therefore followed up young women visiting the same youth clinic in 2017-2018 and analyzed cervical prevalence of 27 different HPV types. Obtained data were then compared to data acquired from samples collected 2008-2010 and 2013-2015 during the introduction of the HPV-vaccination program the past decade (Ramqvist et al., 2011; Grun et al., 2016). In addition, we investigated the influence of school-based HPV-vaccination program and catch-up vaccination on the prevalence of the four vaccine types as well as non-vaccine HR-HPVs. Finally, HPV-prevalence was compared according to vaccination status and between different age groups and compared to HPV-prevalence found 2003-2008 in cervical cancer in the Stockholm region (Du et al., 2011). This study therefore provides an overall map of the influence of school-based HPV-vaccination program and catch-up vaccination program during the period of 2008 to 2018 among the potentially high-risk women, in Stockholm, Sweden.

Materials and methods

Study population

In total 178 self-collected cervical swabs and information of vaccination status and age were obtained from women aged 15-23 years between January 2017 and June 2018 at a youth clinic in central Stockholm, Sweden. Participation of this study was anonymous, and written consent was obtained before sample collection. Most young women were either HPV-vaccinated or catch-up vaccinated through the Swedish national vaccination program with the quadrivalent-Gardasil® vaccine against HPV16, 18, 6 and 11. The data acquired were then compared to data obtained at the same youth clinic in 2008-2010 (615 samples, 10.7% vaccination coverage) and

2013-2015 (338 samples, 71.0% vaccination coverage) and to HPV types occurring 2003-2008 in 143 cervical cancer cases (111 squamous carcinomas and 32 adenocarcinomas), in Stockholm (Du et al., 2011; Ramqvist et al., 2011; Grun et al., 2016). This study was performed according to permissions 2008/813-31/2, 2008/870-31/4 and 2017/725-31 approved by Stockholm Regional Ethics Committee.

Sample collection and DNA extraction

Sample collection was performed as described before (Ramqvist et al., 2011). However, both the sampling buffer and DNA extraction method was exchanged, the latter now performed as reported by Hugerth et al. (Hugerth et al., 2018). Briefly, cervical swabs preserved in DNA/RNA Shield (Zymo Research Corp, Irvine, CA) where beads were beaten with ZR Bashing Bead Lysis Tubes (0.1 and 0.5 mm from Zymo Research Corp, Irvine, CA) and then centrifuged at 1600 rpm with the 96 FastPrep machine (MP Biomedicals, Santa Ana, CA, USA) for 1 minute to briefly separate the buffers and beads. The sample buffer was then spun at 4400 rpm for 4 minutes to further separate out the beads. Thereafter, the supernatant was incubated with lysozyme buffer (20 mM Tris-Cl, 2 mM sodium-EDTA, 100 g/ml lysozyme; Sigma, St. Louis, MO, USA) at 37 °C for 3 hours while centrifuging at 1000 rpm. The DNA extraction was then conducted with the ZR-96 Genomic DNA MagPrep kit (Zymo Research Corp, Irvine, CA) according to the manufacturer's instruction. Finally, the extracted DNA was eluted from the magnetic beads with 70 µl Elution Buffer (10 mM Tris-Cl, pH 8.5; Qiagen, Venlo, Netherlands) and the purified DNA was stored at -20°C before HPV genotyping. For each extraction, DNA-free water samples were included as negative controls for potential contamination.

HPV genotyping

In total, 27 subtypes of HPV were assayed for by a multiplex bead-based assay, as reported before (Schmitt et al., 2006; Ramqvist et al., 2011; Nordfors et al., 2014; Dalianis et al., 2015). These included 15 HR-types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82), 5 potentially HR-types (26, 30, 53, 66, 67 and 69), and 6 LR-types (HPV6, 11, 42, 43, 44 and 70) (Schmitt et al., 2006; Bihl et al., 2017). Notably, typing of HPV30, 67 and 69 was not included in the corresponding assays of the initial sample collection 2008-2010 from the youth clinic nor the 2003-2008 cervical cancer sample collection (Du et al., 2011; Ramqvist et al., 2011). Briefly, for samples tested 2017-2018, PCR amplification with broad-spectrum BGP5+/6+ primers targeting the L1 region was carried out on 5µl of DNA in a 25µl reaction mix containing biotinylated primers from Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany). For HPV16 and HPV33 primers and probes targeting the E6-1F region were also included. The samples were analyzed by a MAGPIX instrument (Luminex Inc., TX, USA) in 96 well plates following manufacturer's instructions. Samples positive for L1 (and/or E6 for HPV16 and 33) were regarded as HPV-positive. For each run, a positive control and negative extraction control were added. Values with only water, before the PCR run were considered as background. The cut-off was set as: raw median fluorescent index (MFI) - 15 - 1.5 × background, except for the following cases: Firstly, a higher cut for HPV33-L1, HPV16-L1, HPV59, 69 and HPV82, (MFI - 25 - 1.5 × background) were used due to a higher background variation. Secondly, for HPV82 a cut-off of: 125-1.5 × background, in cases with an HPV51 value >100, due to a potential cross-reaction.

Statistical analysis

Any HPV infection/overall HPV infection, defined as all 27 HPV types, HR-HPV infection defined as the 15 HR-HPV types, infection with quadrivalent-Gardasil® HPV types (HPV16, 18, 6 and 11) together or alone, were compared between vaccinated and unvaccinated groups using Chi-square analysis. Differences of HPV types collected from this study were also compared with former studies using Chi-square analysis.

Results

HPV cervical prevalence 2017-2018 is high in women irrespective of vaccination status

In total, 172/178 samples had sufficient material for HPV analysis. Total cervical HPV-prevalence, irrespective of HPV-vaccination status, was 72.1% (124/172) and HR-HPV prevalence was 65.1% (112/172) (data not shown). Figure 1 displays all HPV types in HPV-prevalence order. Most common HR-HPV types: HPV56 (24.4%), HPV51, HPV59 (both 19.8%), HPV52 (18.6%) and HPV39 (15.1%) were all none quadrivalent-Gardasil® vaccine types. The prevalence of the quadrivalent-Gardasil® vaccine types HPV16, HPV18, HPV6, and HPV11, were 7.6%, 0.6%, 1.7%, and 0%, respectively. Most common LR-HPV and potentially HR-HPV types were HPV42, 53, 66, 67 and 44 (20.9%, 14.0%, 12.2%, 8.7%, and 5.8% respectively).

HPV cervical prevalence 2017-2018 is generally lower in vaccinated women

Vaccination status was available for 168/172 women above. Of these 138/168 (82.1%), had received at least one HPV-vaccination. Figure 2 shows HPV-prevalence in relation to vaccination status. Any HPV-prevalence (all 27 HPV types) and HR-HPV prevalence were both lower in vaccinated than in non-vaccinated women (67.4% vs. 93.3%, p= 0.0031 and 60.1% vs. 86.7%, p=0.0057, respectively). The four specific HPV-vaccine types combined were also less common in vaccinated than in unvaccinated women (5.8% vs. 26.7%, p=0.002), while for the 13 non-vaccine HR-HPV types the same trend was not statistically significant 60.1% vs. 76.7%, p=0.099).

HPV type specific cervical prevalence according to vaccine status is presented in Figure 3. HPV16 and HPV18 prevalence, included in all HPV-vaccines, tended to be lower in HPV-vaccinated than in non-vaccinated women (5.8% vs. 16.7%, p= 0.058 and 0% vs. 3.3%, p=0.179 respectively), but did not reach statistical significance (Figure 3). Quadrivalent-Gardasil® type HPV6 was significantly lower in vaccinated compared to non-vaccinated women (0% vs. 10.0%, p=0.0052), while HPV11 was not detected at all (neither in vaccinated or non-vaccinated women) in this study (Figure 3).

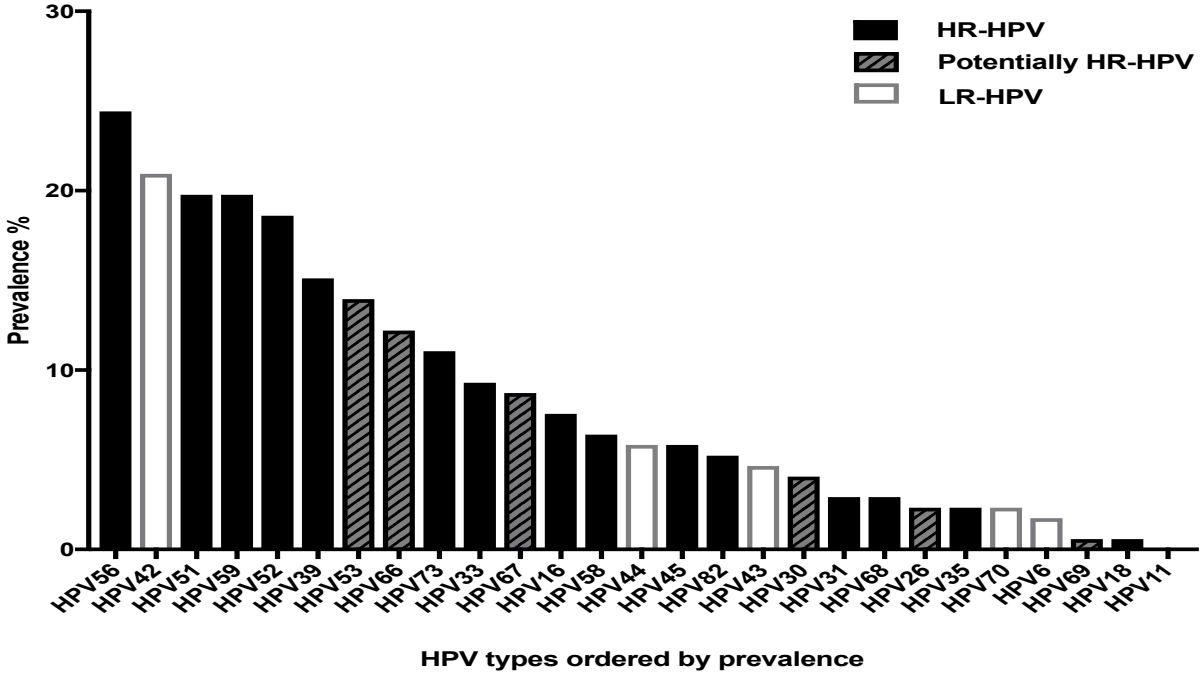


Figure 1. Cervical human papillomavirus (HPV) prevalence of all tested HPV types irrespective of vaccination status.

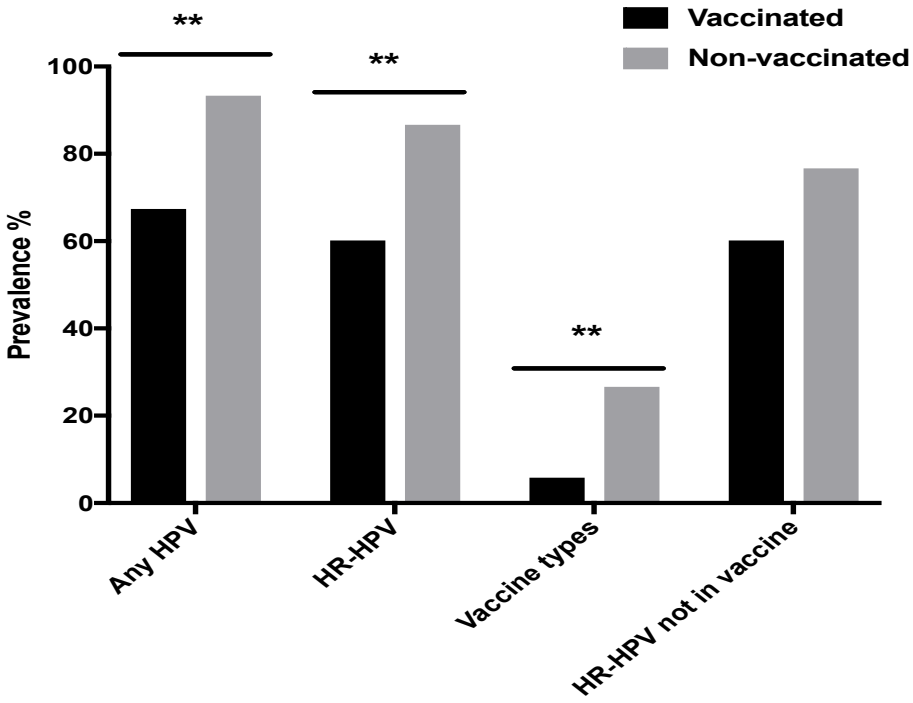


Figure 2. Cervical human papillomavirus (HPV) prevalence in vaccinated and non-vaccinated women with regard to: all 27 assayed HPV types (Any HPV); high-risk HPVs (HR-HPV); quadrivalent-Gardasil vaccine types HPV16, 18, 6 and 11 (Vaccine types); and HR-HPVs not in the vaccine (HR-HPV not in vaccine). ***p*<0.01. *HPV type specific cervical prevalence 2017-2018 according to vaccination status show significant decreases of HPV6, 33, 45 and 44 in HPV-vaccinated women*

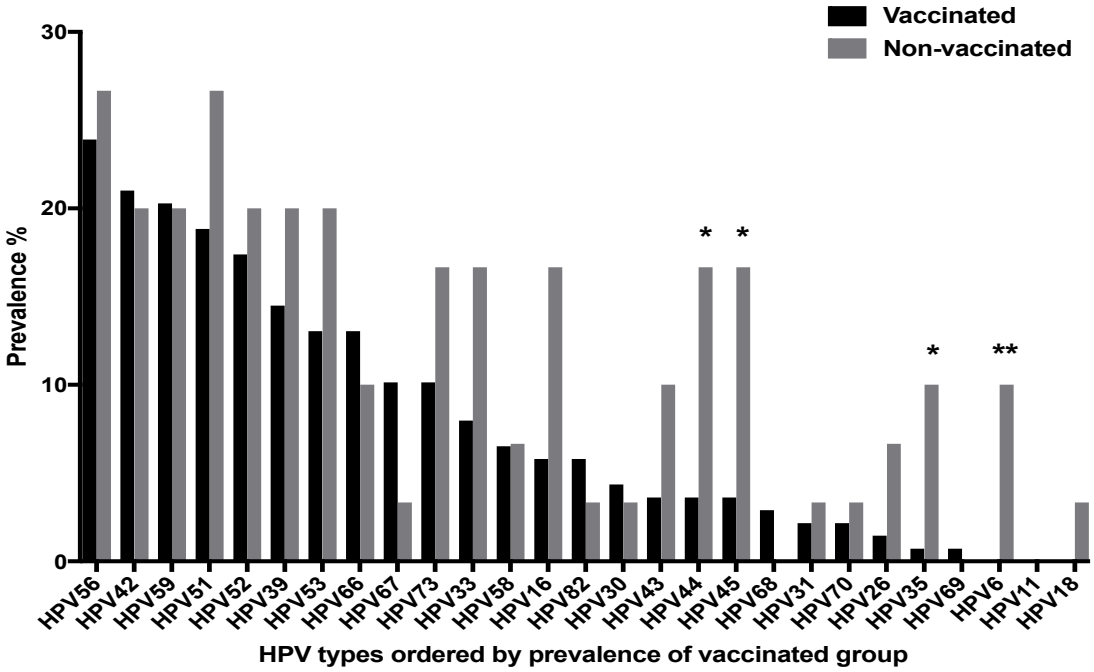


Figure 3. Cervical human papillomavirus (HPV) prevalence of all tested HPV types according to vaccination status (Vaccinated or non-vaccinated). **p*<0.05, ***p*<0.01.

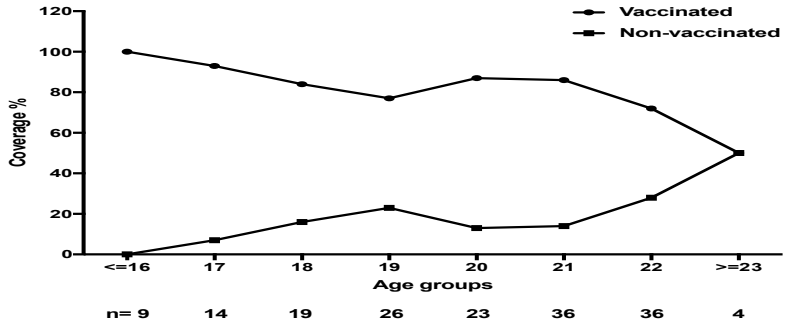
Notably, the prevalence of both HR-HPV35 and 45 also decreased significantly (p=0.0184 and p=0.0172, respectively) in vaccinated as compared to non-vaccinated women, while this was not the case for the remaining HR-HPV types (Figure 3). The five most common non-vaccine HR-HPV types were HPV39, 51, 52, 56 and 59 irrespective of vaccination status (Figure 3). In vaccinated women, they had the following prevalence: HPV56 (23.9%), HPV59 (20.3%), HPV51 (18.8%), HPV52 (17.4%) and HPV39 (14.5%), and in non-vaccinated women corresponding figures were: HPV51 and 56 (both 26.7%) and HPV39, 52 and 59 (all 20.0%) (Figure 3).

There were no significant differences in four of the five most common non-HR-HPV types, HPV42, 53, 66 and 67, between vaccinated and non-vaccinated women. However, LR-HPV 44 was significantly less common in the vaccinated group, p=0.0172 (Figure 3).

Cervical HPV prevalence according to vaccination status and age

Vaccine status and age were obtained from 167/172 women with cervical samples analyzed for HPV DNA. HPV-vaccination coverage was 100% in women ≤16 years, and ~ 80% coverage in those ≤19 years (Figure 4A). There were 169 samples from women with information on HPV-prevalence and age (Figure 4B). Data from women ≤16 years, as well as ≥23 years were grouped together due to small sample sizes. Irrespective of vaccination status, HPV-prevalence increased from ≤16 years of age (33.3%), peaked at 21 years of age (83.8%), and then slowly decreased (77.8%) and HR-HPV types accounted for most cases, especially in younger women (Figure 4B). The prevalence of the HPV types covered by the vaccine was relatively low with <10% in those ≤ 20 years of age but tended to be somewhat higher (13.5-25.0%) in those >20 years of age (Figure 4B).

A



B

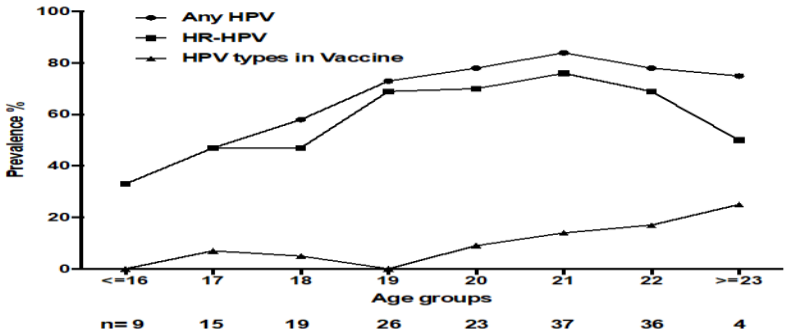


Figure 4. Vaccination status and human papillomavirus (HPV) status according to age.
A) HPV-vaccination status (Vaccinated and Non-vaccinated respectively) according to age.
B) Presence of any of the 27 assayed HPV types (Any HPV), high-risk HPV (HR-HPV) and the combined prevalence of vaccine HPV types 16, 18, 6 and 11 (HPV types in vaccine) according to age.

HPV types per cervical sample 2017-2018

Most women had an infection with at least one HPV type, and mainly a HR-HPV type. The average number of any of the assayed 27 HPV types (any HPV) or 15 HR-HPV types per sample tended to be lower in vaccinated than in unvaccinated women (2.1 vs. 3.1 and 1.4 vs. 2.1, respectively). However, when only considering HPV infected women, the average number of any HPV or HR-HPV type per sample was similar in vaccinated and non-vaccinated women (3.2 vs. 3.3 and 2.1 vs. 2.2, respectively). The maximum HPV types per sample was 11, (9/11 HR-HPVs), and was detected in a sample of a non-vaccinated woman.

Cervical HPV-prevalence 2017-2018 compared to previous data from 2013-2015 and 2008-2010

HPV-vaccination coverage increased from 10.7% (2008-2010) to 71.0% (2013-2015) and 82.1% (2017-2018). Initially most women were HPV catch-up vaccinated, while in 2017-2018, most women aged 16-18 years were vaccinated, likely through the school-based vaccination program. HPV-prevalence was generally high irrespective of time period. More specifically, when comparing vaccinated with non-vaccinated women, it was 53.8% (earlier unpublished data) vs. 69.5% in 2008-2010, 64.6% vs. 74.5% in 2013-2015, and most recently in 2017-2018, it was 67.4% vs. 93.3% (Ramqvist et al., 2011; Grun et al., 2016). HR-HPVs infections were also common, in non-vaccinated women (61.6%) 2008-2010, and in vaccinated vs. non-vaccinated women, 50.8% vs. 62.2% respectively in 2013-2015, and 60.1% vs. 86.7% respectively in 2017-2018 (Ramqvist et al., 2011; Grun et al., 2016).

HPV types included in the vaccination program substantially decreased over time especially in vaccinated women, but also in non-vaccinated women as shown for HPV16 in further detail in Figure 5. During 2017-2018 and 2013-2015 HPV16 prevalence was similar with 5.8% and 5.4% respectively in vaccinated vs. 16.7% and 18.4% respectively in non-vaccinated women, while 2008-2010, 35.4% of vaccinated (earlier unpublished data) vs. 34.7% of unvaccinated women were HPV16 positive (Ramqvist et al., 2011; Grun et al., 2016). Prevalence of HPV18, 6 and 11 also tended to decrease over time. HPV18, 6 and 11 were not found in vaccinated women 2017-2018, while in non-vaccinated women, HPV18 and 6 prevalence was 3.3% and 10.0% respectively, with HPV11 not detected. Notably, 2008-2011, in unvaccinated women prevalence of HPV18, 6 and 11 was 10.1%, 8.1% and 2.0% respectively, while during 2013-2015, corresponding figures were 4.1%, 5.1% and 2.0% in non-vaccinated vs. 1.3%, 0.4% and 0.4% in vaccinated women (Grun et al., 2016).

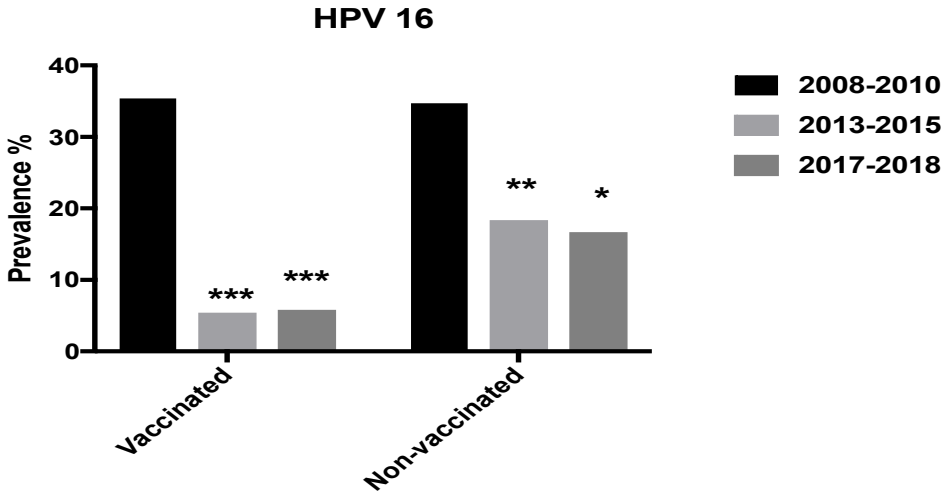


Figure 5. Prevalence of HPV16 in vaccinated and non-vaccinated women over the years 2008-2010, 2013-2015 and 2017-2018. *p<0.05, **p<0.01, ***p<0.001.

Prevalence of top five non-vaccine HR-HPVs in 2017-2018 compared to 2008-2010 and cervical cancer in 2003-2008

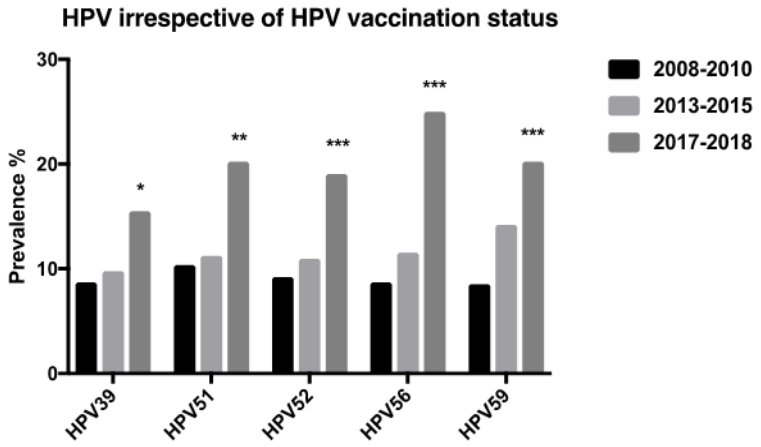
When comparing (irrespective of vaccination status) HPV-prevalence 2017-2018 of the five most common HR-HPVs (HPV39, 51, 52, 56 and 59) with their corresponding prevalence in 2008-2010, we observed significant increases for all of them (p=0.0138, p=0.0014, p=0.0008, p<0.00001 and p=0.0001, respectively) (Figure 6A). As for the vaccinated group, comparing data obtained during 2008-2010 (previously unpublished data) with data obtained during 2017-2018, the increase was significant for HPV52 and 56 (p=0.0032 and p=0.0132 respectively), while in unvaccinated women, the corresponding rise was significant for HPV51, 56 and 59 (p=0.0147, p=0.0047 and 0.0411, respectively) (Figure 6B and C). Finally, notably, HPV39, 51, 52, 56 and 59 were detected in 14/143 (9.8%) cervical cancer cases 2003-2008, in Stockholm, Sweden (Du et al., 2011).

Discussion

HPV vaccination has been shown to have a clear impact on HPV related diseases, reducing the burden of HPV infection, genital warts and cervical disease in countries with high vaccine coverage (Schmitt et al., 2006; Nordfors et al., 2014; Dalianis et al., 2015; Dehlendorff et al., 2018). Here, HPV-prevalence was examined in cervical samples collected during 2017-2018 at a Stockholm youth clinic, with previously documented very high pre-vaccine HR-HPV-prevalence (62%) (Ramqvist et al., 2011). Acquired data were then compared to HPV-prevalence data obtained at the same clinic the past 10 years, (more specifically 2013-2015 and 2008-2010), throughout the introduction of the quadrivalent-Gardasil® vaccine, as well as to HPV-prevalence data in cervical cancer cases 2003-2008, in Stockholm (Du et al., 2011; Ramqvist et al., 2011; Grun et al., 2016). Between 2008-2018, the coverage of HPV vaccinated women increased from 10.7%, 2008-2010, to 82.1%, 2017-2018, while the prevalence of HPV16, 18, 6 and 11, the four vaccine types decreased substantially (Ramqvist et al., 2011). During 2017-2018, their combined presence was also significantly lower in vaccinated as compared to that in non-vaccinated women. Notably, HPV16 prevalence had also declined in unvaccinated women in 2013-2015 and 2017-2018 as compared to 2008-2010 (Grun et al., 2016). Finally, 2017-2018, HR-HPVs as a whole, and especially HR-HPV35 and 45 decreased in vaccinated as compared unvaccinated women. This did however not apply to the most common HR-HPVs HPV 39, 51, 52, 56, and 59, which instead tended to increase in prevalence 2017-2018 compared to 2008-2010 (Ramqvist et al., 2011). Finally, in the present study, similar to that performed 2008-2010, HPV-prevalence peaked at 21 years of age, and most women with HPV-positive samples had more than one HPV type per specimen (Ramqvist et al., 2011).

These data emphasize the importance of HPV-vaccination and show similar to others studies that high vaccine coverage prevents HPV infection, especially of the four quadrivalent-Gardasil HPV types, and potentially prevents a considerable proportion of HPV related diseases e.g. cervical cancer (Cutts et al., 2007; Carozzi et al., 2018; Dehlendorff et al., 2018; Feiring et al., 2018; Herweijer et al., 2018; Patel et al., 2018; Saccucci et al., 2018; Wei et al., 2018). This is supported by the fact that the combined presence of the four vaccine types was less than 10% in women below 21 years of age, where vaccine coverage was high (>80%). Furthermore, during 2017-2018, HPV16 was detected in 5.8% of vaccinated women, while HPV 18, 6 and 11 were not detected at all. In addition, due to herd immunity, the prevalence of HPV16 had decreased substantially also in non-vaccinated women when compared to 2008-2010. The latter may along with the limited number of cases partially also explain why the difference in cervical HPV16 prevalence 2017-2018, although lower in vaccinated women than in non-vaccinated women, did not reach statistical significance.

A



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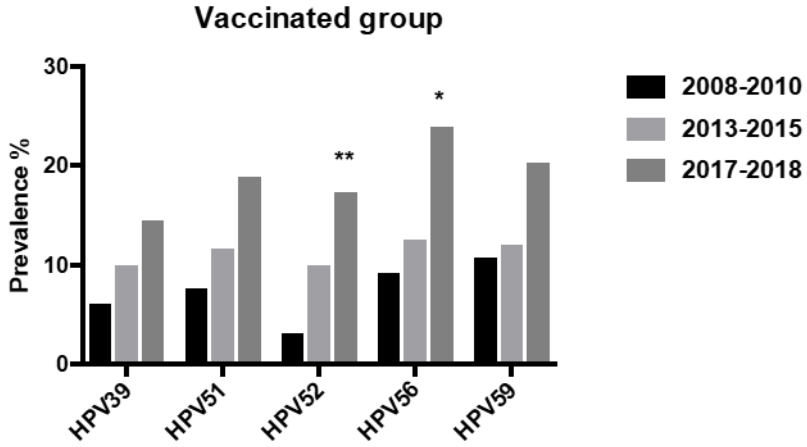

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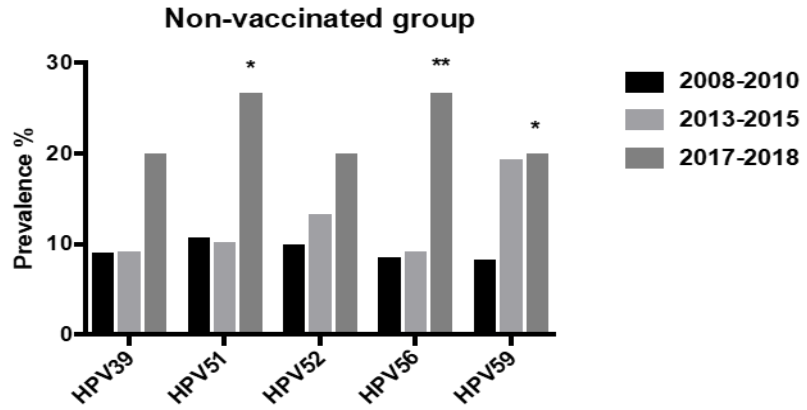

Figure 6. Prevalence of specific human papillomavirus (HPV) types over the years 2008-2018.
A) Cervical prevalence of HPV39, 51, 52, 56 and 59 irrespective of vaccination status over the years 2008-2010, 2013-2015 and 2017-2018.
B) Cervical prevalence of HPV39, 51, 52, 56 and 59 in vaccinated women over the years 2008-2010, 2013-2015 and 2017-2018.
C) Cervical prevalence of HPV39, 51, 52, 56 and 59 in non-vaccinated women over the years 2008-2010, 2013-2015 and 2017-2018.
*p<0.05, **p<0.01, ***p<0.001.

The fact that some HR-HPV types seem to have increased could be due to various reasons of which one possibly could be an increased sexual activity the past decades. Alternatively, potential cross-immunization induced by naturally occurring HPV-vaccine types e.g. HPV16, 18, was better against these non-HR HPV types than that induced by the vaccine, and therefore more efficiently helped to reduce their prevalence as compared to after vaccination was initiated (Saccucci et al., 2018). Elsewise, as suggested by others, the elimination of HPV16, that often produces a strong signal, may have increased the detection rate of non-vaccine HR-HPV types competing for the same primers (Tota et al., 2013; Machalek et al., 2018).

Both this study, and our study from the period 2008-2010, showed that HPV-prevalence peaked at 21 years of age indicating a relation to sexual activity in line with that observed by others, where HPV-prevalence is high in women younger than 25 years of age (Ramqvist et al., 2011; Machalek et al., 2018). Notably, there was higher vaccine coverage in the younger than in the older groups, likely due to the school-based vaccination program, as reported by others [23, 30]. Moreover, in younger women, the combined prevalence of the HPV-vaccine types was lower (<10%) than in older women, even though the total number of HPV infections dropped after 21 years of age, underscoring the importance of the school-based HPV-vaccination program (Feiring et al., 2018; Machalek et al., 2018).

Earlier, cross-protections induced by the HPV-vaccines were reported against HPV31 and 45, types closely related to HPV16 and 18, respectively (Saccucci et al., 2018; Wei et al., 2018). In this study, HPV35 and 45 showed a much lower prevalence in vaccinated compared with that in non-vaccinated women, suggesting the possibility of cross-immunization also for HPV35 (Saccucci et al., 2018; Wei et al., 2018). Other HPV types with a tendency of lower prevalence in the vaccinated group were HPV 26, 31, 33, 43, 53, 70, and 73, possibly also related to cross-reaction or protection by the vaccine.

Gardasil®9 is the latest HPV-vaccine and covers HPV6, 11, 16, 18, 31, 33, 45, 52, and 58 (Schiller and Lowy, 2018). It includes one (HPV52), but not the other four (HPV39, 51, 56 and 59) most common HR-HPV types found in 2017-2018 at the youth clinic, but it does include five of the six most common HR-HPV types (HPV16, 18, 31, 33, 45 and 56) identified in cervical cancer 2003-2008 in Stockholm (Du et al., 2011). Irrespective of whether HR-HPV types HPV39, 51, 52, 56 and 59, have increased or not from 2008-2010 to 2017-2018, their presence rules for some caution, since they together accounted for 9.8% of cervical cancer cases 2003-2008 in Stockholm (Du et al., 2011). Therefore, even if introducing Gardasil®9 one must bear in mind that four of these five HPV types HPV39, 51, 56 and 59 (not included in Gardasil®9), during 2003-2008 contributed to 11/143 (7.7%) of the cervical cancer cases in Stockholm (Du et al., 2011). Notably, although only 6/11 of these were single infections and others were co-infection with another HPV type, even in tumors with co-infections, viral oncogenes of several HPV-types can be transcribed simultaneously (Du et al., 2011; Halec et al., 2013). Furthermore, some potential HR-types not included in Gardasil®9, e.g. HPV53 and HPV66 had a prevalence above 10%, and have as shown for HPV66, been demonstrated as a single infection and actively transcribed in cervical cancer (Halec et al., 2013).

Finally, this study has some bias and limitations that need to be taken into consideration. The young women subjected for this study visit the healthcare center for birth control advice and for the treatment of sexually transmitted diseases (Ramqvist et al., 2011; Grun et al., 2016). Our study, may therefore, be based on a selected group of sexually active young women with possible symptoms for sexually transmitted diseases. Moreover, the potential HR-HPV types HPV 30, 67, 69 assayed in this study, were not tested in our initial study (Ramqvist et al., 2011). Therefore, the total HPV infection rate may have been affected in the investigation over time, while the remaining analyses concerning specific HPV-vaccine or non-vaccine HR-HPV types should not have been influenced by the introduction of these additional types, since these were included previously (Du et al., 2011; Ramqvist et al., 2011).

To conclude, quadrivalent-Gardasil® vaccination has significantly decreased the vaccine specific HPV-types HPV16, 18, 6 and 11, but five non-vaccine specific HR-HPV types HPV39, 51, 52, 56 and 59 still remain high in potentially high-risk women at a youth clinic in Stockholm. Of these five HR-HPV types, only HPV52 is included in Gardasil®9. It is therefore important to follow up HPV infections in the future and possibly consider the most prevalent HPV types in this study for the next generation of HPV-vaccines.

2. HPV & head and neck cancers: scientific context

Epidemiology & natural history of HNSCC

In recent years, an unexpected development in HPV cancer epidemiology has been observed with the increasing contribution of HPV to head and neck cancers. While traditionally these cancers were almost uniquely attributed to tobacco and alcohol exposure, it is now recognised that HPV is also in the aetiological pathway, particularly in the oropharynx. HPV-related oropharyngeal cancers constitute an epidemiological, molecular, and clinically distinct form as compared to non-HPV-related cancer. In 2007, human papillomavirus (HPV) type 16 was recognized as a risk factor, besides smoking and alcohol, for oropharyngeal squamous cell carcinoma (OPSCC), including tonsillar squamous cell carcinoma (TSCC), by the International Agency for Research against Cancer (1. IARC. A review of human carcinogens, 2007).

Globally, 456,000 head and neck cancer cases are diagnosed every year worldwide, and 37,200 cases are attributable to HPV, specifically 29,000 in the oropharynx, 4,400 in the oral cavity and 3,800 in the larynx (2. de Martel, C., et al. 2017. Int J Cancer).

HPV-associated cancers of the head and neck are more commonly seen in high-income countries, while they are rarely diagnosed in low-resource settings. The natural history of HPV-related cancers remains poorly understood, but evidence is being accumulated that HPV infection through oral sex is one of the most consistent independent risk factors with an additional synergistic effect of smoking or alcohol consumption (3. Farsi, N.J., et al. 2017. Carcinogenesis). Incidence rates for oral HPV infections in the literature range from 0.5 - 44 per 100 person-years (cumulative incidence 5/per 12 person-months) (Wood ZC., J Gen Virol 2017). Risk factors for oral HPV persistence include older age, male gender, current smoking, and HIV co-infection. Immunologic susceptibility potentially explains the lack of age-related decline in oral HPV incidence, increased persistence with older age, and the observed associations of oral HPV incidence and persistence with male gender, current smoking, and HIV infection (Giuliano AR, Int J Cancer 2015).

Screening

Importantly, in the oropharynx, there is no recognized HPV-induced precancer, analogous to CIN2/3 or AIN2/3. This lack of a pathologically - defined precancerous state may be related to the unique architecture of the tonsillar crypt epithelium - a non-differentiating reticulated epithelium with large pockets of exposed basal layer and discontinuous basement membrane. Due to the lack of large, long-term natural history studies and an HPV-induced intermediate disease state, screening for HPV-positive oropharyngeal cancer is not feasible at this time. Likewise, estimates for many important questions remain unknown including time from acquisition of causal HPV infection to HPV-OPC, disease latency, and transition probabilities for progression from persistent infection to precancer and invasive cancer.

However, the natural history of oral HPV infection, spanning the initial establishment of infection and the later development of cancer, remains poorly characterized, because few natural history studies have been conducted to date. Nonetheless, initial longitudinal studies evaluating HPV DNA in oral rinse samples show key similarities, as well as some differences, in the epidemiology of oral HPV infections compared to cervical/anogenital HPV infection.

There is a great need in the field for large natural history studies in diverse populations to characterize the steps from acquisition of oral HPV infection to progression to HPV-positive oropharyngeal cancers. Such knowledge remains critical for both primary prevention through prophylactic HPV vaccination (i.e. determination of the appropriate upper age-limit for vaccination) as well as secondary prevention and early detection through screening. (Castellsagué X, J Natl Cancer Inst 2016).

HNC elimination

Prophylactic HPV vaccines have been introduced worldwide since 2006 with a high efficacy and safety record in the prevention of vaccine-type HPV infection and disease. Currently, three HPV vaccines are commercially available (bivalent, tetravalent and nonavalent). All three contain virus-like particles (VLPs) of HPV types 16/18. The tetravalent vaccine also contains VLPs from HPV types 6/11. The nonavalent vaccine additionally protects against HPV types 31/33/45/52/58. The World Health Organisation (WHO) recognises the importance of HPV-related diseases as global public health threats and has reiterated the recommendation to include HPV vaccines in national immunisation programmes (7. Human papillomavirus vaccines WHO position paper. Geneva: World Health Organization, 2017).

Overall the HPV type specific distribution does not differ for the most common types across world regions and account for the vaccine types (HPV16,18,31,33,45,52,58,6,11) for 90% or more in cervical, anal, penile, oral cavity and oropharyngeal cancers, and more than 80% for other anogenital and head and neck HPV positive cancers. It is expected that HPV vaccines will have a significant impact in reducing not only cervical cancer burden but also the burden of other HPV-related cancers.

HPV vaccination of girls has been initiated in many countries, and the vaccines have been efficient and their side effects limited. HPV vaccination of boys has, however, been the exception, but should definitely not be delayed any further. It would benefit both girls and boys directly, and result in better and more robust herd immunity. Today, we have the possibility to eliminate several high-risk HPV types in the younger generations and avoid more than 600 000 cancer cases annually worldwide, and this possibility should be embraced by offering global pan-gender HPV vaccination (Näsman A, J Intern Med. 2020).

This research direction has been realized by publishing the following articles:

- Ursu RG, Ivanov I, Popescu E, Costan V, Stamatin O, Ghetu N, Palade D, Martu C, Andrese E, Danciu M, Spiridon IA, Salceanu SO, Jelihovsky I, Iancu LS. Human papilloma virus genotyping in fresh head and neck tumors - our first experience, Rev Med Chir Soc Med Nat Iasi., 2015, 119(3): 676-680 (Pubmed, Web of Science Core Collection

a. The IARC collaboration

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[1] Linnea Haeggblom and Ramona Gabriela Ursu contributed equally to this work.

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ABSTRACT

Limited information is available about the involvement of human papillomavirus (HPV) in head and neck squamous cell carcinomas (HNSCCs) in Romanian patients.

We evaluated the HPV-attributable fraction in HNSCCs collected in Northeastern Romania. In total, 189 formalin-fixed paraffin-embedded tissue samples (99 oral cavity tumors, 28 oropharynx, 48 pharynx, and 14 larynx/hypopharynx) were analyzed for HPV DNA and RNA using Luminex-based assays, and for overexpression of $p16^{INK4a}$ (p16) by immunohistochemistry.

Of the 189 cases, 23 (12.2%) were HPV DNA-positive, comprising half of the oropharyngeal cases (14/28, 50.0%) and 9/161 (5.6%) of the non-oropharyngeal cases. HPV16 was the most prevalent HPV type (20/23, 86.9%), followed by HPV18 (5/23, 21.7%) and HPV39 (1/23, 4.3%). Only two (2/189, 1.1%) HNSCC cases were HPV-driven, i.e. positive for both HPV DNA and RNA.

In conclusion, a very small subset of HNSCC cases within this cohort from Northeastern Romania appeared to be HPV-driven.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with an estimated annual burden of 355,000 deaths and 633,000 incident cases. Romania ranks second in mortality from HNSCCs in all-age males (32.4/100,000) among European countries (Ferlay et al., 2015).

HNSCCs are etiologically heterogeneous, being caused by tobacco use, alcohol consumption, poor oral hygiene, exposure to certain chemicals, and genetic features (Barul et al., 2017; Stornetta, Guidolin and Balbo , 2018; Riaz et al., 2014) as well as viral infections (Marur et al., 2010; Raab-Traub, 2002). High-risk (HR) human papillomavirus (HPV) infections have been associated with a subset of HNSCCs. (Gillison et al., 2000; Gillison et al., 2014). HPV16 is the most common type, being present in more than 80% of HNSCCs (Bratman et al., 2016; Ndiaye et al., 2014). Chaturvedi et al. (2013) reported that the incidence of oropharyngeal cancer increased significantly in developed countries from 1983 to 2002 (Chaturvedi et al., 2013).

The proportion of HPV-positive oropharyngeal cancers among HNSCCs has been increasing over the past decades in many parts of the world, whereas the overall incidence of HNSCC is decreasing, consistent with declines in tobacco use (Sturgis and Cinciripini, 2007). Several studies reported a steady increase in the proportion of HPV-driven oropharyngeal cancer cases in the United States (Chaturvedi et al., 2018), in Sweden (Nasman et al., 2009; Attner et al., 2010), in Australia (Hong et al., 2010), and in New Zealand (Lucas-Roxburgh et al., 2017). HPV has also been associated, to a much lesser extent, with non-oropharyngeal cancers such as oral or laryngeal cancer. In central India, less than 2% of these cancers were HPV-driven (Gheit et al., 2017).

The prevalence of HPV DNA in HNSCCs varies greatly by study, cancer site, and geographical area (Chaturvedi, 2012; Gillison et al., 2015), being high in oropharyngeal cancer cases from the United States (71.0%) (Goodman et al., 2015), eastern Denmark (62%) (Carlander et al., 2014), and the Czech Republic (57.0%) (Tachezy et al., 2005), whereas several studies reported the absence of HPV DNA in oropharyngeal cancer cases from Mozambique (Blumberg et al.,

2015) and China (Chen, Sun and Jiang, 2016), or a low or intermediate HPV prevalence in Germany (34.4%) and Brazil (15.5%) (Hauck et al, 2015). All these studies are based on HPV DNA detection techniques. However, several independent studies have highlighted that the detection of HPV DNA alone is not sufficient to accurately define HPV-driven HNSCCs (Gheit et al., 2017; Halec et al., 2013; Jung et al., 2010; Chernock et al., 2013).
The use of additional markers, such as viral RNA and $p16^{INK4a}$ (p16) expression as a surrogate for HPV-induced transformation, allows a more precise classification of HNSCC.
In a recent study, the HPV-attributable fraction based on positivity for HPV DNA and for either HPV E6*I mRNA or p16, was 22.4%, 4.4%, and 3.5% for cancers of the oropharynx, oral cavity, and larynx, respectively (Castellsague et al., 2016).
Similar rates have been obtained in Kazakhstan, where 25.7% of oropharyngeal cancer cases tested positive for HPV DNA and p16 (Adilbay et al., 2018), and in Northeastern Italy, where 20% of oropharyngeal cancer cases tested positive for HPV DNA and HPV RNA (Baboci et al., 2016).
In central India, HPV DNA/RNA double positivity was found in only 9.4% of oropharyngeal cancer cases (Gheit et al., 2017). HNSCCs from the Philippines all tested negative for both HPV DNA and HPV RNA (Albano et al., 2017).
In addition, in a recent study (Castellsague et al., 2016), based on 3680 HNSCCs from Europe, Africa, Asia, and the Americas, 22.4% of the oropharyngeal cancers tested positive for HPV DNA and for either HPV RNA or p16, and 18.5% were positive for all three markers. South America had the highest HPV-attributable fraction (53.6%) in oropharyngeal cancer, followed by Central and Eastern Europe (50.0%), Northern Europe (50.0%), Eastern Asia (22.4%), Central America (19.7%), Western Europe (19.4%), and Southern Europe (9.4%).
In Romania, limited information is available about the involvement of HPV in HNSCC. In this study, we aimed to determine the HPV-attributable fraction in HNSCC by analyzing HPV DNA and HPV RNA status, as well as by determining the p16 expression level, within a large retrospective cohort of HNSCC cases from Northeastern Romania.

MATERIALS AND METHODS

Patients and samples

Two hundred and three HNSCC patients were identified in the Departments of Oral and Maxillofacial Surgery, Otorhinolaryngology, and Plastic Surgery at the University of Medicine and Pharmacy "Grigore T. Popa" (Iași, Romania), from January 2010 to September 2014. All specimens were fixed for 18–24 hours in 10% neutral buffered formalin, at room temperature. The formalin-fixed, paraffin-embedded (FFPE) HNSCC blocks included squamous cell carcinoma of the oropharynx (International Classification of Diseases for Oncology [ICD-O] C01 – base of tongue, C02.4 – lingual tonsil, C09 – tonsil, C10 – oropharynx), pharynx (ICD-O C14 – other and ill-defined sites in the lip, oral cavity and pharynx, C14.8 – overlapping lesion of lip, oral cavity and pharynx), oral cavity (ICD-O: C00.0–C00.9, C01, C02.0–C02.9, C03.0–C03.9, C04.0–C04.9, C05.1–C05.9, C06.0–C06.9, C09.1–C09.9, C10), and hypopharynx and larynx (ICD-O: C13, C32). The FFPE tissue samples were retrieved from the hospital archives and comprised 34 HNSCC cases from the oropharynx and 169 HNSCC cases outside the oropharynx (16 larynx/hypopharynx, 51 pharynx, and 102 oral cavity cancer samples). All patients were diagnosed with keratinizing or non-keratinizing squamous cell carcinomas. Histological analyses on hematoxylin and eosin (H&E) stained slides were performed in order to confirm that all FFPE blocks contain cancer tissues. Clinical and epidemiological information was collected from the hospital databases using a form and questionnaire developed in the context of a European and Indian case study (HPV-AHEAD; http://hpv-ahead.iarc.fr). Ethical clearance for the investigations reported in this study was

obtained from the Institutional Ethical Committee of the University of Medicine and Pharmacy "Grigore T. Popa", Iași, Romania (reference number 7150). The study implied the use of archival material only, and it did not envisage any contact with the patients. Adequate measures to ensure data protection, confidentiality, patients' privacy, and anonymization were taken into account. No informed consent was available, due to the retrospective design of the study and the large proportion of deceased and untraceable patients. All data were fully anonymized before access.

Preparation of paraffin sections and DNA extraction

Each FFPE block was sectioned according to the HPV-AHEAD protocol, which includes the preparation of 31 sections from each FFPE tissue block. Sections 1, 10, and 31 (S1, S10, and S31) were used for histology, S2 and S9 were used for p16 immunohistochemistry (IHC), and S11–S30 were stored for future IHC analyses in independent studies. In addition, S3–S5 and S6–S8 were collected in two different vials and subsequently used for DNA and RNA analysis. (Mena et al., 2017). To minimize the risk of cross-contamination during sectioning, a new blade was used for each FFPE block and the microtome was extensively cleaned after each block with ethanol 70% and DNA Away (Dutscher, Brumath, France). In addition, to monitor possible cross-contamination during the sectioning, empty paraffin blocks were processed every 10th cancer specimen. DNA was extracted by an overnight incubation of the paraffin tissue sections in a digestion buffer (10 mM Tris/HCl pH 7.4, 0.5 mg/ml proteinase K, and 0.4% Tween 20) (Gheit et al., 2009). The percentage of tumor cells (0%, <10%, 10–50%, 50–90%, >90%) was estimated by two pathologists (MD, IAS) on H&E-stained slides (S1 and S10) (Mena et al., 2017).

HPV DNA genotyping

HPV DNA positivity was determined by using a type-specific multiplex genotyping (TS-MPG) assay, which combines multiplex PCR and bead-based Luminex technology (Luminex Corporation, Austin, TX) as previously described (Gheit et al, 2006; Schmitt et al, 2010).
This assay detects 19 HR or probable high-risk (pHR) HPV types (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a and b, 70, 73, and 82) and two low-risk (LR) HPV types (HPV6 and 11), as well as cellular beta-globin gene, which is used to control for DNA quality. After PCR amplification, 10 µl of each reaction mixture was analyzed by multiplex HPV genotyping (MPG) using Luminex technology (Luminex Corporation, Austin, TX) as described previously (Schmitt et al., 2010; Gheit et al, 2014). All HPV DNA-positive FFPE specimens and a randomly selected subgroup of approximately 10% of HPV DNA-negative specimens were further analyzed for the presence of HPV E6*I mRNA and for overexpression of the cell-cycle inhibitor p16, which is considered a surrogate marker for HPV infection. The 10% of HPV DNA-negative cases were selected randomly and blindly, while the study was still anonymized.

HPV RNA analysis

Total RNA was purified from three pooled sections of the same tissue block using the Pure Link FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, CA) as described previously (Halec et al., 2013). RT-PCR was carried out using the QuantiTect Virus Kit (Qiagen, Hilden, Germany), in a total volume of 25 µl containing 5 µl of 5xQuantiTect Virus Mastermix, 0.25 µl of 100xQuantiTect Virus RT Mix, 0.4 µM of each oligonucleotide, and 1 µl RNA as described previously (Halec et al., 2013). The HPV type-specific E6*I mRNA assay developed

for 20 HR- or pHR-HPV types [39] was applied for the detection of viral transcripts. The assay amplifies a 65–75 base pair amplicon of HPV and an 81 base pair amplicon of ubiquitin C (ubC) cDNA. Biotinylated amplification products are hybridized to ubC and HPV type-specific probes representing splice junction sequences on Luminex beads, followed by staining with streptavidin-phycoerythrin, and quantified in a Luminex analyzer. The use of a splice product sequence as detection probe makes this assay absolutely specific for RNA and avoids false positivity from residual viral DNA in the RNA preparation, which is a risk in RNA assays assessing unspliced RNA sequences. The analytical sensitivity of the respective assays per reaction is 10,000 copies for HPV70, 1,000 copies for HPV67, and 10–100 copies for the remaining 19 HPV types and for ubC (Halec et al., 2013). The HPV RNA assay has been widely applied and validated as a marker for HPV transformation in carcinoma of the anogenital region, such as the cervix (Halec et al., 2013, Halec et al., 2014), vulva (Halec et al., 2017), penis (Alemany et al., 2016), lung (Anantharaman et al., 2014), and scrotum (Guimera et al., 2017), as well as carcinoma of the head and neck (Castellsague et al., 2016; Albano et al., 2017) and specifically oropharynx (Holzinger et al., 2017, Baboci et al., 2013),unknown primary of the neck (Schroeder et al., 2017; Schroeder et al., 2018), larynx, (Halec et al., 2013) and esophagus (Halec et al., 2016).

All HPV DNA-positive specimens and the randomly selected 10% of HPV DNA-negative specimens were analyzed for the presence of (i) HPV16 E6*I mRNA and (ii) ubC mRNA as a cellular mRNA positive control. Tissues positive for DNA of a non-HPV16 type were, in addition, analyzed for E6*I mRNA of the respective type. Specimens that were HPV E6*I and/or ubC mRNA-positive (RNA+) in RNA analysis were considered HPV RNA valid.

p16 immunohistochemistry

Expression of p16 was evaluated manually by IHC in FFPE sections using the CINtec p16 Histology kit (Roche mtm laboratories AG, Mannheim, Germany) according to the instructions of the manufacturer. Briefly, slides were de-paraffinized in xylene and rehydrated in graded alcohol. The antigens were retrieved for 10 minutes using a pH 9.0 epitope retrieval solution (95–99 ºC), followed by a 20 minute cool-down period at room temperature. Different from the instructions of the manufacturer, specimens were then microwaved in preheated Vector H-3300 unmasking solution (Vector Laboratories, Burlingame, CA) for 15 minutes. This step was followed by incubation of the p16 primary mouse anti-human antibody (clone E6H4) for 60 minutes. The samples were subsequently incubated with the goat anti-mouse IgG secondary antibody/peroxidase conjugate reagent, followed by signal generation using DAB. Finally, slides were counterstained with hematoxylin, dehydrated, mounted with permanent mounting medium, and cover-slipped. Immunoreactivity was visualized by light microscopy. Expression of p16 was evaluated by IHC in all HPV DNA-positive FFPE specimens and in a randomly selected subgroup of approximately 10% of HPV DNA-negative specimens. A continuous, diffuse staining for p16 within the cancer area of the tissue sections was considered as positive, and a focal staining or no staining was considered negative. Positive p16 expression was defined as diffuse nuclear and cytoplasmic staining in 70% or more of the tumor cells. The validity of the p16 IHC staining result was assessed by evaluating the presence of p16 internal control staining. IHC slides were evaluated by RR, FM, and DH blinded to any other clinical information or HPV DNA or RNA status, as specified in the HPV-AHEAD protocol (Mena et al., 2017) Discrepant cases were re-checked by a pathologist, and the final classification of the staining was based on the majority consensus of the working group.

RESULTS

Of the 203 HNSCC cases, 2 cases (1 oral cavity and 1 pharyngeal) were excluded due to insufficient DNA quality as evidenced by negative ß-globin results, and 11 cases were excluded due to invalid RNA and/or p16 data. One case was excluded as the tissue block did not contain cancer tissue. The final study therefore comprised 189 HNSCC patients, with a median age of 62.5 years (range, 35–89 years). The vast majority of the patients were male: n=171 (90.5%) (Table1). Only FFPE blocks where the first and last H&E sections reflected tumor tissue were included in the study. More than 36% of the samples showed >50% of invasive carcinoma in the section, while 47.6% and 15.9% of the samples showed respectively 10–50%, and <10% of tumor cells.

Table 2 shows the HPV DNA, RNA, and p16 detection in HNSCC cases. HPV DNA was detected in 23 of the 189 (12.2%) HNSCC cases. HPV16 was the most prevalent type, being present in 20 of the 23 HPV DNA-positive tumors (86.9%), followed by HPV18 (5/23, 21.7%) and HPV39 (1/23, 4.3%). The oropharynx cases showed higher HPV DNA prevalence (14/28, 50.0%), followed by cancers of the larynx (5/14, 35.7%) and of the oral cavity (4/99, 4.0%). Multiple HPV type infections were detected in 3 HNSCC cases; 2 cases were positive for both HPV16 and HPV18 (1 larynx case and oropharynx case), and 1 oral cancer was positive for both HPV18 and HPV39 (Tables 1 and 2). One larynx case was positive for a low-risk HPV type (HPV6).

Table 1: Description of Romanian HNSCC cases by HR-HPV DNA status

Description	n	HPV DNA-positive n (%)	HPV DNA-negative n (%)
Number of cases	189	23 (12.2)	166 (87.8)
Median age (range), years	62.5 (35–89)	62 (43–86)	63 (35–89)
Sex			
Female	18	2 (11.1)	16 (88.9)
Male	171	21 (12.3)	150 (87.7)
Cancer site			
Oropharynx	28	14 (50.0)	14 (50.0)
Non-oropharynx	162	9 (5.6)	153 (94.4)
Oral cavity	99	4 (4.0)	95 (96.0)
Pharynx	48	0 (0.0)	48 (100)
Larynx*	14	5 (35.7)	9 (64.3)

Row percentages are shown; n, number
*includes hypopharyngeal cancer (n=2)

Table 2: HR-HPV DNA, RNA and p16 positivity in HNSCC subsites by HPV status

		All HNSCC (N=189)	Oropharynx (N=28)	Non-oropharynx (N=161)
HPV type	**Marker positivity**	Positive N (%)	Positive N (%)	Positive N (%)
Any HR-HPV	DNA	23 (12.2)	14 (50.0)	9 (5.6)
	DNA & RNA‡	2 (1.1)	1 (3.6)	1 (0.6)
	DNA, RNA & p16‡	0 (0.0)	0 (0.0)	0 (0.0)
HPV16	DNA	20 (10.6)	12 (42.9)	8 (5.0)
	DNA & RNA	1 (0.5)	0 (0.0)	1 (0.6)
	DNA, RNA & p16	0 (0.0)	0 (0.0)	0 (0.0)
Non-HPV16 HR types	DNA	5 (2.6)	3 (10.7)[1]	2 (1.2)[2]
	DNA & RNA	1 (0.5)	1 (3.6)[3]	0 (0.0)
	DNA, RNA & p16	0 (0.0)	0 (0.0)	0 (0.0)

‡HPV RNA and p16 expression was examined in all HR-HPV DNA-positive cases (n=23) and a randomly selected subset of HR-HPV DNA-negative cases (n=13). All HPV DNA-negative cases were RNA-negative. One case was p16-positive.
[1]Coinfection, HPV16 plus HPV18 (n=1), and single infections, HPV18 (n=2).
[2]Coinfections, HPV16 plus HPV18 (n=1), and HPV18 plus HPV39 (n=1)
[3]HPV18 (n=1)

HPV RNA and p16 expression was examined in all HPV DNA-positive cases (n=23) and a randomly selected subset of HPV DNA-negative cases (n=13). The percentage of HPV-related HNSCCs was 1.1% (2/189) for both HPV DNA and RNA positivity. The highest percentage of combined HPV DNA and RNA positivity was found in the oropharynx (1 of the 28 HPV DNA-positive cases, 3.6%). The corresponding tonsil case tested positive for HPV18. Only one non-oropharyngeal case (1/161, 0.6%) was positive for both HPV DNA and RNA. The corresponding posterior hypopharyngeal wall case tested positive for HPV16 (Table 2).

The p16 IHC data were stratified per HR-HPV DNA and RNA status (Table 3). Only one HPV DNA-positive case (1/23; 4.3%) was p16-positive, and 7.7% (1/13) of HPV DNA- and RNA-negative cases were p16-positive, regardless of the anatomical sub-localization. In addition, none of the HNSCC cases that were HPV DNA- and RNA-positive tested positive by p16 IHC. Moreover, 2 of the 34 HPV RNA-negative cases (5.9%) were p16-positive (Table 3). Among the cases that tested positive for HPV DNA, the smoking status was available for only 10 patients, among whom 8 were current smokers and 2 were former smokers. Most importantly, the clinical information was available for the HPV-driven HNSCCs (n=2). Both tumors (1 tonsil case and posterior hypopharyngeal wall case) were late-stage (III and IV) and were from current smokers: patients aged 54 years (female) and 55 years (male), respectively.

Table 3: p16 IHC data stratified per HR HPV DNA and RNA status

HPV type		**Any HR-HPV DNA-positive** (n=23)				**Any HR-HPV DNA-negative*** (n=13)	
		Any RNA+ (n=2)		Any RNA- (n=21)		Any RNA- (n=13)	
		p16+	p16-	p16+	p16-	p16+	p16-
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
	HNSCC	0 (0)	2 (100)	1 (4.8)	20 (95.2)	1 (7.7)	12 (92.3)
Subsite	Oropharynx	0 (0)	1 (50)	1 (4.8)	12 (57.1)	0 (0)	1 (7.7)
	Non-oropharynx	0 (0)	1 (50)	0 (0)	8 (38.1)	1 (7.7)	11 (84.6)
HPV type		**HPV16 DNA-positive** (n=20)				**HPV16 DNA-negative*** (n=16)	
		HPV16 RNA+ (n=1)		HPV16 RNA- (n=19)		HPV16 RNA- (n=16)	
		p16+	p16-	p16+	p16-	p16+	p16-
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
	HNSCC	0 (0)	1 (100)	1 (5.3)	18 (94.7)	1 (6.3)	15 (93.8)
Subsite	Oropharynx	0 (0)	0 (0)	1 (5.3)	0 (0)	0 (0)	3 (18.8)
	Non-oropharynx	0 (0)	1 (100)	0 (0)	18 (94.7)	1 (6.3)	12 (75)
HPV type		**Other HR-HPV DNA-positive** (n=5)				**Other HR-HPV DNA-negative*** (n=31)	
		Other HR-HPV RNA+ (n=1)		Other HR-HPV RNA- (n=4)		Other HR-HPV RNA- (n=31)	
		p16+	p16-	p16+	p16-	p16+	p16-
		n (%)	n(%)	n (%)	n(%)	n (%)	n(%)
	HNSCC	0 (0)	1 (100)	0 (0)	4 (100)	2 (6.5)	29 (93.5)
Subsite	Oropharynx	0 (0)	1 (100)	0 (0)	2 (50)	1 (3.2)	12 (38.7)
	Non-oropharynx	0 (0)	0 (0)	0 (0)	2 (50)	1 (3.2)	17 (54.8)

*None of the HPV DNA-negative cases were RNA-positive; represents column percentages.

DISCUSSION

It is now well demonstrated that mucosal HR-HPV types, mainly HPV16, are causally involved in a significant proportion of oropharyngeal cancers and to a much lesser extent in a subset of other HNSCCs (Castellsague et al., 2016). However, the contribution of HR-HPV to the carcinogenesis of HNSCC appears to be subject to major geographical variability (Ndiaye et al., 2014). Compared with other European countries, cervical cancer rates are highest in Romania (28.6/100,000), highlighting the importance of HPV infections in this population. However, limited information is available about HPV-associated HNSCC in Romania (Bleotu et al., 2010). Here, we have evaluated the contribution of HPV to HNSCC development in a study in Northeastern Romania by analyzing HPV DNA and HPV RNA status within a large retrospective cohort of HNSCC cases, as well as by determining the p16 expression status. The most frequent HR-HPV type was HPV16, followed by HPV18 and HPV39. The high HPV16 DNA prevalence in this study was similar to other findings published in the literature (Dayyani et al., 2010; Michaud et al., 2014).Approximately 12% of HNSCCs tested positive for HPV DNA, but only 1.1% of all cases (n=2) *were positive for both* HPV DNA and RNA and thus considered as being HPV-driven (Gheit et al., 2017; Boscolo-Rizzo, Pawlita and Holzinger

2016; Rietbergen et al., 2013; Smeets et al., 2007; Boscolo-Rizzo et al., 2015). According to a recent review (Rietbergen et al., 2013), the term HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) refers to carcinomas of the oropharynx presumed to be associated with HPV, on the basis of positivity to HPV DNA and p16 IHC. In this study, only one case tested positive for both markers: HPV DNA and p16. Thus, the fraction of HNSCCs attributable to transforming HPV infections in this Romanian region appeared to be considerably lower compared with various other geographical regions (Ndiaye et al., 2014; Castellsague et al., 2016; Saraiya et al., 2015).

However, the low HPV prevalence in the FFPE samples is in line with the analysis of fresh tumor tissues from Romania, all being HPV-negative (Ribeiro et al., 2011). Both HPV DNA- and RNA-positive cases tested negative for p16. In addition, the great majority (95.6%) of HPV DNA-positive cases were p16-negative. The lack of expression of p16 in HPV DNA- and RNA-positive cases has been reported in other studies (Gheit et al., 2017; Castellsague et al., 2016).

These data contrast with the scenario observed in the Netherlands, where a double positivity for p16 and HPV DNA was shown to be valid to identify HPV RNA-positive cases (Rietbergen et al., 2013; Saraiya et al., 2015), and in Italy, where a fair agreement between HPV16 RNA-positivity and p16 overexpression in oropharyngeal cancer has been reported (Bussu et al., 2013). The absence of p16 expression in HPV DNA-positive HNSCC could be due to the fact that HPV, despite its presence in the tumor, is biologically inactive and is present in the tumor as a passenger virus or viral contaminant. Loss of p16 expression is a frequent event in cancer, and it occurs by deletion, point mutation, or hypermethylation (Kamb et al., 1994; Cairns et al., 1994; Merlo et al., 1995). The inactivation of p16 by hypermethylation of its promotor is common in HNSCC (Choudhury et al., 2015, Rosas et al., 2001, Riese et al., 1999). Hypermethylation of p16 promotor has been reported to be an early event in the development of oral cancer (Sinha et al., 2009; Ruesga et al., 2007). Exposure to certain carcinogens, such as tobacco, may lead to alterations of p16 expression (Gheit T et al., 2017).

Indeed, hypermethylation of the p16 promoters was observed in several smoking-related human cancers, for example in non-small cell lung carcinoma (Georgiou et al., 2007; Zhang et al., 2011; Kim et al., 2001) and cervical squamous cell carcinoma (Lea et al., 2004). In addition, increased methylation of p16 was observed in laryngeal squamous cell carcinoma (Pierini et al., 2014), and in normal oral mucosa (von Zeidler et al., 2004), in smokers. Therefore, loss of p16 expression by hypermethylation in HNSCC, due to smoking or to other exposure factors (Takeshima et al., 2008; Tran et al., 2005), could precede HPV infection, which would not induce p16 accumulation in this specific circumstance. Alternatively, Halec et al. (Halec et al., 2014) suggested that increasing chromosomal instability induced by HPV oncoproteins may lead to the loss of p16 in these cancers. Moreover, one HPV DNA- and RNA-negative case tested positive for p16. A similar result was reported in a recent worldwide HNSCC study, thus suggesting that p16-positivity is not a perfect surrogate for HPV (Castellsague et al., 2016).

A limitation of our study is that information on other HNSCC risk factors (e.g. alcohol consumption, smoking) was available for only a few patients. This limitation was mainly due to the fact that the study implied the retrieval of archived HNSCC specimens, which were often not associated with detailed clinical information. From the available data in clinical questionnaires, 75.6% of the patients declared that they were smokers and 81.3% that they were users of alcohol. According to the latest WHO Report on the Global Tobacco Epidemic, 2015, the smoking prevalence in Romanian male adults was 37.4% (WHO report on the global to bacco epidemic – Romania 2017). This high percentage supports the idea that smoking can be an important risk factor for HNSCC in our study.

Tobacco smoking and alcohol consumption are important risk factors for HNSCC (Sturgis and Cinciripini, 2007;Hashibe et al., 2007). More than 70% of HNSCCs are attributable to tobacco use and alcohol consumption (Hashibe et al., 2007). Cigarette smoking is a strong risk factor

for HNSCC independent of alcohol consumption (Hashibe et al., 2007). The risk of developing laryngeal cancer was 10–20-fold higher in current smokers compared with non-smokers, and a 4–5-fold increased risk was observed for cancers of the oral cavity, oropharynx, and hypopharynx (Tobacco smoke and involuntary smoking. IARC monographs on the evaluation of carcinogenic risks to humans; Vineis et al., 2004; Tuyns et al., 1988). Alcohol consumption alone plays an independent role in approximately 4% of HNSCCs only (Hashibe et al., 2007). However, pooled data from 17 case–control studies in Europe and the USA highlighted a multiplicative joint effect, rather than an additive effect, of tobacco use and alcohol consumption on HNSCC risk (Hashibe et al., 2009).

Another limitation of the study was the limited number of oropharyngeal cancers analyzed. This was mainly due to the fact that the majority of archival HNSCCs were from the oral cavity. The results of this study warrant additional analyses, to describe the risk factors, the natural history and the clinical role of oral HPV infections in Romania.

In conclusion, a very small subset of HNSCC cases within this cohort from Northeastern Romania appeared to be HPV-driven, as evidenced by a low concordance between HPV DNA status and HPV RNA or p16 status of the analyzed HNSCC cases. Our study provides novel insights into the contribution of mucosal HR-HPV types in the development of HNSCC from Northeastern Romania and highlights potential differences in the carcinogenesis of HNSCC in this region compared with other European and non-European countries.

*Bersani C, Haeggblom L, **Ursu RG**, Giusca SE, Marklund L, Ramqvist T, Näsman A, Dalianis T. Overexpression of FGFR3 in HPV-positive tonsillar and base of tongue cancer is correlated to outcome, Anticancer Res, 2018, 38(8): 4683-4690. IF=1.935*

Abstract

Background/Aim. Human papillomavirus positive (HPV^+) tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC) have better outcome than corresponding HPV^- cancers. To better individualize treatment, additional predictive markers are needed. Previously, we have shown that mutated fibroblast growth factor receptor 3 protein (FGFR3) was correlated to poorer prognosis and here FGFR3 expression was analyzed further.

Patients and methods: One-hundred-fifteen HPV^+TSCC/BOTSCC biopsies were analyzed for FGFR3 by immunohistochemistry (IHC), and 109/115 were analyzed for FGFR3 mutations by Ion Proton sequencing, or by Competitive Allele-Specific Taqman PCR **(**CAST-PCR). Disease specific survival (DFS) was then calculated according to FGFR3 IHC expression.

Results: CAST-PCR was useful for detecting the three most common FGFR3 mutations. Focusing especially on the 98/115 patients with HPV^+TSCC/BOTSCC and wild-type FGFR3, high FGFR3 expression correlated to significantly better 3-year DFS, $p<0.043$.

Conclusion: In patients with HPV^+TSCC/BOTSCC, with wild-type FGFR3, overexpression of FGFR3 by IHC was correlated to better DFS.

Introduction

Patients with human papillomavirus positive (HPV^+) tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC) have much better disease free survival (DFS) than those with HPV negative (HPV^-) cancer and most other head neck squamous cell carcinoma (HNSCC) (Mellin et al., 2000; Dahlgren et al., 2004; Attner et al., 2014; Sivars et al., 2014; Dalianis, 2014; Marklund et al., 2014). Also, in many countries the incidences of TSCC/BOTSCC, the oropharyngeal squamous cell carcinoma (OPSCC) subtypes where HPV is most common, have increased (Dalianis , 2014; Marklund et al., 2014; Robinson and Macfarlane , 2003; Hammarstedt et al., 2006; Sturgis and Cinciripini, 2007; Nasman et al., 2009; Braakhuis, Visser and Leemans, 2009; Attner et al., 2010; Chaturvedi et al., 2011; Nasman et al., 2015).

In parallel, HNSCC treatment has been intensified with hyperfractionated radiotherapy, chemoradiotherapy, targeted therapy and surgery. Most HPV^+ TSCC/BOTSCC patients do not need aggressive treatment, and to reduce side effects individualized therapy would of benefit (Dalianis, 2014; Mirghani et al., 2015; 6 Nasman et al., 2017).
To better tailor therapy attempts have been made to find prognostic markers (Ang et al., 2010; Nasman et al., 2013(1); Nasman et al., 2013(2); Tertipis et al., 2014(1); Lindquist D et al., 2014; Tertipis et al., 2014(2); Nordfors et al., 2013; Ramqvist et al, 2015; Rietbergen et al., 2014).
In HPV^+ TSCC/BOTSCC examples of such markers are e.g. age, stage, smoking, high $CD8^+$ tumor infiltrating lymphocyte (TIL) counts, HPV16 E2 mRNA expression, absent/low HLA class I, CD44, LMP10 expression, high expression of LRIG1 or CD98, and absence of HLA-A*02 (Nasman et al., 2013(1); Nasman et al., 2013(2); Tertipis et al., 2014(1); Lindquist D et al., 2014; Tertipis et al., 2014(2); Nordfors et al., 2013; Ramqvist et al, 2015; Rietbergen et al., 2014).
In mathematical models, they can alone, or combined identify 40-56% of patients with high probability for a 3-year DFS (Tertipis et al., 2015, Bersani et al., 2017(1)). However, to recognize more patients that to respond to therapy and to introduce de-escalated or targeted therapy more markers are needed (Dalianis, 2014, Nasman et al., 2017).

To find new prognostic biomarkers, we performed next-generation sequencing (NGS) of hot spot mutations in 50 cancer related genes and found discrepancies between HPV^+ and HPV^- TSCC/BOTSCC (Bersani et al., 2017). HPV^+ TSCC/BOTSCC more frequently had PIK3CA and FGFR3 mutations, while TP53 mutations dominated in HPV^- cancer, similar to data by Tinhofer et al and in HPV^+ TSCC/BOTSCC having FGFR3 mutations was correlated to worse prognosis (Bersani et al., 2017, Tinhofer I et al., 2016)

Here, FGFR3 expression was examined by immunohistochemistry (IHC), in parallel with whether FGFR3 was mutated or not and in relation to outcome. A Competitive Allele-Specific Taqman PCR (CAST-PCR) was used to detect the three most common FGFR3 mutations obtained previously in new samples, and to validate their relation to clinical outcome in another cohort.

Material and methods

Patients and tumor characteristics. Patients diagnosed 2000-2011 at Karolinska University Hospital, with TSCC (ICD-10 code C09.0-9) or BOTSCC (ICD-10 code C01.9), treated with curative intent, were analyzed for FGFR3 IHC (Table 1). Having an HPV^+ TSCC/BOTSCC was defined as having an HPV DNA positive tumor with 16^{INK4A} ($p16^+$) overexpression, or expressing HPV16 E7 mRNA (Smeets et al., 2007). Totally, 115 HPV^+ TSCC/BOTSCC fulfilling these criteria according to our previous studies (Nasman et al., 2009; Nasman et al., 2015; Nordfors et al., 2013; Bersani et al., 2017), were analyzed for FGFR3 IHC and 98 of them had already been tested for their FGFR3 status (Bersani et al., 2017).
To obtain the FGFR3 status of the remaining samples in the IHC analysis, and to compare two methods for detection of FGFR3 mutations (amplification by PCR using the Ion AMpliSeq Cancer Hotspot Panel v2 and CAST-PCR), as well as to test the validity of FGFR3 mutations as a poor prognostic marker, 228 HPV^+ TSCC/BOTSCC samples from Karolinska University Hospital (2000-2016) were tested by CAST-PCR. Furthermore, 20 HPV^- TSCC/BOTSCC cases (from Iasi, Romania) were added for comparison. The study was performed according to permission 2009/1278-31/4 from the Ethical Committee at Karolinska Institutet and permission 3953 (2018) from the University of Medicine and Pharmacy, Grigore T Popa, Iasi, Romania.
Analysis of HPV DNA, and p16 overexpression. HPV DNA status was assayed by a PCR-based bead-based multiplex-assay on a MagPix instrument (Luminex Inc.) as described before (Nordfors et al., 2013, Dalianis et al., 2015).

p16 had been tested for previously using the monoclonal antibody (mAb) clone JC8 (Santa Cruz Biotech, Santa Cruz, California, USA), or the E6H4^{TM} mouse mAb clone (CINtec®, Ventana, Tucson, Arizona, USA) (Nordfors et al., 2013, Dalianis et al., 2015).

Library preparation and sequencing using the Ion Ampliseq cancer hotspot panel v2. Hotspot regions in 50 cancer related genes had been tested for by us in 325 TSCC/BOTSCC by targeted amplification by PCR using the Ion AMpliSeq Cancer Hotspot Panel v2 (CHPv2 - Thermo Fisher Scientific), and of these 98 were included in the FGFR IHC analysis below (Bersani et al., 2017).

Table 1. Patient and tonsillar and base of tongue squamous cell carcinoma characteristics

Patient and tumor characteristics		***Absent/low* FGFR3 expression (N=28)**		***Medium* FGFR3 expression (N=39)**		***High* FGFR3 expression (N=48)**		**All patients with FGFR IHC staining (N=115)**	
		N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Age	*Mean (years)* *Median (years)* *Range (years)*	60 60 42-78		61 60 30-90		58 59 39-78		59.6 59.5 30-90	
Diagnosis	*Base of tongue cancer ICD-10: C01.9*	7	25%	11	28%	13	27%	31	27%
	Tonsillar cancer ICD-10: C09.0, C09.1, C09.8, C09.9	21	75%	28	72%	35	73%	84	73%
Sex	*Male*	22	80%	28	72%	34	71%	83	72%
	Female	6	20%	11	28%	14	29%	32	28%
Tumor differentiation	*Poor*	19	64%	28	72%	33	69%	80	70%
	Moderate	9	36%	11	28%	12	25%	32	28%
	Well	0	0%	0	0%	3	6%	3	3%
Tumor size	*T1*	4	14%	7	18%	18	38%	29	25%
	T2	11	39%	16	41%	18	38%	45	39%
	T3	8	29%	8	21%	1	2%	17	15%
	T4	5	18%	8	21%	11	23%	24	21%
Nodal disease	*N0*	3	11%	7	18%	2	4%	12	10%
	N1	4	14%	5	13%	16	33%	26	23%
	N2a	5	18%	5	13%	4	8%	14	12%
	N2b	13	64%	12	31%	21	44%	46	40%
	N2c	3	11%	5	13%	4	8%	12	10%
	N3	0	0%	2	5%	1	2%	3	3%
	NX	0	0%	2	5%	0	0%	2	2%
Distant metastasis	*M0*	26	93%	39	100%	47	98%	112	97%
	M1	2	7%	0	0%	0	0%	2	2%
	MX	0	0%	0	0%	1	2%	1	1%
Tumor stage (AJCC 8th Edition)	*I*	4	14%	4	10%	18	38%	26	23%
	II	17	61%	22	56%	19	40%	58	50%
	III	5	18%	10	26%	10	21%	25	22%
	IV	2	7%	1	3%	0	0%	3	2.5%
	Unknown	0	0%	2	5%	1	2%	3	2.5%
Treatment	*Induction chemotherapy and radiation (conventional/accelerated)*	9	32%	13	33%	20	42%	42	37%
	Radiation only (conventional/accelerated)	19	68%	26	67%	28	58%	73	63%
Smoking	*Never*	8	29%	18	46%	16	33%	42	37%
	Former (>15 years ago)	4	14%	6	15%	6	13%	16	14%
	Former (<15 years ago)	4	14%	8	21%	7	15%	19	17%
	Current upon diagnosis	12	43%	7	18%	19	40%	38	33%
FGFR3 status	*Wild type*	22	78%	37	95%	39	81%	98	85%
	Mutated	3	11%	1	3%	7	15%	11	10%
	Not tested	3	11%	1	3%	2	4%	6	5%

Competitive allele-specific Taqman PCR (CAST-PCR)

Detection of FGFR3 mutations was performed by Competitive Allele-Specific Taqman® PCR technology (Applied Biosystems). The analysis was performed in 384-well plates, in 10 μL comprising 5 μL 2X Taqman Genotyping Mastermix (Applied Biosystems), 0.2 μL 50X Exogenous IPC template DNA, 1 μL 10X Exogenous IPC mix, 1 μL Mutation Detection Assay, 1.8 μL deionized water and 20 ng DNA (in 1 μL). Runs were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System using the following set of reaction conditions: 95ºC, 10 min followed by 5 cycles at 92ºC, 15 sec and 58ºC, 1 min and 40 cycles at 92ºC for 15 sec and 60ºC for 1 min. The PCR result was analyzed with the SDS 2.3 software program and Mutation Detector Software 2.0 (Life Technologies). Ct was determined for exogenous IPC (Internal Passive Control) reagents added to each reaction to evaluate PCR failure or inhibition in a reaction. The Mutation Detection Assays were Hs00000811_mu, Hs00000812_mu, Hs00001342_mu, which detects variants p.R248C, p.S249C and p.K650Q in FGFR3 gene respectively, and reference assay Hs00001015_rf was used for detection of wild-type FGFR3.

Immunohistochemistry for FGFR3. Evaluation of FGFR3 protein expression by IHC was done on 4 μm FFPE sections with the mouse mAb FGFR3 clone B-9 (Santa Cruz Biotechnology, Heidelberg, Germany), using an avidin-biotin peroxidase method as previously described (Lindquist et al., 2014; Nordfors et al., 2013).

Staining was evaluated by two researchers, including one pathologist, blinded for clinical data. Tumor staining intensity was scored as 0, absent; 1, weak; 2, moderate; and 3, strong, and tumor percentage of staining was assessed to the nearest 10%. For cases where the evaluation differed, a consensus was reached. Cases where the staining was not possible to evaluate were excluded.

Statistical analysis. Categorial variables (FGFR3 IHC and FGFR3 mutation data) were compared with the Chi2-test. Clinical outcome was measured as 3-year DFS or 3-year overall survival (OS). DFS was defined as time from diagnosis until date of relapse in disease and OS defined as time from diagnosis until date of death. Patients never tumor-free were censored on day 0, while patients dying without prior recurrence were censored at the time-point, when assessing DFS. Survival curves with 3-year DFS and OS were calculated using the Kaplan-Meier method and differences in survival were tested using the log-rank test. All statistical tests were performed in STATA (STATA v11, StataCorp, 4905 Lakeway Dr, College Station, TX 77845, USA).

Results

Analysis of FGFR3 mutations in HPV$^+$ TSCC/BOTSCC by CAST-PCR. Totally 115 HPV$^+$ TSCC/BOTSCC samples were evaluated for FGFR3 expression by IHC and the characteristics of the patients and their tumors are depicted in Table 1. Of the HPV$^+$ TSCC/BOTSCC, 98 had been analyzed for by targeted amplification using the Ion AMpliSeq Cancer Hotspot Panel v2, and of these 10 had mutations within the FGFR3 region (Bersani et al., 2017(2)). Of the remaining 17 samples, 1/11 successfully analyzed for FGFR3 mutations by CAST-PCR had an FGFR3 mutation, while for six samples FGFR3 status could not be determined due to lack of material. Thus, by sequencing or CAST-PCR, 11/109 HPV$^+$ TSCC/BOTSCC exhibited FGFR3 mutations, while 98/109 samples were regarded as having wild-type FGFR3.

To analyze the prognostic importance of FGFR3 mutations in a larger set, and since CAST-PCR for analysis of FGFR3 has to our knowledge not been compared to sequencing with the Ion AMpliSeq Cancer Hotspot Panel v2, 228 new HPV$^+$ TSCC/BOTSCC samples (not analyzed for FGFR3 mutations before Bersani C et al., 2017) were tested by CAST-PCR. Totally 196/228 (86%) samples passed the quality requirements, and of these 16/196 (8.6%) exhibited FGFR3 mutations, similar to the 5.7% obtained by sequencing (Bersani et al., 2017(2)), for details see Table 2. The COSM715-p.S249C mutation dominated with a frequency of 5% in the CAST-PCR analyzed cohort as compared to (4.3%) previously obtained by sequencing (Bersani et al., 2017) (Table 2).

Table 2. Samples tested for presence of FGFR3 mutations by CAST-PCR compared to prevalence obtained using sequencing with the Ion AMpliSeq Cancer Hotspot Panel v2

Samples and mutants	No of samples	Percentage by CAST-PCR	Percentage by the Ion AmpliSeq Cancer Hostpot Panel v2[ref]
Samples tested	228		297
Non passed	32	14%	6%
Passed	196	86%	
COSM714 - p.R248C	6	3%	1%
COSM715 - p.S249C	9	5%	4,3%
COSM726 - p.K650Q	1	0,6%	0,35%
Total	16/196	8%	6%

Furthermore, when analyzing previously sequenced HPV^+ TSCC/BOTSCC with 15 FGFR3 mutations identified by CAST-PCR, 13/15, (87%) of these mutations were confirmed, while none of the 16 previously sequenced samples with wild-type FGFR3 had any mutations detected by CAST-PCR (data not shown).

Finally, to confirm that FGFR3 mutations were less common in HPV^- TSCC/BOTSCC, CAST-PCR was performed also in 20 HPV^- TSCC/BOTSCC from Iasi, Romania and all these exhibited wild-type FGFR3. This finding was in concordance with the previous sequencing data showing a very low frequency mutated FGFR3 (1/46, 2.1%) among HPV^- samples (Bersani et al., 2017).

FGFR3 expression in HPV^+ TSCC/BOTSCC analyzed by immunohistochemistry. FGFR3 staining was evaluated as percentage of tumor cells per intensity group (defined as absent/low, medium or strong expression) (Table 1 and Figure 1). Tumor FGFR3 staining was heterogeneous and different staining intensities could sometimes be observed in the same tumor. Tumors were therefore grouped together by their highest intensity staining to create three intensity groups (absent/low, medium and strong). Totally 48 samples were defined having high, 39 samples having medium, and 28 samples having absent/low FGFR3 expression. Among those with wild-type FGFR3, 39 had high, 37 had medium, and 22 had absent/low expression. Among those with mutated FGFR3, seven samples had high, one medium, and three had absent/low FGFR3 expression. There were no major differences in high FGFR3 expression between samples with wild-type or mutated FGFR3 ($p=0.2$).

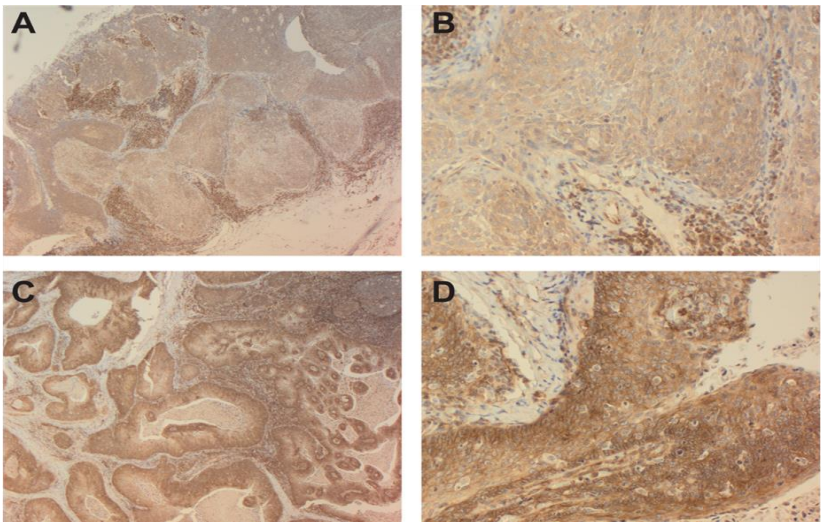


Figure 1. Immunohistochemistry of FGFR3. A and B. High FGFR3 expression in HPV$^+$ TSCC, 4x and 20x respectively; C and D. Low FGFR3 expression in HPV$^+$ TSCC, 4x and 20x respectively.

FGFR3 expression in relation to clinical outcome in HPV$^+$ TSCC/BOTSCC. FGFR3 expression defined by IHC was correlated to outcome defined as 3-year DFS or OS. Initially, attempts were made to evaluate survival for all 115 patients separated into three groups with low, medium and high FGR3 expression in the tumors. This analysis indicated similar survival for patients with low and medium FGFR3 expression, and therefore the latter two were grouped together. Furthermore, it seemed reasonable to analyze survival only among the 98 patients with HPV$^+$ TSCC/BOTSCC and wild-type FGFR3 and exclude the 11 cases with FGFR3 mutations and the six patients with unknown FGFR3 mutation status.

Upon this stratification (39 patients with high and 59 with medium/low FGFR3 expression) a clear picture emerged disclosing a significant correlation between high FGFR3 expression as compared to having medium/low FGFR3 expression and better DFS (log-rank test, p=0.043) (Figure 2). There was however no significant difference between the groups with regard to OS (data not shown). A similar correlation was attempted for the 11 mutated FGFR3 cases, but no specific differences were revealed (log-rank test, p = 0.5).

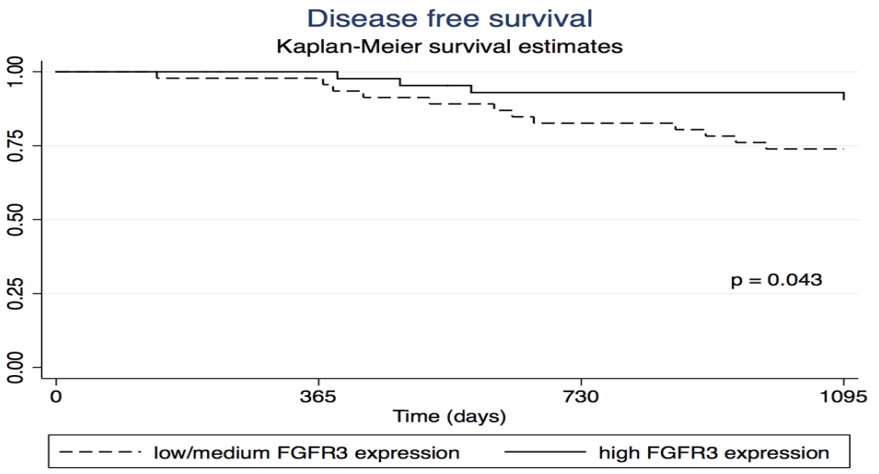


Figure 2. Disease free survival (DSF) for patients with HPV$^+$ TSCC/BOTSCC stratified for low/medium and high FGFR3 expression

Cumulative DSF for HPV$^+$ TSCC/BOTSCC stratified for medium/low FGFR3 and high expression. Differences in survival analyzed with the log-rank test.

FGFR3 mutation status in relation to clinical outcome. Clinical outcome was also examined for the 109 patients with HPV^+ TSCC/BOTSCC, with known FGFR3 wild-type or mutant status. Notably, however 98 of these 109 patients with HPV^+ TSCC/BOTSCC had already been included in a survival analysis, in our previous report, showing that having mutated FGFR3 was correlated to worse DFS (Bersani et al., 2017(2)). After analyzing clinical outcome for all 109 patients with HPV^+ TSCC/BOTSCC in this study, it was still clear that patients having wild-type FGFR3 in their tumors had better DFS than those with FGFR3 mutations in their tumors (log-rank test, p=0.007). An analogous survival analysis was performed for 121 patients having completed a 3-year follow up period among the 196 patients with adequate data obtained in the new CAST-PCR tested cohort. However, no significant correlation between FGFR3 mutation status and survival could be observed.

Discussion

Here FGFR3 expression was analyzed by IHC in a cohort of 115 HPV^+ TSCC/BOTSCC and correlated to outcome in patients with tumors with wild-type FGFR3. High FGFR3 expression was found in 40% of the tumors, and for those with wild-type FGFR3, high expression correlated to better DFS as compared to those with medium/low FGFR3 expression. A similar analysis was not possible to perform in the FGFR3 mutated group, due to few patients.

There is limited information on FGFR3 expression in OPSCC/TSCC/BOTSCC. One report by Koole et al analyzed overexpression of FGFR3, (using the same FGFR3 antibody as in this study) in oral squamous cell carcinoma (OSCC) and OPSCC, and found that FGFR3 was overexpressed in 48% and 59% of OSCC and OPSCC respectively, which is similar to our data (Koole et al., 2016). Nevertheless, there, FGFR3 expression was not correlated to DFS or OS irrespective of whether OPSCC was separated into HPV^+ and HPV^- cases (Koole et al., 2016). Consequently, our two studies differ. However in that study there were only 18 HPV^+ OPSCC cases, and the analysis did not take into consideration whether FGFR3 was of wild-type origin or mutated (Koole et al., 2016). Nonetheless, the authors concluded, similar to us in our sequencing report, that FGFR3 could serve as a therapeutic target for FGFR3-directed therapies (Bersani et al., 2017, Koole et al., 2016). FGFR3 expression has been investigated in more detail in other tumors, especially in e.g. bladder cancer (Bertz et al., 2014; Tomlinson et al., 2007; Gust et al., 2013). Bertz et al. examined the role of angiogenesis and FGFR protein expression in bladder cancer and showed that high FGFR3 expression was correlated to better survival in a multivariate analysis (Bertz et al., 2014). Also there, the authors suggested FGRF3 could be a potential therapeutic target also from the angiogenesis perspective (Bertz et al., 2014). FGFR3 has been studied in brain tumors, and high expression of FGFR3 has been shown to be common in aggressive ependymomas, although it was not likely driven by genetic changes (Lehtinen et al., 2017).

In gliomas strong FGFR3 expression has been detected upon FGFR3 fusions and in another report FGFR3 expression was mainly correlated to the presence of squamous cell carcinoma, but FGFR3 expression was not correlated to survival in any of these tumors (Granberg et al., 2017).

Here, using CAST-PCR we evaluated the presence of the three most common FGFR3 mutations previously observed by sequencing using the Ion AMpliSeq Cancer Hotspot Panel v2 in a new cohort, including also some tumors tested for FGFR3 IHC (Bersani et al., 2017).

In this new cohort we could show that 8.6% of the tumors exhibited FGFR3 mutations. This was insignificantly higher than in our previous study (5.4%) and similar to that (10%) recently published by Mirghani et al. (Nordfors et al., 2013; Mirghani et al., 2018).

The method discovered 87% of the mutations detected before when using the Ion AMpliSeq Cancer Hotspot Panel v2 and did not reveal any additional FGFR3 mutations in "negative control samples", so the compatibility was relatively good between the two methods. Presently

however, we do not have explanation for the discrepancy between these methods with regard to mutations in some tumors.

There are some limitations in this study. First of all, here only three of the most common FGFR3 mutations were tested and although other FGFR3 mutations are rare, they may still be present in the analyzed material. Furthermore, we do not know if some tumors have other genetic changes in FGFR3 e.g. amplifications or translocations. In addition, FGFR3 mutations are not very frequent, and since HPV^+ TSCC/BOTSCC in general have a very favorable outcome, analysis of HPV^+ TSCC/BOTSCC from a large number of patients is required to fully evaluate survival in relation to FGFR3 mutation status. Therefore, with a larger cohort and a longer follow up time it is possible that the obtained results could differ. Nonetheless, the finding that high FGFR3 expression among patients with wild-type FGFR3 was correlated to better DFS may be of clinical value.

FGFR3 has, as indicated above, been suggested to be useful for targeted therapy. However, given the fact that overexpression of wild-type FGFR3 is a prognostic favorable factor some caution may be called for, and further studies are needed before initiating clinical studies on HPV^+ TSCC/BOTSCC.

To conclude, in patients with HPV^+ TSCC/BOTSCC and wild-type FGFR3, overexpression of FGFR3 by IHC was correlated to better DFS. CAST-PCR was efficient in detecting the majority of FGFR3 mutations also detected by Ion AMpliSeq Cancer Hotspot Panel v2, but upon validation of the role of FGFR3 mutations as a prognostic marker, our previous findings were not possible to confirm.

*Haeggblom L[3], **Ursu RG**[2], Mirzaie L, Attoff T, Gahm C, Nordenvall LH, Nasman A. No evidence for human papillomavirus having a causal role in salivary gland tumors, Diagn Pathol, 2018, 13: art. no 44. IF=2.528*

Abstract

Background: Salivary gland malignancies are a very heterogeneous group of cancers, with histologically >20 different subtypes, and prognosis varies greatly. Their etiology is unknown, however, a few small studies show presence of human papillomavirus (HPV) in some subtypes, although the evidence for having a causal role is weak. The aim with this study was to investigate if HPV plays a causal role in the development of different parotid salivary gland tumor subtypes.

Methods: DNA was extracted from 107 parotid salivary gland formalin fixed paraffin embedded tumors and 10 corresponding metastases and tested for 27 different HPV types using a multiplex bead based assay. HPV DNA positive tumors were stained for $p16^{INK4a}$ overexpression by immunohistochemistry.

Results: One of the 107 malignant parotid salivary gland tumors and its corresponding metastasis on the neck were positive for HPV16 DNA, and both also overexpressed $p16^{INK4a}$. The HPV positive primary tumor was a squamous cell carcinoma; neither mucoepidermoid nor adenoid cystic tumors were found HPV positive.

Conclusions: In conclusion, HPV DNA analysis in a large number of malignant parotid salivary gland tumors, including 12 different subtypes, did not show any strong indications that tested HPVs have a causal role in the tested salivary gland tumor types.

Key words: Salivary gland tumors, salivary gland malignancies, human papilloma virus, HPV, tumor viruses

[2] Linnea Haeggblom and Ramona Gabriela Ursu contributed equally to this work.

1. Introduction

Salivary gland malignancies are a very heterogeneous group of cancers, where histologically more than 20 different subtypes have been identified and prognosis varies greatly (Gillespie, Albergotti and Eisele, 2012). These tumors are rare, yet affect patients in all age groups and are mainly found in the parotid gland, but can also be found in submandibular, sublingual and accessory parotid glands (Barnes, Everson and Peter Reichart, 2005; Cancercentrum R. Nationellt vårdprogram Huvud- och halscancer. 2015). In Sweden about 100 new cases of salivary gland cancers are diagnosed each year, making up about 8% of head and neck cancers (Cancercentrum R. Nationellt vårdprogram Huvud- och halscancer. 2015), which is similar in the United States with 6% of head and neck cancers (Barnes, Everson and Peter Reichart, 2005). Today the determination of tumor subtype is mainly based on the pathologist's histological evaluation and is, due to the large histological variability in this group of tumors, a difficult task and the diagnoses are often set with uncertainty. As a supportive measure genetic profiling is used in some cases, however the sensitivity of the tests vary greatly. Moreover, it may often be with great uncertainty the prognostic tumor grade is determined, which is directly correlated to treatment strategy. The most common treatment strategy is surgery sometimes followed by postoperative radiation therapy (Barnes, Everson and Peter Reichart, 2005; Cancercentrum R. Nationellt vårdprogram Huvud- och halscancer. 2015). It has been well established by us and others that human papillomavirus (HPV) plays a causative role in a large proportion of oropharyngeal squamous cell carcinomas (OPSCC) (Dahlstrand and Dalianis, 2005; International Agency for Research on Cancer. IARC monographs on the evaluation of carcinogenic risks to humans, 2007; Gillison et al., 2000). It is also well established that HPV can be valuable as a prognostic tool, since patients with HPV-positive OPSCC have a much better clinical outcome compared to patients with HPV-negative OPSCC (Bersani et al., 2017).

However, whether HPV has a causal effect in salivary gland carcinomas is not as certain. A recent study indicated that there are major differences in mutation frequencies between different salivary gland tumor subtypes, where none or few mutations were found in mucoepidermoid, adenocystic and acinic cell subtypes (Lindquist et al., 2007), suggesting that other factors such as e.g. a viral infection may be the causative factor. There are a handful small studies that have shown an association between HPV infection and certain salivary gland malignancies, however, reported HPV prevalence varied to a great extent (Grünewald et al., 2015; Isayeva et al., 2012; Boland et al., 2012; Vageli et al., 2007; Skalova et al., 2013; Hafed et al., 2012; Lin et al., 2014; Qian et al., 2016). Moreover, these studies included only a small sample size, and most studies only included one or few specific subtypes of salivary gland tumors with very few samples in each subgroup, thus missing proper control groups. In this study we investigated the occurrence of HPV (defined as being HPV DNA positive and overexpressing $p16^{INK4a}$) in, to our knowledge, the largest cohort of parotid salivary gland tumors, including 12 different subtypes, in patients diagnosed between 2000-2009 in the counties of Stockholm and Gotland.

2. Patients, materials and methods

2.1. Patients and tumor samples

Between 2000-2009, 145 patients in the counties of Stockholm and Gotland were identified and diagnosed with the following types of malignant salivary gland tumors: acinic cell carcinoma, adenocarcinoma not otherwise specified (NOS), adenoid cystic carcinoma, basal cell adenocarcinoma, epithelial-myoepithelial carcinoma, mucoepidermoid carcinoma, myoepithelial carcinoma, oncocytic carcinoma, poorly differentiated carcinoma, salivary duct carcinoma, secretory carcinoma and squamous cell carcinoma (ICD-10: C07.9). From these,

haematoxylin and eosin-stained tumor sections were histologically evaluated to confirm presence of malignant cells and diagnosis. In total 117 biopsies (including 10 corresponding metastases) from 107 unique patients had sufficient tumor material for inclusion in this study and were collected as formalin fixed paraffin embedded (FFPE) tumors from the Pathology Department at Karolinska University Hospital. Sections from the FFPE tumors were taken for DNA extraction and immunohistochemistry (IHC) staining.

2.2. DNA extraction and detection of HPV

DNA extraction and detection of HPV

DNA was extracted from 10 μm FFPE tumor tissue sections using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's suggested protocol. In short, the FFPE sections were dissolved in Xylene followed by rehydration in absolute ethanol. After protein degradation the samples were purified in several steps using spin columns, thereafter the purified DNA was stored at − 20 °C. For each tumor sample a blank paraffin section was included as a negative control in order to detect possible cross-contamination between samples. Presence of HPV DNA was detected, following a 40 cycle PCR amplification using the QIAGEN multiplex PCR kit (Qiagen, Hilden, Germany) together with broad-spectrum GP5 +/6+ primers (Cybergene, Stockholm, Sweden) (Schmitt et al., 2008). PCR products were then hybridized to specific probes for 27 different HPV types (HPV 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73 and 82) and evaluated on a Magpix instrument (Luminex Corporation, Austin, USA) for the different HPV types as previously described (Nordfors et al., 2013; Du et al., 2012). β-globin was included as a positive control for presence of DNA as described previously (Dalianis et al., 2015). In addition, negative controls with distilled water and positive controls with SiHa cells corresponding to 5, 50 and 500 HPV genomes were also included and treated per protocol.

p16INK4a overexpression

The tumor that tested positive for HPV was also evaluated for p16INK4a tumor suppressor protein (p16) overexpression. p16 overexpression was determined by IHC using the mouse monoclonal antibody CINtec® p16 Histology (clone E6H4, Ventana, Roche Diagnostics, Basel, Switzerland), as previously described (Dalianis et al., 2015). The sections were evaluated by a pathologist, where a ≥ 70% stained tumor was considered as positive for p16 overexpression, and < 70% as negative.

2.3. Statistical analysis

Fishers exact test was used to compare HPV numbers per sub-group of salivary gland tumors.

3. Results

In total 117 tumor samples, including 10 corresponding metastases, from 12 different salivary gland tumor types, were analyzed for presence of HPV DNA (Table 1). All samples were positive for the β-globin gene used as a positive control, indicating successful DNA purification. One out of the 107 tested malignant salivary gland primary tumors was positive for HPV16, which was 1/7 salivary gland squamous cell carcinomas included in this study. This tumor also showed p16 overexpression. HPV was not significantly more common in squamous cell carcinomas as compared to the other subgroups (Table 1). Moreover, the HPV16 and p16

positive tumor had a corresponding metastasis located on the neck, which also was positive for HPV16 and p16.

Table 1. Patient and tumor characteristics

Histological subtype[1]	**Tumors** (N)	**Age** (mean)	**TNM-stage**[2] *T*				*N*			*M*		**Available regional metastasis**	**HPV DNA + primary tumors**[3]	**HPV DNA + regional metastasis**[3]
			T1	**T2**	**T3**	**T4**	**N0**	**N1**	**N2**	**M0**	**M1**			
Acinic cell carcinoma	22	54	9	10	2	1	22	0	0	22	0	-	0	0
Adenocarcinoma, UNS	19	66	3	8	2	6	13	2	4	17	2	4/6	0	0
Adenoid cystic carcinoma	13	55	5	6	1	1	13	0	0	1	2	-	0	0
Basal cell adenocarcinoma	3	75	2	0	1	0	3	0	0	3	0	-	0	0
Epithelial-myoepithelial carcinoma	4	75	1	2	1	0	4	0	0	4	0	-	0	0
Mucoepidermoid carcinoma	22	47	11	9	0	2	21	1	0	21	1	0/1	0	0
Myoepithelial carcinoma	2	53	0	2	0	0	1	0	1	1	1	1/1	0	0
Oncocytic carcinoma	1	65	0	1	0	0	1	0	0	1	0	-	0	0
Poorly differentiated carcinoma	3	85	0	1	1	1	1	1	1	3	0	1/2	0	0
Salivary duct carcinoma	10	57	2	3	1	4	4	2	4	9	1	2/6	0	0
Secretory carcinoma	1	85	1	0	0	0	1	0	0	1	0	-	0	0
Squamous cell carcinoma	7	56	2	2	3	0	4	1	2	7	0	2/3	1[4,5]	1[4]
Total			107		59								10	1

[1] Histological subtype according to WHO 2017
[2] TNM-stage according to AJCC 7th Ed
[3] Human papillomavirus DNA positivity (HPV DNA+) as assessed by presence of HPV DNA by Luminex Multiplex PCR
[4] Primary tumor and regional metastasis obtained from the same patient
[5] Fischer exact test for HPV positivity in Squamous cell carcinoma v.s, all other sub-types: p = 0.06

4. Discussion

In this study, 107 malignant salivary gland tumors, including 12 different subtypes, with the addition of 10 corresponding metastases were tested for the presence of 27 different HPV types (including all high-risk HPV types) using a multiplex bead-based assay. One of the 107 malignant salivary gland tumors and its corresponding metastasis on the neck were positive for HPV16 DNA, and both also overexpressed p16 and therefore could be defined as being HPV positive. All other subtypes tested were HPV DNA negative. In total, only one of seven squamous cell carcinoma salivary gland tumors (6,5%) was HPV positive and due to the small sample size of squamous cell salivary gland tumors it was not possible to draw any safe conclusion to whether HPV was involved in the development of this tumor type. Furthermore, all other subgroups lacked high risk HPV DNA and did not express presence of any of the other tested HPV types, suggesting that HPV does not play a major role as a causative agent in these subgroups. The latter was shown in a more limited study by Skalova et al. (Skalova et al., 2013). Nonetheless, HPV positive head and neck cancers often present with an HPV positive neck metastasis, and it has been shown that HPV positive cancer of unknown primary of the head and neck region often originate from an HPV positive OPSCC (Sivars et al., 2014; Begum et al., 2003).

Hence there is also a possibility that the HPV16 positive squamous cell salivary gland carcinoma found in this study was instead a primary tumor, or a cancer of unknown primary originating from an undetected OPSCC. This would further support that HPV does not play a major role for the development of salivary gland tumors. However, the clinical evaluation in this case did not support such an assumption.

There are some limitations to this study. Firstly, salivary gland tumors are a rare and heterogeneous group of tumors including more than 20 different subtypes, making it difficult to gather representable amounts of material for this type of analysis. Furthermore, although, to our knowledge, the presence of HPV was analyzed in the largest cohort published so far, the number of samples per subgroup was still limited, which makes it difficult to draw any clear conclusions. Secondly, the presence of p16 overexpression in samples was only tested on the one sample, and its corresponding metastasis, that both had tested positive for HPV DNA. Nevertheless, previous studies have shown p16 overexpression in malignant salivary gland tumor cohorts, still lacking or having a low correlation to presence of HPV (Isayeva et al., 2012, Vageli et al., 2007).

These findings may also be indicative of HPV not playing the cancer-driving role and that p16 in these tumors is a bad surrogate marker for an active HPV infection. Lastly, the HPV16 positive tumor found in this study was one out of seven squamous cell carcinomas, which is a very rare type of salivary gland tumor. With a larger cohort of squamous cell salivary gland carcinomas, perhaps more conclusive results could be drawn to whether HPV is a causative agent in the development of these tumors or not.

5. Conclusions

In conclusion, HPV DNA analysis in a large number of malignant salivary gland tumors, including 12 different subtypes, did not show any strong indications that tested HPV types have a causal role in the tested salivary gland tumor type.

*Haeggblom L, Ahrlund-Richter A, Mirzaie L, da Silva PFN, **Ursu RG**, Ramqvist T, Nasman A. Differences in gene expression between high-grade dysplasia and invasive HPV+ and HPV- tonsillar and base of tongue cancer, Cancer Med-US, 2019, 8(14): 6221-6232. IF=3.491*

Abstract

Background: Human papillomavirus (HPV) is a causative agent for tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC), as well as for cervical cancer. Premalignant stages in cervical cancer have been studied extensively, while little is known about premalignant stages in TSCC/BOTSCC and the role of HPV. Here we analyzed differences in gene and protein expression between high-grade dysplasia and invasive cancer in both HPV-positive (HPV+) and HPV-negative (HPV−) TSCC/BOTSCC.

Methods: High-grade dysplasia and invasive carcinoma were laser microdissected from HPV+ and HPV− TSCC/BOTSCC tumor sections. Differential gene expression was studied utilizing nanoString RNA-panels and genes of interest were validated on the protein level by immunohistochemistry.

Results: Forty genes in the HPV+ tumors showed significantly different expression between high-grade dysplasia and invasive cancer and 33 genes in the HPV− tumors. Five out of the nine most significant pathways showed similar increased activity in invasive cancer as compared to high-grade dysplasia in both HPV+ and HPV− tumors. Lastly, significant differences in protein expression was confirmed for SPARC, psoriasin, type I collagen and galectin-1 in both HPV+ and HPV− tumors.

Conclusions: This is to our knowledge the first study disclosing differences and similarities in gene expression between dysplastic and invasive HPV+ and HPV− TSCC/BOTSCC.

KEYWORDS

base of tongue cancer, cancer in situ, cancer progression, high-grade dysplasia, human papillomavirus, invasive carcinoma, oropharyngeal cancer, premalignant stages, tonsillar cancer

BACKGROUND

Previous studies by us and others have demonstrated an epidemic increase in the incidence of tonsillar and base of tongue squamous cell carcinoma (TSCC and BOTSCC), and this has been attributed to an increase in human papillomavirus (HPV) infection.1-8

IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 90, Human Papillomaviruses, 2007; Gillison et al., 2000; Lindquist et al., 2007; Attner et al., 2011; Nasman et al., 2015; Nasman et al., 2009; Hocking et al., 2011; Chaturvedi et al., 2013). Moreover, patients with HPV positive (HPV+) TSCC and BOTSCC are generally younger and do not usually present with the classical head and neck squamous cell carcinoma (HNSCC) risk factors, for example, smoking and alcohol abuse (Grisar et al., 2016; Blitzer et al., 2014). However, despite an epidemic increase in a younger patient population, no screening methods, in line with those for cervical cancer, have been developed.

The absolute majority of all carcinomas develop in a similar manner, starting with dysplasia followed by highgrade dysplasia/cancer in situ, which eventually progresses into an invasive cancer and thereafter becomes metastatic. High-grade dysplasia/cancer in situ refers to the earliest stage of cancer when the cancerous growth is still contained by the basal membrane and has not invaded the surrounding tissues or spread to other organs in the body (Weinberg, 2007). In cervical carcinoma, the premalignant and malignant stages are very well characterized and divided into low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) (former cervical intraepithelial neoplasia [CIN] 1-3 grades) and invasive cancer. Analogous stages are also found in other HPV-associated anogenital carcinomas (Waxman et al., 2012).

A similar, yet less studied, scenario is also observed in the epithelium coating the tonsils and the base of tongue, where occasionally premalignant lesions are found close by the invasive tumor. However, it has been debated if pure premalignant lesions occur in HPV+ TSCC or BOTSCC (Fossum et al., 2017, Begum et al., 2015, Palmer et al., 2014, Holmes and Wenig, 2019).

Increased knowledge of genes involved in tumor invasion mechanisms and identification of new progression markers could potentially in the future improve and personalize treatment and/or facilitate development of new strategies for early detection and preventative measures. Due to its rarity, no studies have, to our knowledge, been performed on differences between premalignant and invasive lesions in HPV+ TSCC and BOTSCC. Instead, a handful of studies have been performed on premalignant changes in HPV+ cervical carcinomas. In two independent studies by Gius et al and den Boon et al, differences in gene expression in the different stages of premalignant lesions (CIN1-3) leading up to cervical cancer were examined, and both studies show specific changes displayed from stage-to-stage (Gius et al., 2007; den Boon et al., 2015).

However, no comparisons between HPV+ and HPV negative (HPV−) precancerous and invasive tumors were possible in these studies, since >99% of all cervical carcinomas are HPV+ (Herrington, 1999, Burd, 2003).

Moreover, it is imperative for the patient treatment and prognosis to distinguish dysplasia/cancer in situ from an invasive carcinoma. Whearas an invasive tumour diagnosis most often will result in heavy oncological treatment and/or radical surgery, a diagnosis of cancer in situ/severe dysplasia diagnosis may lead to surgical extirpation, but not with extensive margins, and only clinical follow-up. Normally, the distinction between premalignant and invasive disease is unproblematic, but there are cases, especially in head neck cancer pathology when the diagnosis is vague. Therefore, some invasion markers, for example, Laminin-5 (Skyldberg et al., 1999) have been proposed, but these markers have all very poor sensitivity and specificity in the head and neck area. Therefore, there is an urgent need for new clinical invasion markers in head and neck pathology, for better and safer diagnosis of invasive disease.

In this study we aimed, for the first time, to examine and better understand differences between HPV+ and HPV− preinvasive TSCC and BOTSCC lesions and invasive tumors by laser capture microdissection (LCM) of the malignant epithelial cells followed by gene analysis utilizing the PanCancer Progression Panel (NanoString). In addition, we also aspired to identify usable clinical invasion markers by visual verification of obtained RNA data on protein level by immunohistochemistry (IHC).

2. METHODS

2.1 Patients and tumor samples

In this study 24 TSCC and BOTSCC (ICD-10: C09.0, C09.1,C09.8, C09.9, C01.9) biopsies were selected from a larger consecutive cohort diagnosed at Karolinska university hospital 2007-2015.5,22 All biopsies, previously tested for both presence of HPV DNA and p16INK4a (p16) expression, (Nasman et al., 2015; Haeggblom et al., 2018) were histologically evaluated and selected for containing both high-grade dysplasia/cancer in situ and invasive carcinoma within the same formalin fixed paraffin embedded (FFPE) section. Thirteen HPV driven tumors, being both HPV DNA positive and p16 positive (HPV+p16+) were identified from the cohort and 11 HPV DNA negative and p16 negative (HPV−p16−) tumors, thus driven by other factors, were selected. From these 24 samples, the six HPV+ and HPV− patient samples with highest quality and satisfactory amount of RNA were used in the RNA expression assay (please see below). Differentially expressed genes were then validated in all 24 samples by IHC. For more details about the study subjects see Supplementary Tables S1 and S2. The study was performed according to approval 2009/1278-31/4 from the Regional Ethics Committee, Karolinska Institutet.

2.2 Tumor selection and microdissection

Hematoxylin/eosin stained FFPE tumor sections were histologically examined by three independent researchers (LH, PFNS, and AN) and selected for containing sufficient amounts of high-grade dysplasia/cancer in situ as well as invasive cancer (Figure 1). FFPE biopsies with distinct differences for dysplasia and invasive carcinoma were sectioned (six 5 µm-sections per sample) by microtome with RNase-free water and mounted on Membrane Slides PENMembrane 2.0 µm (No.11505158) (Leica Microsystems AB). Tissue sections were deparaffinized for 2 × 15 minutes in fresh xylene followed by rehydration in decreasing percentages of fresh ethanol (5 minutes each in 100%, 95%, 70%, 50%, 0% ethanol) and thereafter stained with fresh hematoxylin for 30 seconds. On a Leica LMD 7000 microscope (Leica Microsystems AB), using the Laser Microdissection System (version 7.6.5684) the dysplastic and invasive carcinoma areas of the six replicate tumor sections were laser micro dissected and collected separately in microcentrifuge tubes containing PKD buffer from the RNeasy FFPE kit (Qiagen).

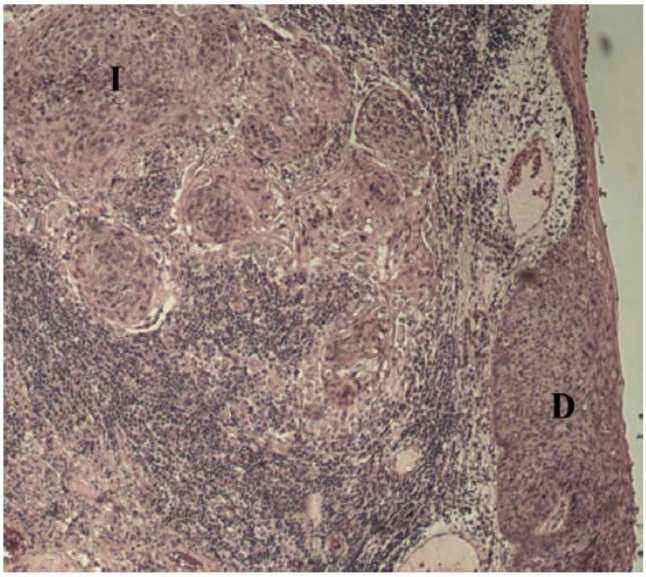


FIGURE 1 Histological representation of contained high-grade dysplasia/cancer in situ (D) and invasive carcinoma (I)

2.3 RNA extraction and multiplex gene expression analysis

Samples were kept on ice whenever possible and RNA was extracted immediately upon laser microdissection to ensure best RNA quality, using the RNeasy FFPE kit (Qiagen) according to manufacturer's instructions. RNA concentration was measured on Qubit 4 Fluorometer (Thermo Fisher Scientific) using the Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific). Twelve samples (six HPV+p16+ tumors and six HPV−p16− tumors) with sufficient RNA concentrations (>2 ng/µL) were selected for the multiplex gene expression assay. Due to low RNA concentrations from FFPE material, the nCounter Low RNA Input Amplification Kit (NanoString Technologies) was used for the cDNA conversion and multiplex target enrichment steps. Thereafter the hybridization reactions were set up using provided master kit and progression primers needed for the nCounter PanCancer Progression Panel (NanoString Technologies). The gene expression assay was performed according to manufacturer's instructions utilizing the nCounter® Sample Prep Station with FLEX configuration (NanoString Technologies) as well as the nCounter® Digital Analyzer 5s (NanoString Technologies) for reading the samples. For data analysis the nSolver Analysis Software (version 4.0) was used, including the nCounter Advanced Analysis add-on software (version

2.0.115). More specifically, fold changes and P-values were calculated using nCounter default settings. Pathway scores were calculated using measurements of genes included in 34 different pathways, using the nCounter Advanced Analysis Software (Tomfohr, Lu and Kepler, 2005).

2.4 Immunohistochemistry evaluation of differentially expressed genes

Six of the top differentially expressed genes between highgrade dysplasia/cancer in situ and invasive carcinoma, identified from the multiplex gene expression analysis, were further evaluated for protein expression by IHC. FFPE sections from the 24 TSCC/BOTSCC biopsies were stained by IHC with six different antibodies as per standard protocol. Further details about antibodies and methodologies are presented in Supplementary Table S3. Stained tissue sections were histologically evaluated by light microscopy by three trained scientists (LH, AN, LM) as described separately for each antibody in Supplementary Table S3. Evaluation scores are presented in Supplementary Table S4.

2.5 Statistical analysis

Nanostring data and pathway analysis are described above. Differences in protein expression were assessed with the Wilcoxon signed rank-test. Two sided P-values were reported.

3 RESULTS

3.1 Gene expression and pathway analysis of high-grade dysplasia and invasive TSCC and BOTSCC

Gene expression was measured in six HPV+ and six HPV−TSCC and BOTSCC samples from a panel of 770 cancer progression related genes (PanCancer Progression Panel, NanoString). In total, 40 genes in the HPV+ tumors and 33 genes in the HPV− tumors showed differential expression ($P < .05$), comparing high-grade dysplastic epithelium and invasive cancer. Significant genes are presented in Figure 2 for both HPV+ and HPV− tumors. Of these genes, 10 were common for both HPV+ and HPV− tumors. More specifically, the genes COL3A1, COL1A1, COL1A2, SPARC, COL6A3, AEBP1, COL6A2, and VIM were significantly upregulated, whereas S100A7 and TACSTD2 were significantly down-regulated, in invasive cancer as compared to the dysplastic epithelium (Figure 2). Consequently, 30 genes differed between high-grade dysplasia vs. invasive cancer uniquely for HPV+ samples. The following five genes, FN1, MS4A4A, POSTN, LUM, and SPP1, were the most significantly up-regulated, in invasive cancer as compared to the dysplastic epithelium. Whereas IL1RN, CXCL8, ID1, ECM1, and IL6, were the five most significantly down-regulated genes in invasive cancer as compared to the dysplastic epithelium. For more detailed data see Figure 2.

Exclusively for HPV− cases 23 genes differed between high-grade dysplasia and invasive cancer. The following five genes, LAMB3, TIMP2, COL5A2, DPYSL3, and LAMC2, were the most significantly up-regulated, in invasive cancer as compared to the dysplastic epithelium. Whereas CEACAM5, CDK14, NOS3, HGF, and PPP1R16B, were the five most significantly down-regulated genes in invasive cancer as compared to the dysplastic epithelium (Figure 2).

Upon further evaluation of the above mentioned genes, a more stringent cut-off was applied in order to exclude samples with a low count value, resulting in that 19 genes in the HPV+ samples (Figure 3A) and 32 in the HPV− samples (Figure 3B) presented with differentially expressed genes. To characterize the effect of altered gene expression on various cancer progression pathways, the pathway analysis tool provided in the nSolver Advanced Analysis Software was utilized. This tool condenses each sample's gene expression profile to calculate a pathway score using a first principal component analysis (Tomfohr, Lu and Kepler, 2005).

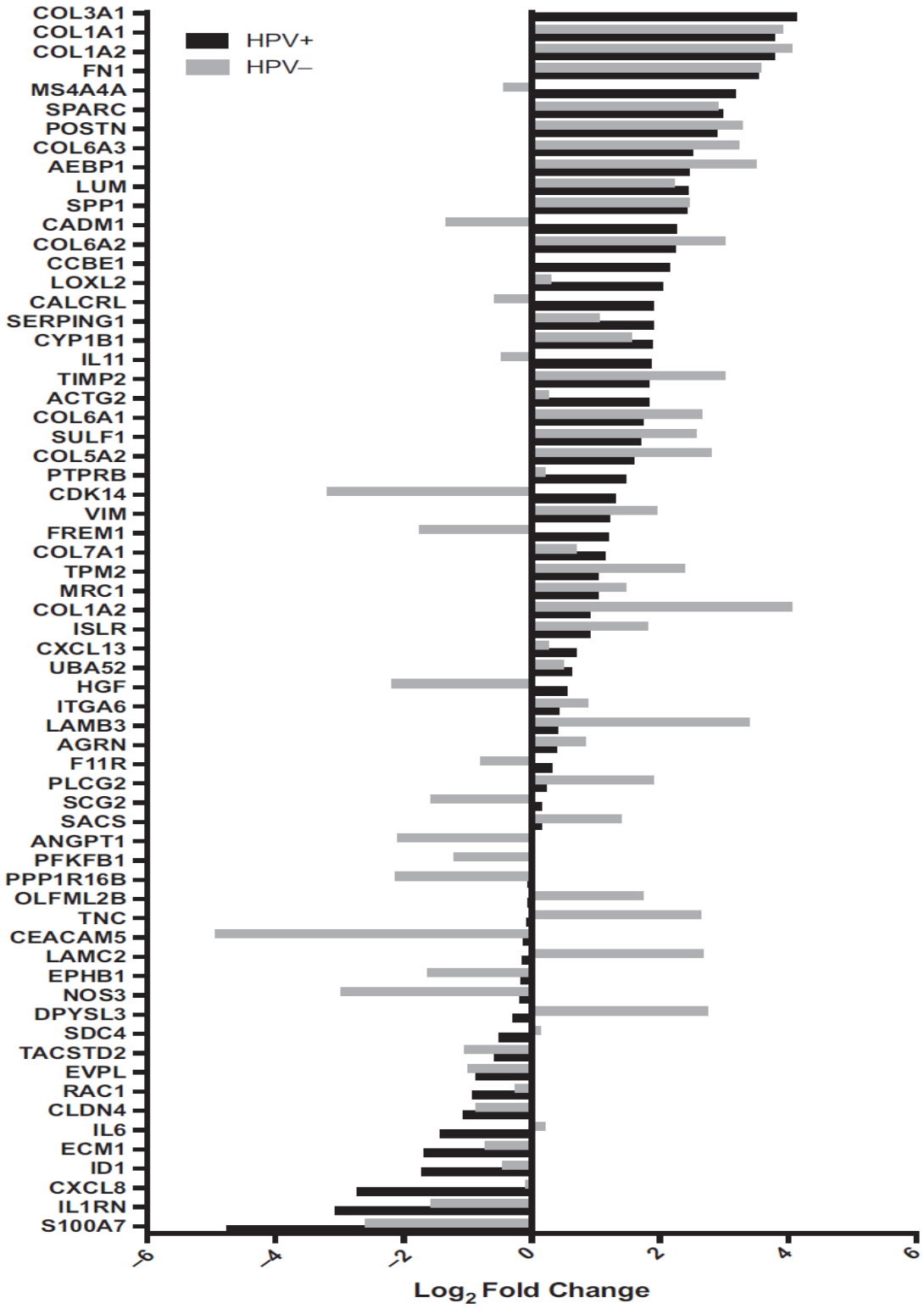
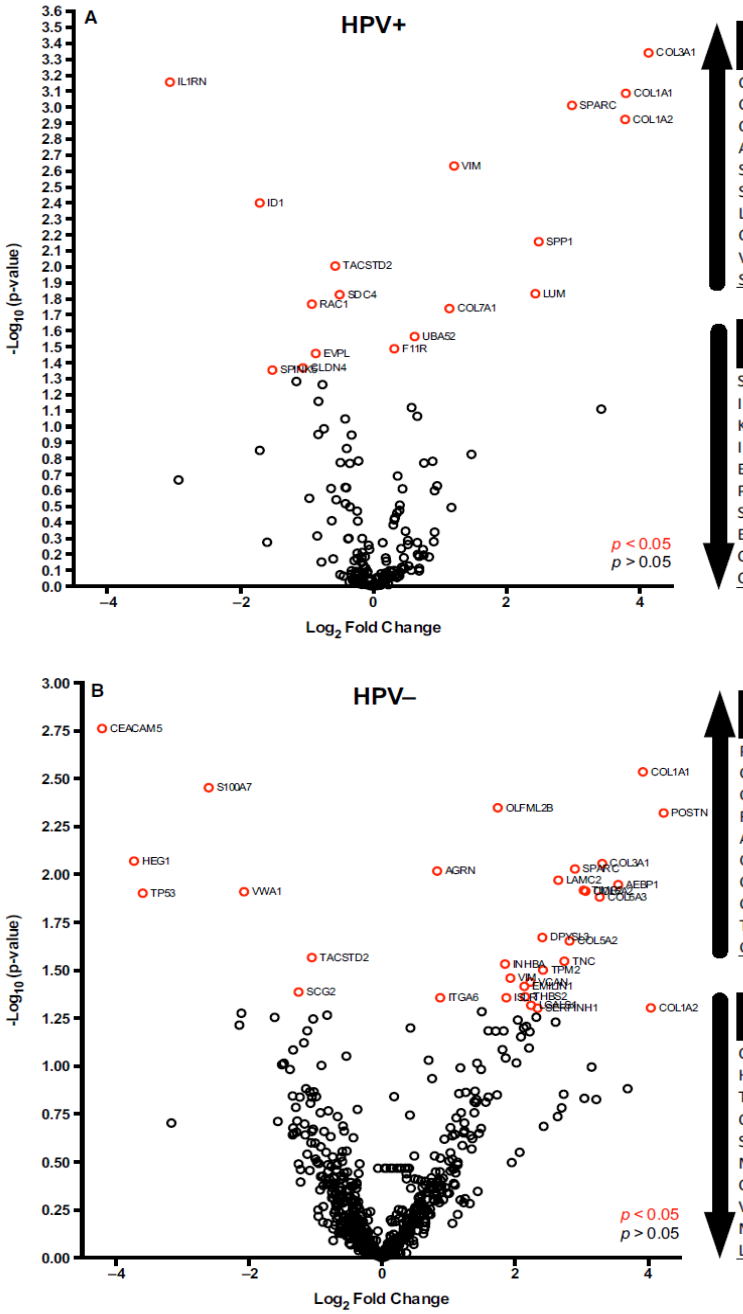


FIGURE 2 Log2 fold change difference between high-grade dysplasia and invasive carcinoma comparing statistically significant differences ($P < .05$) in mRNA expression in the HPV+ and HPV− samples separately. All genes showing a positive fold change value have a higher expression in invasive carcinoma compared to high-grade dysplasia, and genes showing a negative fold change value have a lower expression in invasive carcinoma compared to highgrade dysplasia.



Gene name	In situ vs. Invasive linear fold change	log2 fold change	*P*-value
COL3A1	17.5	4.13	0.000
COL1A1	13.9	3.79	0.001
COL1A2	13.7	3.78	0.001
APOE	10.7	3.42	0.078
SPARC	7.91	2.98	0.001
SPP1	5.58	2.48	0.007
LUM	5.39	2.43	0.015
COL4A1	2.78	1.47	0.149
VIM	2.31	1.21	0.002
SERPINH1	2.26	1.17	0.321

Gene name	In situ vs. Invasive linear fold change	log2 fold change	*P*-value
S100A7	0.03	−4.85	0.031
IL1RN	0.12	−3.06	0.001
KRT14	0.13	−2.93	0.216
ID1	0.31	−1.71	0.004
ECM1	0.31	−1.71	0.141
PPL	0.33	−1.60	0.530
SPINK5	0.35	−1.52	0.044
EPHA2	0.45	−1.16	0.052
CLDN4	0.48	−1.06	0.043
CLU	0.51	−0.97	0.281

Gene name	In situ vs. Invasive linear fold change	log2 fold change	*P*-value
POSTN	18.8	4.23	0.005
COL1A2	16.5	4.04	0.050
COL1A1	15.1	3.92	0.003
FN1	12.9	3.69	0.131
AEBP1	11.7	3.55	0.011
COL3A1	9.89	3.31	0.009
COL6A3	9.65	3.27	0.013
COL5A1	9.32	3.22	0.149
TGFBI	8.91	3.15	0.101
COL6A2	8.36	3.06	0.012

Gene name	In situ vs. Invasive linear fold change	log2 fold change	*P*-value
CEACAM5	0.05	−4.20	0.002
HEG1	0.08	−3.72	0.009
TP53	0.08	−3.59	0.013
CXCL17	0.11	−3.16	0.198
S100A7	0.17	−2.60	0.004
MS4A4A	0.23	−2.14	0.061
CEACAM1	0.23	−2.11	0.053
VWA1	0.24	−2.07	0.012
MUC1	0.33	−1.61	0.056
LRG1	0.34	−1.56	0.194

FIGURE 3 Gene expression in high-grade dysplasia and invasive carcinoma in HPV+ and HPV− tonsillar and base of tongue squamous cell carcinoma separately. (A) Volcano plot showing differentially expressed genes between high-grade dysplasia and invasive carcinoma by log2 fold change (x-axis) and −log10 of P-value (y-axis) in HPV+ tonsillar and base of tongue squamous cell carcinoma cases. The top 10 genes with increased and decreased linear fold change as well as log2 fold change expression between high-grade dysplasia and invasive carcinoma are together with P-values presented in the tables to the right. (B) Same as in (A), here however with HPV− tonsillar and base of tongue squamous cell carcinoma cases.

Notably, among the nine most significant pathways, there was a similar increased activity in invasive cancer as compared to high-grade dysplasia in both HPV+ and HPV− tumors in five

pathways: metastasis response, extracellular matrix (ECM) receptor interaction, cellular growth factor, collagen family, and ECM structure (Figure 4).

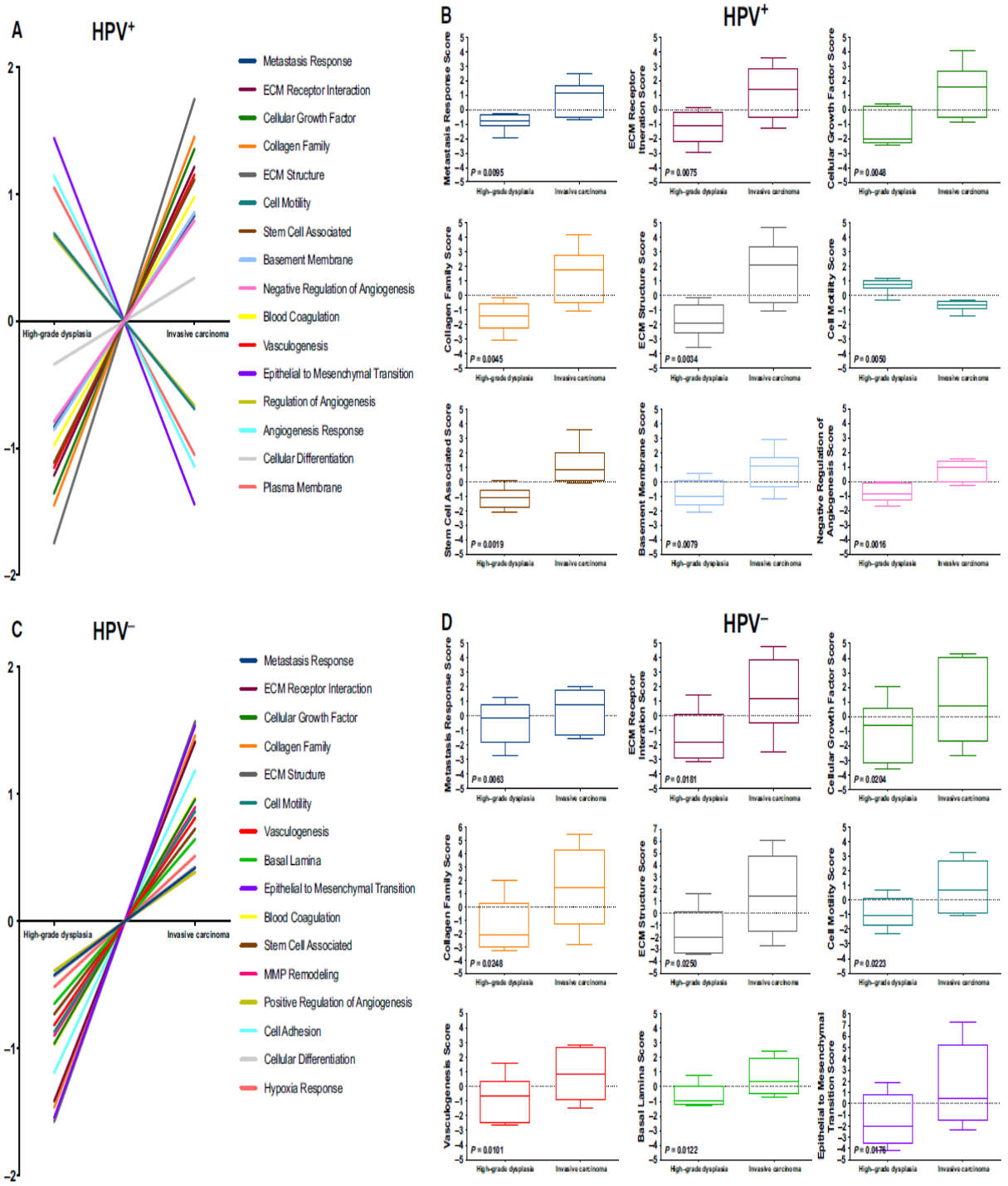
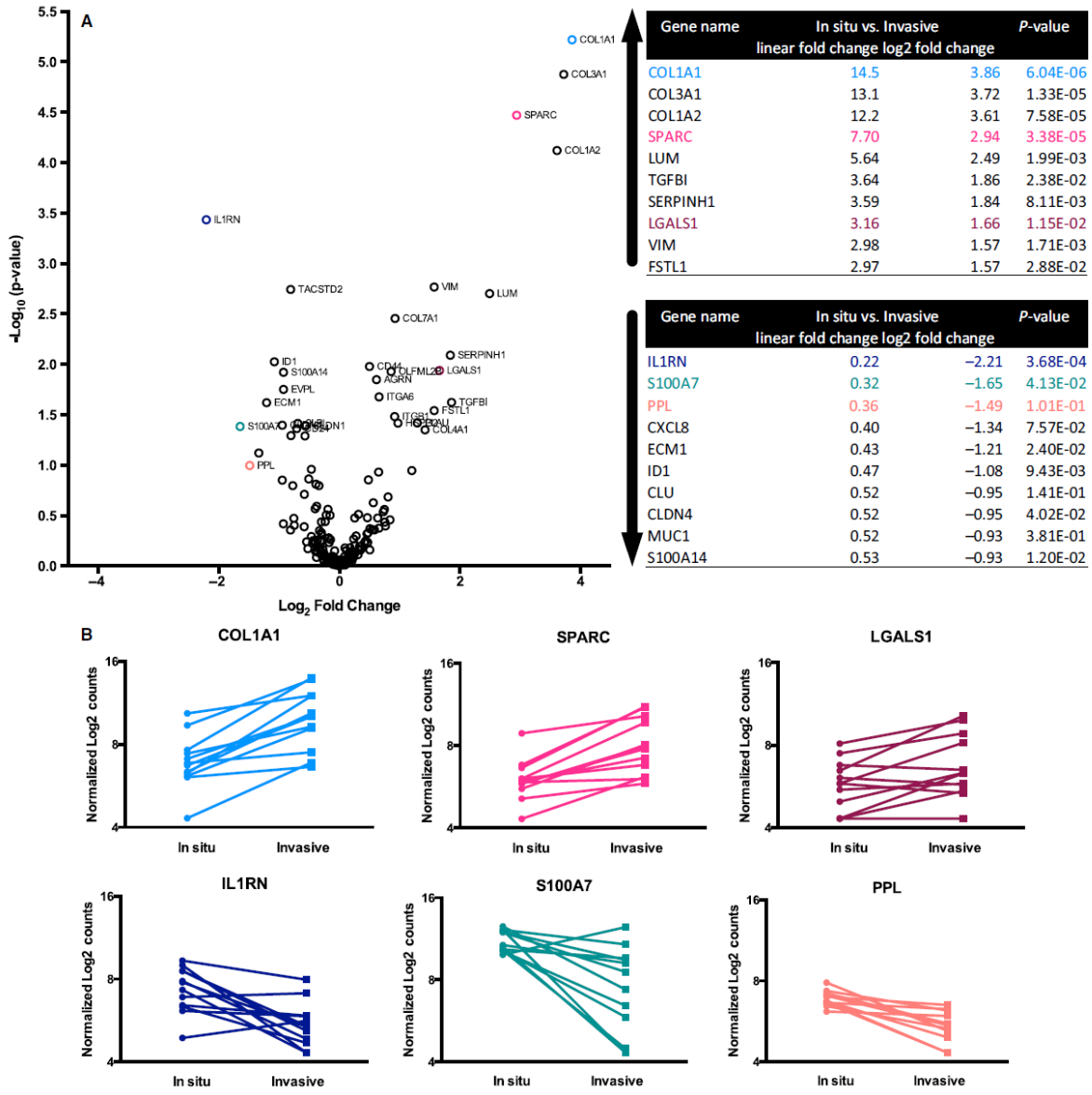


FIGURE 4 Pathway differences between high-grade dysplasia and invasive carcinoma. Individual pathway scores for high-grade dysplasia and invasive carcinoma are shown for (A) and (C) respectively in HPV+ and HPV− tonsillar and base of tongue squamous cell carcinoma respectively. Boxplots for individual pathway scores for the nine most statistically significant different pathways are illustrated in (B) and (D) respectively for HPV+ and HPV− tonsillar and base of tongue squamous cell carcinoma

In the search of new clinical invasion biomarkers in head and neck cancer, and because of low sample number, the HPV+ and HPV− groups were combined to investigate gene expression, adjusted for HPV status (Figure 5A).



Gene name	In situ vs. Invasive linear fold change	log2 fold change	*P*-value
COL1A1	14.5	3.86	6.04E-06
COL3A1	13.1	3.72	1.33E-05
COL1A2	12.2	3.61	7.58E-05
SPARC	7.70	2.94	3.38E-05
LUM	5.64	2.49	1.99E-03
TGFBI	3.64	1.86	2.38E-02
SERPINH1	3.59	1.84	8.11E-03
LGALS1	3.16	1.66	1.15E-02
VIM	2.98	1.57	1.71E-03
FSTL1	2.97	1.57	2.88E-02

Gene name	In situ vs. Invasive linear fold change	log2 fold change	*P*-value
IL1RN	0.22	−2.21	3.68E-04
S100A7	0.32	−1.65	4.13E-02
PPL	0.36	−1.49	1.01E-01
CXCL8	0.40	−1.34	7.57E-02
ECM1	0.43	−1.21	2.40E-02
ID1	0.47	−1.08	9.43E-03
CLU	0.52	−0.95	1.41E-01
CLDN4	0.52	−0.95	4.02E-02
MUC1	0.52	−0.93	3.81E-01
S100A14	0.53	−0.93	1.20E-02

FIGURE 5: Gene expression in high-grade dysplasia and invasive carcinoma of both HPV+ and HPV− tonsillar and base of tongue squamous cell carcinoma samples combined, adjusted for HPV+ status. (A) Volcano plot showing differentially expressed genes between high-grade dysplasia and invasive carcinoma by log2 fold change (x-axis) and −log10 of P-value (y-axis) in HPV+ and HPV− tonsillar and base of tongue squamous cell carcinoma cases. The top 10 genes with increased and decreased linear as well as log2 fold change expression between high-grade dysplasia and invasive carcinoma are together with P-values presented in the tables to the right. Genes marked with a color were selected for immunohistochemical protein analysis. (B) Graphs of selected genes showing normalized log2 counts for tonsillar and base of tongue dysplastic lesions and invasive carcinoma (HPV+ and HPV−) for each individual sample

In invasive cancer vs. high-grade dysplasia, COL1A1, COL3A1, COL1A2, and SPARC were the most up-regulated genes, while IL1RN, S100A7, and PPL were the most down-regulated genes (Figure 5A). After analyzing the fold-change values for mRNA expression, P-values and published gene functions, six genes: COL1A1, SPARC, LGALS1, IL1RN, S100A7, and PPL (Figure 5B), were chosen as potential pathological biomarker candidates for further evaluation by IHC for protein expression.

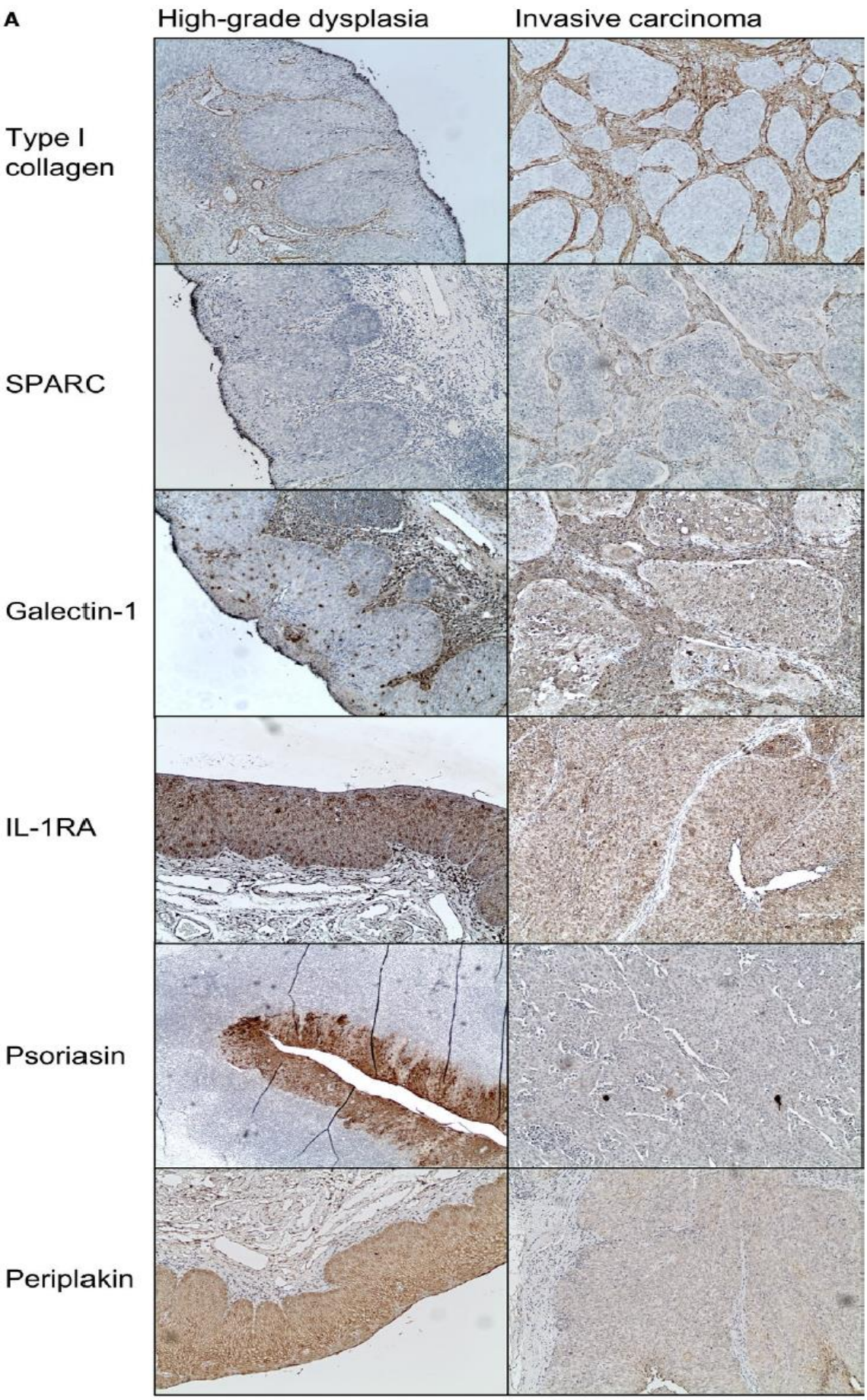
3.2 Protein expression in high-grade dysplasia and invasive TSCC and BOTSCC

Based on the results from the gene expression assay for the combined cohort of HPV+ and HPV− TSCC, six proteins (type I collagen (COL1A1), SPARC (SPARC), galectin-1 (LGALS1), IL1-RA (IL1RN), psoriasin (S100A7) and periplakin (PPL)) were selected for evaluation by IHC as potential invasion marker candidates. The transcripts of these si proteins showed with few exceptions either an increase (type 1 collagen, SPARC and galectin-1) or decrease (IL1-RA, psoriasin and periplakin) between high-grade dysplasia and invasive cancer (Figure 5B).

Protein expression of the above genes was therefore evaluated on the extended cohort with 24 different FFPE tumor sections, but due to lack of sufficient tumor material one sampl was excluded for type I collagen, SPARC, galectin-1, and periplakin. Details of the staining evaluation and scores are shown in Figure 6 and Supplementary Tables S3 and S4 respectively, and examples of the stainings are presented in Figure 6.

Neither type I collagen nor SPARC were found to be expressed at all in dysplastic epithelium or in invasive carcinomas. However, type I collagen was often expressed in the immediate ECM surrounding the cancer, but mostly to a lesser extent around high-grade dysplasia as compared to invasive tumor areas ($P = .0002$) (Figure 6). Similarly, SPARC was often expressed in the peritumoral stroma in peritumoral fibroblasts, mostly with absent or little expression around dysplasia, whereas higher expression was found around 17/23 invasive cancer areas, while 6/23 samples showed no difference in expression ($P < .0001$) (Figure 6).

Galectin-1 was mainly highly expressed in most tumors, with similar expression in high-grade dysplastic epithelium and invasive tumor areas in 13/23 cases, while 9/23 cases showed higher expression in the invasive component, and 1/23 showed lower expression ($P = .02$) (Figure 6). Since galectin- 1 is variably expressed on immune cells (Cedeno-Laurent and Dimitroff, 2012), we also assessed tumor infiltrating lymphocytes (TILs) in dysplasia and invasive components, but no differences were observed in galectin-1 positive TILs in the different components (Figure 6). Notably, 14/24 tumors showed that psoriasin was highly expressed in epithelial dysplasia, while the remaining samples showed no difference in expression ($P = 0001$) (Figure 6). Periplakin was expressed very heterogeneously in both dysplasia and invasive tumor areas, often with no clear differences between dysplasia and invasive tumor, with a tendency toward having higher expression in invasive carcinoma. Likewise, IL1-RA expression was mostly strongly expressed in cancer cells, however, a significant difference between high-grade dysplasia and invasive cancer was not observed. (Figure 6).


A
High-grade dysplasia
Invasive carcinoma
Type I collagen
SPARC
Galectin-1
IL-1RA
Psoriasin
Periplakin

B

	Number of samples with higher expression in high-grade dysplasia	Number of samples with higher expression invasively	Number of samples with no difference between high-grade dysplasia and invasive carcinoma	P-value
SPARC	0	17	6	<0.0001
Psoriasin	14	0	10	0.0001
Type I Collagen	0	13	10	0.0002
Galectin-1	1	9	13	0.0215
IL1-RA	5	0	19	0.0625
Galectin-1 TILs	1	6	16	0.1250
Periplakin	8	3	12	0.2266

FIGURE 6 (A) Examples of immunohistochemistry stainings of type I collagen, SPARC, galectin-1, IL-1RA, psoriasin, and periplakin, in high-grade dysplasia/carcinoma in situ an in invasive carcinoma. Expression of type I collagen and SPARC is higher in the tissue surrounding invasive carcinoma, and galectin-1 has a higher expression in invasive carcinoma, whereas IL-1RA, psoriasin, and periplakin show a higher expression in high-grade dysplasia compared to invasive carcinoma. (B) Summary of immunohistochemical protein evaluation scores and P-values for selected proteins, HPV+, and HPV− tumors combined

4 DISCUSSION

In this study we report, for the first time, differences in gene expression between high-grade dysplasia/cancer in situ and invasive HPV+ and HPV− TSCC/BOTSCC. Some of the most significant differential mRNA expressions were found for COL1A1, SPARC, and LGALS1 that were stronger expressed in invasive carcinoma compared to high-grade dysplasia, and the opposite was shown for S100A7, which was stronger expressed in high-grade dysplasia. These findings were also confirmed on a protein level by IHC. Moreover, we show that there are many similarities in gene expression and signal pathway activities between HPV+ and HPV− tumors— especially in genes related to ECM, highlighting the importance of the tumor micro milieu. As noted in the introduction, malignant invasive epithelial tumor develop from a dysplastic epithelium and detection of dysplasia has been utilized in different screening programs, for example, in cervical cancer. However, dysplasia in HPV+ TSCC/BOTSCC is rarely detected and pure premalignant stages have not been found, but common in HPV− TSCC/ BOTSCC. Some authors have even postulated that HPV+ premalignant phases do not exist in TSCC/BOTSCC,16 but have been described b others (Begum et al., 2005; Mooren et al., 2014; Masterson et al., 2015). Subsequently, only two studies have to our knowledge investigated HPV+ TSCC/BOTSCC dysplasia on a molecular level. Mooren et al, studied presence of p16INK4a expression in tonsillar dysplasia and found that p16INK4a positivity correlated to HPV status (Mooren et al., 2014). Additionally, another study by Masterson et al confirmed increased SYPC2 expression in premalignant carcinoma in situ and invasive carcinoma vs. normal epithelium by LCM and qPCR (Masterson et al., 2015). However, in this study we aimed to examine a wider range of genes and their differential expression between dysplastic and invasive HPV+ and HPV− TSCC/BOTSCC. Notably, numerous similarities in differential expression were here observed between HPV+ and HPV− TSCC/BOTSCC dysplasia and invasive cancer, suggesting that HPV+ dysplasia in tonsils/ base of tongue is a distinguishable entity from invasive HPV+ TSCC/BOTSCC. Therefore, it is very likely that premalignant stages of HPV+ TSCC/BOTSCC exist. Why such stages have not been described

so far, (Begum et al., 2005; Palmer et al., 2014) may be due to that tonsillar/base of tongue neoplasias are uncommon or possibly due to a more aggressive behavior of HPV+ dysplasia, an assumption that is not quite supported by data presented above. Hence, larger cohorts may be needed to identify premalignant phases in tonsils/base of tongue—possibly also by utilizing markers described here, such as psoriasin. On the signaling pathway level, it is not surprising that pathways involved in tumor progression, such as metastasis response, ECM receptor interaction, ECM structure, collagen family, and cellular growth factor pathways are up-regulated both in HPV+ and HPV− invasive carcinoma. Interestingly, we also observed a higher stem cell associated score in invasive cancer compared to dysplasia, which was especially observed in the HPV+ samples while present to a lesser extent in the HPV− samples. Notably, a study by Zhang et al showed that the cancer stem cell pool was much higher in HPV+ OPSCC as compared to HPV− cancer (Zhang et al., 2014).

However, high expression of stem cell markers have been shown to be associated with a poor prognosis in HNSCC, (Fan et al., 2017; Joshua et al., 2012; Nasman et al, 2013) yet most patients with HPV+ tumors in general have a very good prognosis. On the transcript level, the collagen genes COL1A1, COL1A2, COL3A1, where in our study found to be the most significantly increased ones in invasive cancer as compared to dysplasia in both HPV+ and HPV− tumors. Moreover, the proteins encoded by these genes belong to a family of proteins that strengthen and support many tissues in the body, being the most abundant part of the ECM (Ricard-Blum, 2011). Their expression has however also been linked to cell proliferation and tumor progression and the correlation to prognosis in various cancer types (Kirkland , 2009). Thus, collagen has been suggested as a double- edged sword in tumor progression, in one way acting as a passive barrier to resist tumor cells, in another way sending out biochemical and biophysical signals that affect, for example, cell adhesion, migration, angiogenesis, and repair systems that in cancer typically can be deregulated causing tumor progression (Fang et al., 2014).

Another matricellular protein, SPARC (secreted protein acidic and rich in cysteine), which regulates cell-matrix interactions and signaling pathways in cells, was also observed to increase in invasive cancer as compared to dysplasia. SPARC, which is considered to be solely produced by cancer associated fibroblasts in the tumor stroma, is suggested to regulate tumor cell growth and metastasis (Framson and Sage, 2004, Paulsson J et al., 2014). In a study by Witkiewicz et al, SPARC expression was correlated to tumor recurrence in ductal carcinoma in situ (DCIS) of the breast (Witkiewicz et al., 2010). Moreover, Chang et al show that in HNSCC a higher SPARC expression correlated to a higher tumor grade (Chang et al., 2017), but the prognostic role of SPARC expression is however still inconsistent within and between cancer types (Paulssonand Micke, 2014).

S100A7 encodes the protein psoriasin part of the S100 family containing calcium-binding motifs and is an important cell mediator for, for example, cell survival and maturation and has been associated to tumor progression and survival (Hattinger et al., 2013). In a study by Liu et al, psoriasin was shown to promote invasion and survival of pancreatic cancer cells (Liu et al., 2017). Other studies have shown that psoriasin is more highly expressed in, for example, squamous skin cancer in situ compared to invasive squamous skin cancer (Alowami et al., 2003), and in DCIS compared to invasive breast cancer (Petersson et al., 2007; Emberley Murphy and Watson, 2004), which is in line with our findings. Tripathi et al. have shown that psoriasin is significantly overexpressed in oral leukoplaki lesions with squamous cell hyperplasia or dysplasia as well as in HNSCC, compared to normal tissue (Tripathi et al., 2010).

Moreover, they show that patients with a nuclear accumulation of psoriasin in HNSCC have a reduced disease-free survival (Tripathi et al., 2010). IL1RN encodes the interleukin-1 receptor antagonist (IL-1RA) and is a natural inhibitor of the IL-1 receptor. Expression of IL-1RA

protein has been suggested to be able to decrease tumor growth, metastasis, and angiogenesis in mouse models (Lewis et al., 2006).
In this study, we show that IL1RN gene expression is lower in invasive cancer compared to dysplastic epithelium, especially in HPV+ TSCC/BOTSCC. Interestingly, similar results have previously also been published in CIN3 vs. invasive cervical carcinoma (den Boon et al., 2015). Lastly, in order to validate our data and in search for new pathological invasion biomarkers, we assessed six gene transcripts for protein expression and tissue distribution by IHC and were able to validate four out of six transcripts at the protein level. However, while the relative expression in dysplasia and invasive carcinoma often corresponded to the trend of transcriptional data, the absolute expression varied considerably between samples. Therefore, using any of these markers as markers of invasion in a clinical setting would probably need further tuning of staining interpretation.
There are some limitations in this study. First, tumor areas containing high-grade dysplasia/carcinoma in situ were often scanty and decreased upon serial sectioning in some cases. Hence, the RNA concentration was very low after extraction. Due to low RNA concentrations and fragmented FFPE material, the NanoString platform using tumor progression RNA panel was utilized. However, using a more "global" method, such as RNAseq, would have yielded more data and a better picture of the transition between dysplasia and invasive tumor. Secondly, few patient samples were included in this study, reflecting that dysplasia is uncommonly detected in HPV+ TSCC/BOTSCC.
In conclusion, this is the first study to our knowledge, examining and disclosing differences in gene expression between dysplastic and invasive HPV+ and HPV− TSCC/BOTSCC. However, larger studies are needed to distinguish pure premalignant from invasive tonsillar/base of tongue neoplasias.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval and consent to participate was conducted according to ethical permission 2005/431-31/4 from the Ethics Committee at Karolinska Institute, Stockholm, Sweden. The study was performed in accordance with the Declaration of Helsinki.

*Hammarstedt L, Holzhauser S, Zupancic M, Kapoulitsa F, **Ursu RG**, Ramqvist T, Haeggblom L, Näsman A, Dalianis T, Marklund L. The value of p16 and HPV DNA in non-tonsillar, non-base of tongue oropharyngeal cancer, Acta Otolaryngol, 2020, online. IF=1.157*

Background

Oropharyngeal squamous cell carcinoma (OPSCC) is dominated by tonsillar and tongue base carcinomas (TSCC/BOTSCC), but there are carcinomas at other oropharyngeal sites (other OPSCC). Human papillomavirus (HPV) positive TSCC/BOTSCC have a favorable outcome, and the TNM-classification separates OPSCC into HPV mediated ($p16^{INK4a}$ overexpressing, p16+) and HPV unrelated OPSCC ($p16^{INK4a}$ non-overexpressing, p16-) cancer, but the prognostic role of p16+ in other OPSCC is unclear.

Methods

195 other OPSCC, from patients diagnosed 2000-2018 were tested for p16, and/or presence of HPV DNA and the data correlated to outcome.

Results

Neither overall survival, nor disease free survival correlated to presence of p16+ or HPV DNA in other OPSCC. p16+ and presence of HPV DNA were correlated ($p<0.0001$), but the sensitivity of p16 as a surrogate marker for presence of HPV DNA was low (49%).

Conclusions

Our data suggest that p16+/HPV DNA positive other OPSCC should be analyzed separately and excluded from the HPV mediated OPSCC staging group.

1 INTRODUCTION

In many Western countries, a large proportion of oropharyngeal squamous cell carcinoma (OPSCC), which is dominated by tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC), is human papillomavirus positive (HPV^+) (Nasman et al., 2017; Haeggblom et al., 2017; Sturgis and Cinciripini, 2007; Nasman et al., 2009; Attner et al., 2010).

In addition, patients with HPV^+ TSCC/BOTSCC have a more favorable clinical outcome than those with corresponding HPV negative (HPV^-) cancer (Nasman et al., 2017; Attner et al., 2011; Mellin et al., 2000). This has also been proposed for all HPV^+ OPSCC as compared to HPV^- OPSCC (Gillison et al., 2000).

$p16^{INK4a}$ overexpression (p16+) is used as a surrogate marker for presence of HPV in the latest American Joint Committee on Cancer/Union for International Cancer Control (AJCC-8/UICC-8) staging system for OPSCC, which separates the TNM classification of HPV mediated (p16+) and HPV unrelated (p16-) OPSCC (Amin et al., 2017).

However, an estimated 10–20% of all OPSCCs are p16-positive, but HPV^-, being most apparent in OPSCC arising outside the tonsils and base of tongue (otherOPSCC). [2, 10-13] We and others have previously reported that presence of HPV DNA and p16+ was much less common in otherOPSCC, and that presence of HPV DNA or p16+ in these tumors did not correlate well to each other, or to better clinical outcome (Haeggblom et al., 2017; Ljokjel et al., 2014; Marklund et al., 2012; Gelwan et al., 2016).

It has been suggested that it should be possible to de-escalate today's more intensified treatment, i.e. chemo-radiotherapy, targeted therapy, and surgery either alone or in combination for patients with HPV^+ OPSCC, in order to reduce therapy-related side effects and complications (Nasman et al., 2017). Patients with otherOPSCC are often included into the same studies and treatment protocols as patients with TSCC/BOTSCC, even though earlier studies by us and others have indicated that prevalence, clinical significance and the correlation between HPV and p16+ is markedly lower in otherOPSCC (Haeggblom et al., 2017; Ljokjel et al., 2014; Marklund et al., 2012; Gelwan et al., 2016).

Since TSCC/BOTSCC dominates OPSCC with roughly 90% of all cases, there is an obvious risk that the results from patients with other OPSCC are concealed and misinterpreted. (Haeggblom et al., 2017).

Thus, if the basis of selection i.e. p16+ as a surrogate marker for HPV, does not apply to better clinical outcome for patients with otherOPSCC as indicated before by us and others, this definitely presents a problem (Nasman et al., 2017; Marklund et al., 2012; Smeets et al., 2007; Tham et al., 2020).

Based on the above, we wanted to examine if p16 overexpression, indeed was an adequate surrogate marker for HPV, as well as prognostic favorable factor in OPSCC other than TSCC and BOTSCC. For this purpose, we extended our previous study of patients diagnosed 2000-2008 with OPSCC at sites outside the tonsil and base of tongue (otherOPSCC) (Marklund et al., 2012), and included patients diagnosed from 2000 until 2018 to examine if p16 overexpression is an accurate marker for HPV as well as a prognostic favorable factor in OSCC other than TSCC and BOTSCC.

2. MATERIAL AND METHODS

2.1 Patients and tumor samples

Clinical data were obtained from 195 patients diagnosed 2000-2018 with otherOPSCC (ICD-10: C10.0–C10.9 and C50.1–C50.8) at Karolinska University Hospital. From these, 75 patients, diagnosed 2000-2008, had 69 tumor biopsies available, previously analyzed for p16 expression and presence of HPV DNA, and 61 of these had earlier been incuded in a survival analysis. [11]

Here, for an additional 120 tumor patients, data were either obtained from patient case reports, or tumor biopsies were analyzed as depicted below, for p16 overexpression and/or presence of HPV DNA. The study was performed according to permissions (2009/1278-31/4 and 2018/870-32) from the Stockholm Regional Ethical Review Board.

2.2 Analysis of HPV DNA, p16 overexpression, other OPSCC samples

The previously non-analyzed samples were tested for DNA of 27 HPV types, including all high risk types, by a PCR-based bead-based multiplex-assay on a MagPix instrument (Luminex Corp., Austin, TX, USA) as described before (Dalianis et al., 2015).

p16 overexpression (p16+), i.e. >70% of the tumor cells being strong cytoplasmatic and nuclear p16 positive, was tested by immunohistochemistry using the monoclonal antibody (mAb) clone JC8 (Santa Cruz Biotech, Santa Cruz, California, USA), or the E6H4^{TM} mouse mAb clone (CINtec®, Ventana, Tucson, Arizona, USA) (Dalianis et al., 2015).

2.3 Statistical analysis

Differences in categorical data was examined by Chi^2 test, and continious data was assessed by two-sided student t-test. Outcome was analyzed as disease free survival (DFS) or overall survival (OS). DFS was defined as day of diagnosis until day of any relapse. Patients never tumor-free were censored day 0, and patients dying without recurrence were censored at the time-point, when assessing DFS. OS was defined as day of diagnosis until day of death irrespective of cause of death. Survival curves with DFS, and OS were calculated using the Kaplan-Meier method. Differences in survival were calculated using the log-rank test. In addition, because of treatment differences in these patient groups, we performed a subgroup analysis in patients with WHO performace status 0-1.

All statistical tests were performed using SPSS (SPSS Statistics for Mac, Version 25. Armonk, NY: IBM Corp. USA).

3. RESULTS

3.1 Analysis of p16 and HPV in other OPSCC

Data on either p16 expression, and/or presence of HPV were obtained for totally 195 patients with otherOPSCC, when summing up data obtained earlier for 69 patients (Marklund et al., 2012). The data and patient characteristics are shown in Table 1 according to the p16 status of the tumors. p16 expression was obtained for 148 cases; data on presence/absence of HPV was available for 154 tumors; and data for both was obtained for 128 tumors. p16+ was found in 35/148 (24%) of the cases; HPV DNA was present in 31/154 (20%) cases; and both p16+ and HPV DNA were present in in 17/128 (13%) of the cases, while 82/128 (64%) were both p16- and HPV DNA negative (Table 2). HPV DNA positive tumors were p16+ significantly more often than HPV DNA negative tumors ($p<0.0001$) (Table 2). However, the sensitivity for p16+ as a surrogate marker for presence of HPV DNA was low 17/35 (49%), while specificity was 88%.

3.2 p16, HPV DNA and combined p16 and HPV DNA status in relation to outcome in other OPSCC

Of all the 195 patients, 148/195 (76%) were treated with curative intent and of those 75 (51%) had a 3-year OS.

Among patients with other OPSCC and data on p16, comparing those with p16+ to those with p16-, 18/31 (58%) vs. 40/93 (43%) had a 3-year OS (log-rank test: $p = 0.2$) (Figure 1A). Similarly, no difference in 3-year DFS was observed between patients with p16+ as compared to those with p16- tumors, 77% vs. 78%; log rank test: $p = 0.8$, (Figure 1B).

Analyzing patients with other OPSCC with data on HPV DNA and comparing the ones with HPV DNA positive tumors to those with HPV DNA negative tumors, 16/27 (59%) vs. 44/95 (46%) had a 3-year OS ($p = 0.3$) (Figure 1C). In addition, no differences were observed in 3-

year DFS between patients with HPV DNA positive and negative tumors (82% vs 79%, log rank test: p = 0.6) (Figure 1D).

Table 1. Patient and tumor characteristics separated by tumor p16 expression.

		p16 expression					
		overexpressing		*non-overexpressing*		*missing*	
		Mean	Count	Mean	Count	Mean	Count
Age (diagnosis)		67		67		68	
Sex	*Female*		14		44		14
	Male		21		69		33
T	*T0*		0		0		2
	T1		8		11		6
	T2		0		0		1
	T2		8		37		10
	T3		10		44		5
	T4		9		21		23
N	*N0*		18		49		23
	N1		5		19		5
	N2		10		42		16
	N3		1		0		2
	NX		1		3		1
M	*M0*		32		106		40
	M1		1		3		2
	MX		2		4		5
Treatment	*Intention to cure*		31		93		29
	Palliative		4		20		18
WHO performance status	*0*		15		41		17
	1		14		24		7
	2		3		31		10
	3		1		7		4
	Data missing		1		8		6
HPV DNA status	*Positive*		17		11		3
	Negative		18		82		21
	Data missing		0		19		23

Table 2. Correlation between p16 expression and HPV DNA status

Count	*HPV DNA positive*	*HPV DNA negative*	**TOTAL**
p16 overexpression	**17**	**18**	35
non-p16 overexpression	**11**	**82**	93
TOTAL	28	100	128

For patients with data on both HPV DNA and p16 expression, comparing those with p16+ and HPV DNA positive tumors to the remaining patients, 10/16 (63%) vs. 42/93 (45%) had a 3-year OS (log rank test: p = 0.3) (Figure 1E). Also, for this category, no differences were observed in 3-year DFS between patients with HPV DNA positive and p16+ tumors and the remaining patients (94% vs 77%, log rank test: p = 0.1) (Figure 1F).

Furthermore, no differences were observed in 3-year OS or 3-year DFS between patients younger or older than the mean age of the study population, or with regard to gender (data not shown). Moreover, when adjusting for (WHO) performing status, no changes in statistical significance were observed within any of the groups (data not shown).

4. DISCUSSION

In this study, we show that most other OPSCC, was p16-, lacked presence of HPV DNA and was not p16+ and HPV DNA positive, and despite there was a correlation between p16+ and presence of HPV DNA, the sensitivity of p16+ as a predictor for presence of HPV DNA was poor. More importantly, there were no differences in survival between patients with HPV DNA positive/negative, p16+/p16-, or p16 and HPV DNA positive/remaining other OPSCC treated with curative intent.

Our results thereby confirm previous data by others and us, where we showed that p16 overexpression and presence of HPV DNA was markedly less frequent in OPSCC other than TSCC/BOTSCC (Haeggblom et al., 2017; Marklund et al., 2012; Gelwan et al., 2017).

Moreover, although p16 and HPV status were significantly correlated to each other, the sensitivity of p16 as a surrogate marker of HPV infection was lower here in otherOPSCC, as compared to the sensitivity reported in TSCC/BOTSCC.[16] More specifically, only 61% (17/28) of the HPV DNA positive tumors also overexpressed p16, and only 49% (17/35) of p16+ tumors were also HPV DNA positive. This observation may be of great importance since p16 is regarded as a surrogate marker for active HPV infection in OPSCC, and that the AJCC (8th Ed) TNM-staging system now separates HPV mediated (p16+) OPSCC from HPV unrelated (p16-) OPSCC. While the latter may hold true for most, but not all cases of TSCC and BOTSCC, this is likely not the case for otherOPSCC (Marklund et al., 2012; Smeets et al., 2007).

The low prevalences of p16 overexpression and presence of HPV DNA in otherOPSCC, in this study (24% and 20% respectiely) were very similar to those obtained in our previous study, i.e. 25% and 17% respectively, as well as to the data in the systematic analysis study (Haeggblom et al., 2017; Marklund et al., 2012).

Furthermore, combining HPV DNA and p16 overexpression in other OPSCC, similar to the suggested golden standard (HPV E6 and E7 mRNA positivity) regarded as indicating active HPV positive status (Smeets et al., 2007), the proportion was even lower (13%). Also here the trend was similar to the data obtained in our previous study (12%) as well as to the data in the metaanalysis study (Haeggblom et al., 2017, Marklund et al., 2012).

Nevertheless, the low p16 overexpression and HPV prevalence in other OPSCC, irrespective of how it was assayed for, was not unexpected, since the epithelial tissue of these tumors differs from that of TSCC and BOTSCC, that are mainly lymphoepithelial origin (Haeggblom et al., 2017).

Similar to our previous study, neither p16 overexpression, nor presence of HPV DNA alone, in other OPSCC, were correlated to better clinical outcome, since no significant differences were disclosed for either OS or DFS.[11] Combined presence of p16 overexpression and HPV DNA present in 13% of the samples, was not correlated to better OS or DFS either, whether

this was due to the very low numbers of patients or not, needs likely to be confirmed in an even larger cohort.

It has been suggested that it should be possible to de-escalate today's more intensified treatment, i.e. chemo-radiotherapy, targeted therapy, and surgery either alone or in combination for patients with HPV^+ OPSCC, in order to reduce therapy-related side effects and complications (Nasman et al., 2017). Attempts have also been made to find more predictive markers to use in combination with HPV-status, e.g. age, stage, high $CD8^+$ tumor infiltrating lymphocyte (TIL) counts, HPV16 E2 mRNA expression, absent/weak CD44, or high LRIG1 or CD98 expression to better select patients for de-escalated therapy (Nasman et al., 2017). Today there are several ongoing clinical trials including patients with OPSCC based on p16-status (Fakhry et al., 2019; Jones et al., 2020).

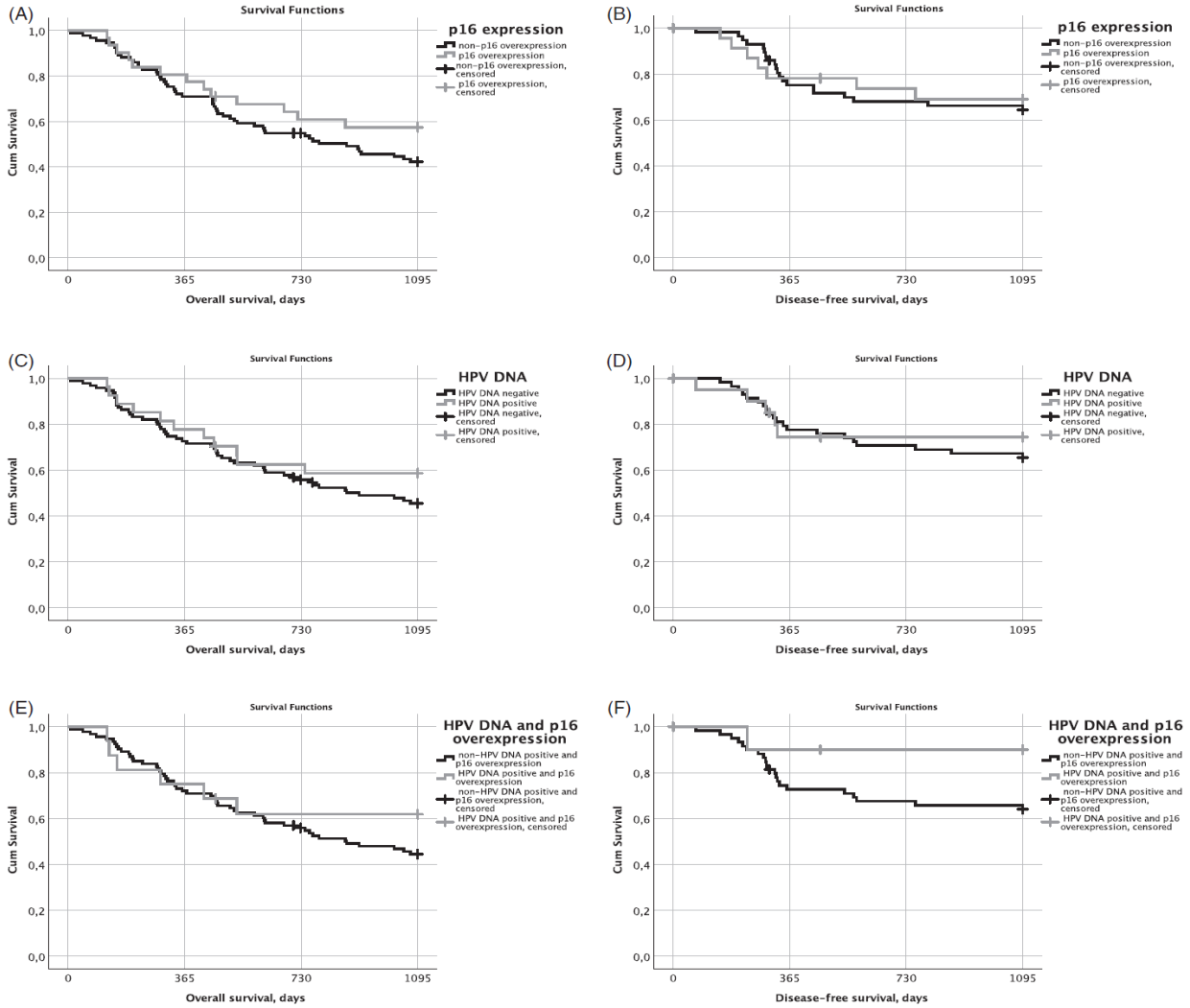


Figure 1. Overall survival (OS) and disease free survival (DFS) in correlation to presence or absence of p16 overexpression and HPV DNA in non-tonsillar and non-base of tongue oropharyngeal squamous cell carcinoma. (A) OS in correlation to p16 overexpression or not; (B) DFS in correlation to p16 overexpression or not; (C) OS in correlation to presence or absence of HPV DNA; (D) DFS in correlation to presence or absence of HPV DNA; (E) OS in correlation to both presence of p16 overexpression and presence of HPV DNA or remaining cases; and (F) DFS in correlation to both presence of p16 and presence of HPV DNA or remaining cases.

If the basis of selection, i.e. HPV status, or p16 status as a surrogate marker for HPV, does not apply to better clinical outcome for other OPSCC, this definitely presents a problem (Nasman et al., 2017, Marklund et al., 2012).

Hence, it may be suboptimal to stratify patients to de-escalation studies based on evaluation of a single biomarker (i.e. p16 alone) due to the risk of misclassification of tumors and thereby misallocation of patients with an undesired prognosis (Craig et al., 2019).

This study has limitations. First of all, it is a retrospective clinical analysis study with prospectively collected patient data. However, given the small number of cases, it would require a large multicenter approach to run this study prospectively. Secondly, the different treatment approaches are not adjusted for. Given the fact that there is a great variation and fairly few cases with few "events", we decided to use low performance status (WHO performance score 0-1) as a proxy for curative treatment in its true sense. This did not however, change the deduction.

To conclude, in this expanded study of other OPSCC, neither p16+ status nor HPV DNA postivie status correlated to OS or DFS in these patients. Moreover, the sensitivity of p16 as a surrogate marker for presence of HPV DNA was low. These data support the notion that other OPSCC differs from TSCC/BOTSCC and should be analyzed separately and be excluded from the p16 overexpressing OPSCC group in the recent AJCC 8th Edition.

Chapter 3: Other oncogenic viruses

1. Scientific context

HBV is one of the etiologic agents widely presented by IARC monograph like one of the the viruses which are having carcinogenic risks to humans (IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 100B). Polyomaviruses are also widely presented in another volume of IARC monograph (Malaria and Some Polyomaviruses, Volume 104).
To consider a virus like having oncogenic properties, there were analysed many types of studies (e.g., cohort and case control studies, correlation and interventional studies). Quality of studies is important, and therefore were analysed meta-analyses and polled analyses, as combined analyses of data from multiple studies are a means of resolving any result difficult to be interpreted by independent epidemiological studies. Other aspects which were taken into account for analysing the oncogenic risk of these viruses were temporal effects, different biomarkers in epidemiological studies, criteria of causality. Studies of cancer in experimental animals have produced positive results in one or more animal species. Mechanistic and other relevant data may provide evidence of carcinogenicity and help in assessing the relevance and importance of findings of cancer in animals and in humans. Here are mentioned the toxicokinetic data, data on mechanisms of carcinogenesis (changes in physiology, functional changes at the cellular level, changes at the molecular level, data on other adverse effects.
Inclusion criteria for skin cancer-inducing viruses: in order to label a virus as causal factor in tumorigenesis, objective criteria of diagnosis are necessary. For example, to conclude that an MCV is the etiological factor in a FFPE tumor samples, it is necessary to get a very high signal by MagPix detection, in comparison with controls (unpublished personal data). The reason for this is that polyomaviruses can be part of normal flora and only the simple detection of DNA is not enough to establish the diagnosis. For HPV HNC driven cases there are also criteria for establishing diagnosis (some authors have published an algorithm of diagnosis which included three biomarkers - DNA, RNA, p16INK4a of HPV [Ursu et al., 2017], while others used only the last two biomarkers) [29, 30]. (
Bersani et al., 2018, Haeggblom et al., 2018)
In some fresh skin and HNC tumors we have detected by multiplex genotyping the DNA of some polyoma and herpesviruses (EBV, HH7, HH8, MCV – unpublished personal data), but for the moment we cannot conclude that these viruses are causally-related with the analyzed tumors.

Ramqvist T, Ursu RG , Haeggblom L, Mirzaie L, Gahm C, Hammarstedt-Nordenvall L, Dalianis T, Näsman A. Human polyomaviruses are not frequently present in cancer of the salivary glands, Anticancer Res, 2018, 38(5): 2871-2874. IF=1.935

Abstract. Background/Aim. Malignant tumors of the salivary glands are rare and heterogeneous, with more than 20 subtypes, and classified mainly by histopathology. Their diagnosis is often challenging and their etiology unknown. Here, the possible association between human polyomaviruses (PyVs) and one or more salivary gland tumor subtypes was examined. Materials and Methods: Ninety-one primary tumors, including 12 subtypes and eight corresponding metastases, were analyzed for the presence of DNA of 10 different human PyV species by a bead-based multiplex assay using polymerase chain reaction and Luminex analyses. Results. Three samples, one adenocarcinoma (not otherwise specified), one adenoid

cystic carcinoma, and one mucoepidermoid carcinoma were found to be positive. However, the amount of MCPyV DNA in these tumors was estimated to be less than one genome per tumor cell. Conclusion. The analysis of DNA from 10 human PyVs in a large number of malignant salivary gland cancers did not implicate any of these human PyVs as an important causative agent in any of the 12 subtypes studied.

Malignant tumors of the salivary glands are rare malignancies and constitute a very heterogeneous group of cancer, currently divided into more than 20 different subtypes, according to the latest WHO classification (El-Naggar et., 2017).

Subtype classification is mainly based on histopathology and to a lesser extent on genetic profiling. To diagnose these tumors is often challenging, especially since they are rare and their histopathology may be indistinct. Moreover, little is known about the etiology of the different subtypes. In addition, few mutation analyses on malignant tumors of the salivary glands have been performed on very large cohorts. One recent analysis indicated major differences in the frequency of mutations between different subtypes (Grunewald et al., 2015). Notably, none or few mutations were found in the adenoid cystic, acinic cell and mucoepidermoid subtypes, indicating that other factors, e.g. viral, may be causative in these tumors. Similar studies were performed on a single subtype (e.g. adenoid cystic carcinoma), with similar results (Stephens et al., 2013). One virus family with oncogenic members that could be causative for the development of these tumors is the polyomavirus (PyV) family. The number of known human PyV species has increased rapidly, from two species (JCPyC and BKPyV) detected in 1971, currently to 13-14 species disclosed since 2007 (Gheit et al., 2017; Calvignac-Spencer et al., 2016). Most of these viruses were isolated from nasal samples, skin, serum or stool samples (Moens, Van Ghelue and Ehlers, 2014). Despite their recent discovery, many human PyVs are common in the population, and several, including MCPyV, and human PyV 6 and 7 are frequently found in the skin (Schowalter et al., 2010). In 2008 the first, and so far only, oncogenic human PyV, Merkel Cell polyoma virus (MCPyV) was discovered as a causative factor of Merkel cell carcinoma, a rare cancer, mostly in elderly or immunosuppressed patients (Feng et al., 2008). Disorders related to human PyV are mostly associated with immunodeficiency or immunosuppression in transplant patients, e.g. progressive multifocal leukoencephalopathy caused by BKPyV and trichodysplasia spinulosa caused by TSPyV (van der Meijden et al., 2010). Other human PyVs, e.g. KIPyV, WuPyV, human PyV6 and human PyV7, have been in implicated as being related to disease in individual cases but further studies on this topic are needed (Moens, Van Ghelue and Ehlers, 2014). Moreover, the early antigens of several of these viruses were shown to bind to the retinoblastoma protein (pRb), as well as other proteins involved in cell growth, which further emphasizes their potential oncogenicity (Cheng et al., 2013; Wu et al., 2016; Delbue Comar and Ferrante, 2017; Baez et al., 2017). Due to their established or suspected oncogenic potential, there have been numerous studies on the occurrence of human PyVs in different tumor types, mostly without any viral association being established (Moens, Van Ghelue and Ehlers, 2014; Ramqvist et al., 2014; Colombara et al., 2016; De Paoli and Carbone, 2013, Haeggblom et al., 2017)

In order to investigate the possible involvement of human PyV in one or more types of salivary gland tumor, we analyzed 91 primary tumors and eight corresponding regional metastases, of 12 different histological types for the presence of 10 different human PyV species.

Materials and Methods

Patients and tumor material. Patients diagnosed in Stockholm 2000- 2009 with malignant salivary gland tumors located in the parotid gland were identified through the Karolinska University Hospital Registry. In total, 145 consecutive patients, (82 female and 63 male) were found. After reviewing slides from available formalin-fixed paraffin-embedded (FFPE) tumors, obtained from surgery, 91 primary tumors and eight corresponding regional metastases from 91 individual patients were obtained and analyzed for the presence of human PyV DNA.

Tumor details are depicted in Table I. The study was performed according to approval 2005/431-31/4 from the Regional Ethics Committee, Karolinska Institute.

Sample preparation and DNA purification. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen) (Qiagen, Sollentuna, Sweden) according to the instructions from the manufacturer. Briefly, FFPE tumors and parallel blanks were treated with xylene and ethanol to remove paraffin. The tissue was then dried, dissolved in 180 µl tissue lysis buffer with proteinase K, incubated for 1 h at 56˚C followed by 1 h at 90˚C, before DNA purification on MinElute columns (Qiagen). After elution, the amount and purity of DNA was evaluated on a NanoDrop instrument (NanoDrop Technology Inc., Wilmington, DE, USA). Bead-based multiplex human PyV assay. For analysis of human PyV DNA a bead-based multiplex assay was utilized, as presented in Gustafsson et al. (Gustafsson et al., 2013) and modified as in Franzén et al. (Franzen et al., 2016). This assay detects the small T (ST) or the capsid protein VP1 regions of 10 different human PyVs: BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, TSPyV, human PyV6, human PyV7, human PyV9 and human PyV10 as well as the primate viruses SV40 and LPyV, with primers and probes described in Gustafsson et al. (Gustafsson B et al., 2013). To evaluate the presence of cellular DNA, β-globin was also assayed with primers as presented in Ramqvist et al. (Ramqvist et al., 2014).

Table I. Patient and tumor characteristics.

0		*Salivary gland tumors*	*Adinic cell carcinoma*	*Adenocarcinoma NOS*	*Adenoid cystic carcinoma*	*Basal cell adenocarcinoma*	*Epithelial-myoepithelial carcinoma*	*Mucoepidermoid carcinoma*	*Myoepithelial carcinoma*	*Oncocytic carcinoma*	*Poorly differentiated carcinoma*	*Salivary duct carcinoma*	*Secretory carcinoma*	*Squamous cell carcinoma*	Total
Total			19	17	11	3	2	18	2	1	3	8	1	6	91
Location	Parotis dx		7	10	6	1	1	9	1	0	0	3	0	2	40
	Parotis sin		12	7	5	2	1	9	1	1	3	5	1	4	51
T-stage	T1		7	3	5	2	1	10	0	0	0	2	1	2	33
	T2		10	6	5	0	0	7	2	1	1	3	0	1	36
	T3		1	2	1	1	1	0	0	0	1	1	0	3	11
	T4		1	6	0	0	0	1	0	0	1	2	0	0	11
N-stage	N0		19	11	11	3	2	18	1	1	1	3	1	3	74
	N1		0	2	0	0	0	0	0	0	1	1	0	1	5
	N2		0	4	0	0	0	0	1	0	1	4	0	2	12
M-stage	M0		19	15	10	3	2	17	0	1	3	7	1	6	84
	M1		0	2	1	0	0	1	1	0	0	1	0	0	6
Age	Median		60	71	53	84	74	43,5	53	65	84	66	85	59,5	62
	Range		17-98	49-76	35-67	57-85	60-88	14-86	51-55	65	76-96	46-84	85	24-78	14-96
Analyzed metastasis			0	4	0	0	0	0	0	0	0	2	0	2	8
MCPyV pos			0	1	1	0	0	1	0	0	0	0	0	0	3
% MCPyV pos			0%	6%	9%	0%	0%	6%	0%	0%	0%	0%	0%	0%	3%

Briefly, the polymerase chain reaction (PCR) was performed on 10 ng of DNA from each sample using the Qiagen Multiplex kit and including PyV and β-globin primers as noted above. For each sample, a corresponding FFPE non-containing sample as negative control was included, and this FFPE was treated, as described above. An additional negative control of 5 µl of distilled water was included in the assay in order to evaluate possible background fluorescence when calculating MFI values. Two positive controls, corresponding to 5 and 50 MCPyV genomes were included in the assay. DNA from an MCPyV-positive Merkel cell carcinoma was included as an additional positive control. After PCR, the presence of human PyV amplicons was assayed with a Luminex MagPix system (Luminex Inc., Houston, TX, USA) as presented previously (Gustafsson et al., 2013; Franzen et al., 2016).

In brief, 5 µl from the 25 µl PCR reaction was incubated together with a mixture of 23 different bead types, each coupled to a specific ST or VP1 probe for one of the 11 different PyVs, as

well as β-globin. The output is presented as the median fluorescent index (MFI) and an MFI value of more than 2 × background + 300 was regarded as a positive value. For MCPyV, this value corresponded to approximately 5 genomes. Values below this were considered of no significance.

Results

Ninety-nine samples, including eight metastases, of 12 malignant salivary gland subtypes were analyzed for the presence of human PyV DNA corresponding to ST or VP1 (Table I). All samples were positive for the β-globin gene, indicating successful DNA extraction, PCR and Luminex analysis. The only human PyV DNA found in the material was from MCPyV (Table I). Three samples were regarded as positive (corresponding to 5-50 MCPyV genomes/sample), while another three samples had signals below the cut-off, corresponding to <5 MCPyV genomes/samples. All six of these samples, with exception of one of those with weaker signals, were positive for both the ST and VP1 region of MCPyV. All three positive samples were of different subtypes, namely adenocarcinoma (not otherwise specified), adenoid cystic carcinoma and mucoepidermoid carcinoma.

Discussion

To our knowledge this is the first study where such an extensive number of malignant salivary gland tumors were analyzed for the presence of human PyVs. Salivary gland tumors constitute a heterogeneous group of tumors, likely with several different, but so far unknown, etiologies. It was therefore of interest to explore the possibility of whether or not any of these human PyVs could be a causative factor for one or more of these tumor types. Here, all major, as well as some more rarely occurring salivary gland tumor subtypes were represented.

The only PyV DNA detected was MCPyV DNA, and only in a limited number of salivary gland tumors. Only three samples were considered MCPyV-positive (>5 MCPyV genomes/10 ng sample DNA) and all three had very low amounts of MCPyV DNA, indicating it unlikely that MCPyV was the causative factor for any of these tumors. The reason for this is that 10 ng DNA, corresponding to roughly 6,000 human cell genomes, was analyzed per sample and therefore even if only 50% of the cells in the samples were tumor cells and some DNA was degraded in the FFPE samples, one would still expect a signal of >1,000 copies to suggest human PyV as a causative factor. Moreover, the three positive salivary gland samples were of different tumor subtypes, strengthening the likelihood that MCPyV is not a major causative factor for any specific subtype. MCPyV is common in the skin (7) and has also been found at low levels in saliva (Loyo et al., 2010). Thus, a low amount of MCPyV genome in a sample may not necessarily originate from the tumor itself, but possibly also from these sources when a patient is operated on. In a recent study by Chen et al., 79 benign and five malignant salivary gland tumors and 28 normal salivary glands were analyzed for the presence of 62 different DNA viruses, including all human PyVs investigated in the present study with exception of human PyV10. They found MCPyV in 20-40 of all sample types irrespective of whether they were benign, malignant or normal (Chen et al., 2017). In comparison to the present study, the study by Chen et al. was mostly focused on benign tumors of the salivary glands. However, taken together, both the study by Chen et al. and the present study indicate that MCPyV is often present in low amounts in salivary glands or in their vicinity, and unlikely to be responsible for the development of these tumors. MCPyV is a causative factor in 80-97% of Merkel cell carcinomas, a rare neuroendocrine tumor (Mui, Haley and Tyring, 2017; Becker, Schrama and Houben, 2009). Chernock et al. analyzed a series of neuroendocrine carcinomas of the salivary glands for the presence of MCPyV, but failed to find MCPyV DNA in any of them (Chernock et al., 2011).

In the present study, only one neuroendocrine tumor (included among the poorly differentiated tumors) was analyzed and was found to be negative for MCPyV.

The results obtained in the present study do not rule out an involvement of human PyVs or other viruses in one or more subtypes of salivary gland tumor. In the PyV family alone, 11-12 new human PyVs have been discovered in the past 11 years, of which eight were included in this analysis, but still more may be discovered. Moreover, viruses from other families, known or so far undiscovered, may be involved.
There are several limitations to this study. Salivary gland tumors are divided into many subtypes based on histology. Although the total number of tumors was reasonably large, the number of samples for some of the included subtypes was quite small. For this reason, we cannot rule out the possibility that one or more of these salivary gland subtypes are associated with the presence of one of the assessed human PyVs, but can only state that they are not likely a major contributing factor. In addition, 3-4 recent human PyV species were not assessed in this study and more members may yet be detected, so a statement with regard to the role of human PyVs in general in the development of salivary gland tumors cannot be made.
In conclusion, the analysis of the presence of DNA from 10 different human PyV species in a large number of malignant salivary gland tumors of different subtypes, did not implicate any of these 10 human PyVs as an important etiological factor in any of these subtypes.

*Popovici O, **Ursu RG**[3], Azoicai D, Iancu LS. HBV genotypes circulation in pregnant women in Romania: a pilot study, Rev Romana Med Lab, 2020, 28(1): 91-98. IF=0.945*

Abstract
Background: The risk of mother to child transmission of hepatitis B virus (HBV) is recognized worldwide, reason for which the World Health Organization aims to reduce this public health issue of major concern in the next ten years. The aim of our study was to detect circulating HBV genotypes in a selected population of pregnant women, as scientific evidence to recommend personalized antiviral therapy and to obtain updated epidemiological information.
Methods: HBsAg positive pregnant women were selected by the National Institute of Public Health Romania. Blood samples were collected after signing the informed consent. The HBV genotypes were tested by INNO LiPA HBV genotyping method.
Results. The D genotype was detected in 9/18 (50%) patients, genotype A in 3/18 (16.7%), and genotype F in 3/18 (16.7%) patients. Three patients had double infection, 11 had unique infection and 4 had no detectable genotype.
Conclusion. This study confirmed previous studies' results regarding HBV genotype circulation in our country, with the mention that F genotype was a new one for our area. These data are useful from epidemiological point of view and for therapeutical reasons, as it is known that the therapy should be genotype guided.

Key words: HBV, genotype, pregnant women, antiviral therapy

Background

The World Health Organisation (WHO) recognized the risk of maternal transmisson of HBV to newborns, therefore the plan is to reduce this risk by the end of 2030 (WHO Regional Committee for Europe, Action plan for the health sector response to viral hepatitis in the WHO European Region, 2017). The *International Agency for Research on Cancer* (IARC) mention HBV as one of the known biological agents involved in human carcinogenesis since 1993 (IARC Working Group, HEPATITIS B VIRUS, International Agency for Research on Cancer,

[3] corresponding author

2012). 240 million people are known worldwide with chronic HBV infection, with variation between 2 and 8%, acording to each regional endemicity levels. In some countries the HBsAg prevalence is decreasing due to vaccination and probably due to efficient therapy. An actual problem is the migration of population which led to prevalence changes in countries with known low endemicity (e.g., Italy, Germany) (Lampertico et al., 2017). The estimated age-standardized incidence rate in 2018, for liver cancer, both genders, all ages for Romania was 8.4, value which is one of the highest in Europe ((Bray et al., 2018). A special category of HBV infected patients are the pregnant women, regarding the risk of transmission to the new borns. Recently the seroprevalence of HBV infection markers in this patients category was evaluated in Romania, and a 5.1 % prevalence of HBsAg was detected and a 7.4% for HBeAg among the HBsAg positive ones (Popovici et al., 2018). The proportion of childbearing age women having natural immunity after HBV infection varied between 6.9 and 19.2% and increased with age group (Popovici et al., 2016). Viral markers as HBeAg and HBV-DNA are known to influence the risk of hepatocellular carcinoma (HCC). Also, the viral genotype seems to modify the risk of HCC, but this data are difficult to analyze because of the global variation in viral genotypes circulation (IARC Working Group, HEPATITIS B VIRUS, International Agency for Research on Cancer, 2012). There have been identified 10 genotypes of HBV, labeled A to J. In comparison with genotypes B and D, genotype A is associated with significantly higher rates of HBeAg and HBsAg loss as a result of IFN therapy. There have beed observed differences regarding natural history of genotpes B and C evolution to HCC, in different area of the world (e.g., Asia, Alaska) (Terrault et al., 2018).

The starting point for our research was the previous studies performed on pregnant women in Romania, aiming to estimate the seroprevalence of HBV markers of infection (Popovici et al., 2018) and also studies which evaluated the HBV genotypes in patients with chronic HBV infection (Ursu et al, 2016, Luca et al., 2016). As HBV perinatal transmission is a problem of major concern, we thought that it is important to provide scientific evidence for personalized antiviral therapy and for public health interventions.

The aim of this pilot study was to evaluate the HBV genotypes circulation in a selected group – pregnant women, from different regions of Romania, to establish personalized therapy and to obtain updated epidemiological information.

Material and methods

The selection of HBsAg positive pregnant women was done by the National Institute of Public Health Romania - National Centre for Communicable Diseases Surveillance and Control. The pregnant women were selected as the first who gave their consent to participate in the study. The number of subjects was imposed by the available financial resources. From all the pregnant women which were tested for HBV markers during routine pregnancy control between May and August 2018, eighteen HBsAg positive pregnant women were selected from 4 different counties of Romania (Iași, Călărași, Sibiu and Suceava).. Sixteen out of 18 pregnant women presented themselves for HBsAg testing at the family doctor, and only 2 were tested in maternity, immediately after admission to give birth. Blood samples were then sent to the Virology Laboratory of "Grigore T. Popa" University of Medicine and Pharmacy, Iași. The same protocol for HBV genotype detection was used for all samples.

Research ethics

All the patients included in this study have signed the informed consent approved by the Research Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy Iași. The study is following the international recommendations on human studies as mentioned in the Declaration of Helsinki.

The INNO LiPA HBV genotyping method followed the next steps: DNA purification, PCR amplification (outer and nested amplification), hybridisation of the amplicons, detection

of HBV genotypes by strip hybridisation. All these steps were described in details previously (Ursu et al, 2016; Luca et al., 2016).

Results

The median age of pregnant women was 31 years (23 – 39), and the median age of pregnancy was 28 weeks (9 – 39). Demographic and laboratory data can be seen in Table I. None of the pregnant women had been vaccinated and none was in antiviral treatment. Three patients had double infection, 11 had unique infection and 4 had no detectable genotype. The D genotype was detected in 9/18 (50%) patients, genotype A in 3/18 (16.7%), and genotype F in 3/18 (16.7%) patients.

Discussion

In this study we confirmed our previous findings in patients with chronic HBV infections, using the same HBV genotyping method: D is the most frequent HBV genotype in our area, followed by A genotype, as unique or in double infections (Ursu et al, 2016; Luca et al., 2016).

Many other authors have published their results regarding HBV genotype circulation worldwide, mentioning also the natural history of HBV infection and the role of HBV genotypes. A research team from Fundeni Clinical Institute, Bucharest, detected the same two genotypes D and A in Romanian chronic infected patients (Constantinescu et al., 2008; Constantinescu et al., 2014). In a recent review, Kmet Lunaček et al. confirmed the A and D genotype distribution in Europe, but also mentioned that 41% of non–A-D genotypes were identified in some European countries (Kmet Lunaček, Poljak and Matičič, 2018).

Thereby, we mention the study of Toy et al in which the Dutch CHB patients were detected positive for genotypes B, C and G beside the known A and D genotypes (Toy et al., 2008). Also, in a study done in the French population, Moussa S et al detected the genotype D, followed by E (Moussa et al., 2016).

Ghany et al identified the distribution of HBV genotypes in a North American cohort (A 18%, B 39%, C 33%, D 8%, E 3% and other 1%) and correlated the genotype with Asian, White and Black races. Interestingly, among Whites they found genotypes A (55%) and D (33%) and this distribution was also correlated with the place of birth - Europe (Ghany et al., 2015).

A recent review is underlying the relations between HBV genotypes and mutants, and hepatitis B vaccine failure, acute and chronic HBV infection, HBeAg seroconversion and HBsAg seroclearance, with the purpose to implement individualized management for HBV infected patients (Lin and Kao , 2017).

Some papers are mentioning the subtypes of HBV and their role in influencing the response to antiviral therapy (Pourkarim et al., 2014; Rajoriya et al., 2017), a relevant reason to test chronic infected patients. In a comprehensive review, Pourkarim et al showed the role of the almost forty subgenotypes of HBV in the natural history of HBV infection. Also, the authors suggested the introduction of the term “immigro-subgenotype” to distinguish exotic (sub)genotypes from local known genotypes in each area of the world. The hope of the authors is that by vaccination, accurate diagnosis method, and monitoring therapy, elimination of HBV could be achieved (Pourkarim et al., 2014). In another review of Rajoriya et al, the genotype of HBV was mentioned as the first viral factor involved in a personalised medicine approach for the future treatment of hepatitis B infection (Rajoriya et al., 2017).

Compared to the previous regional studies carried out in Romania, this study is also mostly a regional one, as 78% of tested pregnant women were from the North-East region, and only a few patients belonged to other counties (Calarași, Sibiu and Suceava). Detection of F genotype of HBV in 3 cases, in double infection with A and D genotypes, was an unexpected result given the known global geographical distribution of HBV genotypes. We reviewed the literature and we found in a recent review that F genotype of HBV is usually present in South

and in Central America, but it was also detected at the Arctic Circle and in Spain (Sunbul , 2014).

Another review established the following genotype distribution within chronic HBV infections: 0.86% for F genotype, 22.1% for D, and 16.9%, for A genotype (Velkov et al., 2018).

There are not many studies published about F genotype, but we can mention that it was detected the second as prevalence in different regions of Brasil (Lampe et al., 2017).

Regarding it's role in pathogenesis, Marciano S et al specify that genotype F is correlated with a higher risk of HCC and mortality, and the respose to interferon is similar with that of genotype A (Marciano et al, 2013).

As a support for our findings is the study of Hirzel et al which detected the genotype F in 5 cases (1.1 %) in a retrospective cohort study on Swiss patients having different countries of birth, using the same genotyping method (e.g., INNO LiPA) (Hirzel et al., 2015).

This study highlights for the first time the F genotype circulation in Romania, which is why these 3 cases have been thoroughly investigated from an epidemiological point of view. No direct or indirect link(s) with the above-mentioned geographical regions/country have been established. One of them could have been exposed, through a transfusion, 20 years before, and another one mentioned a long stay of her husband for work, in another European country.

The risk of mother-to-child transmission of HBV is correlated with an elevated risk of developing chronic B hepatitis (CHB), cirrhosis and HCC, and therefore, many professional associations have developed guidelines for the diagnosis and monitoring of pregnant women in order to reduce this transmission. One of them is the 2018 Guide of *American Association for the Study of Liver Diseases* (AASLD) which recommends the counseling of pregnant women for HBV vaccination, breast feeding, monitorization of HBsAg positive women, the risk of mother to child transmission, the necessity of testing the sexual partners for HBV. The same guideline suggest that HBsAg positive pregnant women should be tested for liver transaminases, HBV - DNA and assessed for the utility of antiviral therapy (Terrault et al., 2018).

The EASL (European Association for the Study of the Liver) 2017 Clinical Practice Guidelines suggest that in pregnant women with high HBV- DNA viral load or high levels of HBsAg, the antiviral therapy should be initiated (Lampertico et al., 2017).

The guidelines are recognising the utility of HBV genotyping, as different responses to peg-IFN therapy were seen for different genotypes of HBV. Perinatal transmission from HBsAg positive pregnant women with high level of HBV-DNA could be reduced by antiviral therapy (Terrault et al., 2018). HBV genotyping in pregnant women was also performed by other authors. In a similar study with ours, 21 HBsAg positive out of 1489 Japanese pregnant women were detected positive for genotype C in 14 cases, D in six cases, and undetermined in one case (Michitaka et al., 2012). In a Chinese pregnant women study group genotypes B and C of HBV were detected, genotype C being considered a risk factor for mother to child transmission (Ding et al., 2013). Denmark is another country which introduced HBV genotyping for pregnant women, genotype C being the most prevalent in their population (Harder et al., 2011). The pregnant women from an antenatal clinic in UK were detected positive for genotypes E (13/40, 32.5%) and B (10/40, 25%) as predominant, and for genotypes A (6/40, 15%), C (9/40, 22.5%), and D (2/40, 5%) in lower proportion (Dervisevic et al., 2007). In a study performed in France, the author did not find any relation between the HBV genotype or origin of the patients and the risk of mother to child transmission (Sellier et al., 2015).

We are confident in our HBV genotyping method, as it is an optimized assay which has amplification, conjugation positive and negative controls. Four patients known to be HBsAg positive had no HBV genotype detectable. We analysed the literature and we found at least two other articles which reported the same untippable HBV genotypes. Possible explanations could

be some immune escape mutants or the lower sensitivity of our genotyping method, so the untypable genotype can be completely identified only by sequencing and phylogenetic analysis of the S gene (Faleye et al., 2015; Baclig et al., 2017) which was not the case in this study. A recent study from Norway supports this information, as the authors mentioned that their molecular epidemiology analysis indicated a geographical clustering of sequences depending on their geographical origin (Pettersson et al., 2019)

Still, there was performed a comparison between the Sanger sequencing assay and INNO Lipa method and the authors have found a very good correlation of the results of both assays (Alidjinou et al., 2018).

Another application of INNO LiPA is to test the antiviral resistance. A multidisciplinary team concluded in a clinical trial that TDF (tenofovir) alone is safe and effective for treatment of patients with lamivudine-resistant, chronic HBV infection (Fung et al., 2014).

From our point of view, the major disadvantage of INNO LiPA assay is that is time consuming (14 hours all procedure), and secondly, the price which cannot be supported by patients. More developed techniques have beed used by other authors for HBV genotyping: Real Time PCR or sequencing (Alidjinou et al., 2018, Chevaliez S et al, 2017).

The future of HBV testing regarding the genotypes worldwide distribution and antiviral resistance is by far the sequencing analysis, for which, in countries such Romania, requires larger studies. Due to financial constraints, this study was limited to only 18 pregnant women.

To achieve the elimination goal (WHO Regional Committee for Europe, Action plan for the health sector response to viral hepatitis in the WHO European Region), building on such pilot studies that bring valuable data on circulating genotypes, personalized therapy, combined with public health actions at national level, could be keys of success.

Conclusions

In this study, we detected the genotypes of HBV in a group of pregnant women. The most frequent genotype was D, followed by A and F. The last one has never been described in our country until now. These data are important from epidemiological point of view, regarding the HBV genotype circulation, and for guiding a personalized antiviral therapy in case of chronic hepatitis B.

Chapter 5: Target cell lines therapy
Scientific context

Treatment of cancers involve surgery, chemotherapy and radiotherapy alone or combined. In some cases, resistance to therapy, with recurrences and metastasis and/or side effects appear. This underlines the need of a new direction of research for cancer targeted therapy.

PI3K inhibitors

Phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway components are key therapeutic targets in cancer, immunity, and thrombosis. In normal cells, the PI3K/mTOR pathway has regulatory roles in cell survival, proliferation, and differentiation. However, aberrant activation of this pathway frequently occurs in human cancer (Filip Janku et al. Cancer Treat Rev. 2017).

PI3K are believed to be one of the key therapeutic targets for cancer treatment based on the observation that hyperactivity of PI3K signaling is significantly correlated with human tumor progression, increased tumor microvessel density and enhanced chemotaxis and invasive potential of cancer cells. Enormous efforts have been dedicated to the development of drugs targeting PI3K signaling, many of which are currently employed in clinical trials evaluation. PI3K inhibitors are subdivided into dual PI3K/mTOR inhibitors, pan-PI3K inhibitors and isoform-specific inhibitors (Yang, J. et al. Mol Cancer 2019).

The most used inhibitors in solid tumors are the **pan-PI3Kis** (Buparlisib - BKM120; Pictilisib - GDC-0941 and Copanlisib - BAY 80-6946) which target each of the four catalytic isoforms of class I PI3K, therefore they have the potential for broad activity in a number of tumor types with a range of molecular alterations. However, such broad inhibition may lead to a potentially higher risk of adverse events, which could limit the use of such agents at therapeutic doses. Also, BEZ235 is a potent, oral, ATP-competitive **dual inhibitor of the four class I PI3K isoforms** and the downstream effectors mTORC1/2. Alpelisib - BYL719 **isoform-specific PI3Kis** have the narrowest profile and may require careful patient selection based on potential biomarkers of sensitivity and resistance (Filip Janku et al. Cancer Treat Rev. 2017).

Lessons learned from studies with PI3Kis

The findings to date suggest that PIK3CA and PTEN alterations are relatively weak biomarkers of clinical activity. However, PIK3CA mutations appear to be more promising as predictive factors for p110a catalytic isoform-specific inhibitors, with PTEN alterations possibly associated with resistance. Second, it is increasingly evident that single-agent targeting of the PI3K pathway has limited activity. Therefore, the identification of appropriate biomarkers of efficacy and the development of optimal combination therapies and dosing schedules for PI3Kis are likely to be required for the graduation of this class of compounds to clinical practice (1).

Acquired amplification and mutation of PIK3CA and PIK3CB, which resulted in a marked upregulation of the PI3K signaling itself, have been shown to cause resistance to selective PI3K inhibitors (Filip Janku et al. Cancer Treat Rev. 2017).

Overall, PI3K inhibition is being investigated as a potential strategy to develop novel therapeutics for cancer management. Although we move forward with the clinical development of PI3K inhibitors, maximizing the utility of these agents in the treatment of patients remains challenging. Certainly, understanding the precise mechanisms of PI3K signaling and PI3K inhibition will be critical. Optimization of the patient selection strategies and combination approaches will help increase the practical efficacy of these agents. Continued work to clarify

the resistance mechanisms and the novel strategies to overcome resistance will also be important (Filip Janku et al. Cancer Treat Rev. 2017).

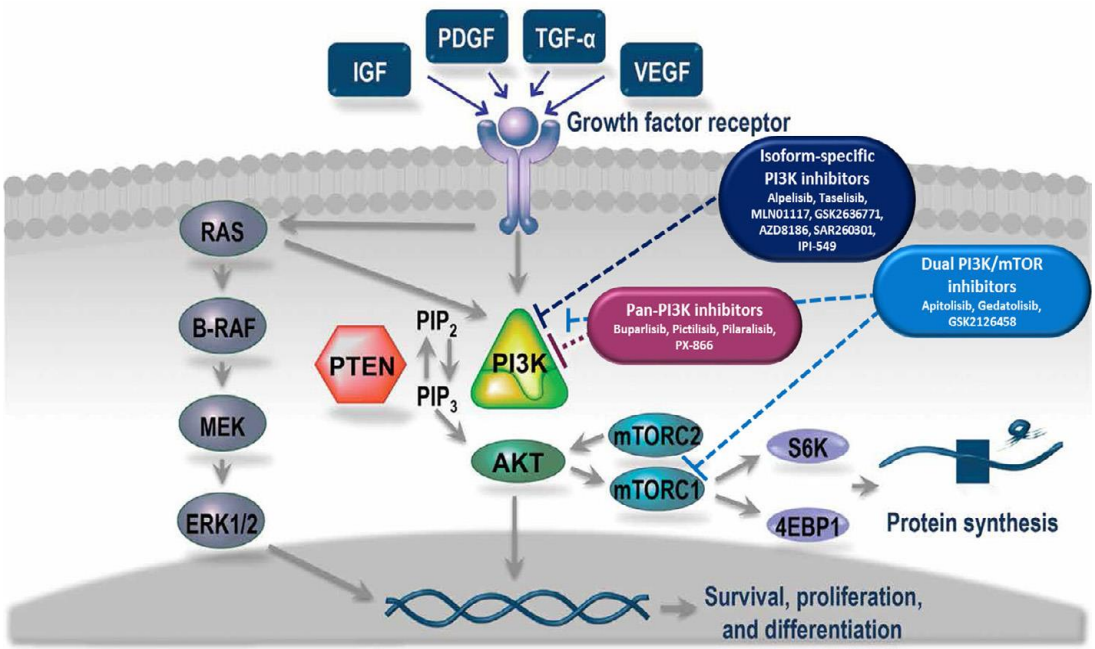


Fig. 1. Mechanism of action of PI3K/mTOR inhibitors. Signaling through the PI3K pathway regulates multiple cellular processes that can contribute to the development of malignancies. Multiple inhibitors are under development that target PI3K and/or mTOR. Abbreviations: 4EBP1 = eIF4E-binding protein 1, ERK1/2 = extracellular signal regulated kinase 1/2, IGF = insulin-like growth factor, MEK = mitogen activated protein kinase kinase, mTOR = mammalian target of rapamycin, mTORC = mTOR complex, PDGF = platelet-derived growth factor, PI3K = phosphoinositide 3-kinase, PIP2 = phosphatidylinositol 4,5-bisphosphate, PIP3 = phosphatidylinositol 3,4,5-trisphosphate, PTEN = phosphate and tensin homolog, TGF-a = transforming growth factor-alpha, VEGF = vascular endothelial growth factor.

The FGFR inhibitors

The fibroblast growth factor receptor (FGFR) family consists of four highly conserved transmembrane receptor tyrosine kinases (FGFR1-4), and one receptor that lacks the intracellular kinase domain FGFR5 (also known as FGFRL1) can bind to fibroblast growth factor (FGF) ligand. Receptors activated by FGFs can trigger a series of intracellular events, so that to activate the main survival and proliferation signaling pathways. FGFs and FGF receptors mediate important physiological mechanisms such as metabolic homeostasis, endocrine function, and wound repair. The deregulation of the FGF signal axis is related to the tumorigenesis, tumor progression and resistance of anti-tumor treatments in many types of tumors (Qi Liang, et al. Eur J Med Chem. 2021 Jan).

Main FGFR genomic alterations found in human cancers are amplification, mutation, translocation, Germline SNP. The FGFR signaling pathway represents a major target for cancer therapeutics as a number of studies indicate that it plays a crucial role in tumor proliferation, angiogenesis, migration, and survival. The FGFR pathway is subject to various somatic aberrations resulting in carcinogenesis. Receptor overexpression can be a result of gene amplification or changes in post transcriptional processing; point mutations may result in constitutive receptor activation or decreased sensitivity to ligand binding; translocations can produce fusion proteins with constitutive activity; and isoform switching and alternative

splicing can reduce specificity to FGFs. These major oncogenic aberrations represent features that make FGFR an ideal therapeutic target for treating a broad scope of malignancies. There are many ongoing clinical trials combining selected anti-FGFR drugs and existing therapies. From the selective FGFR inhibitors we mention AZD4547, BGJ398, JNJ42756493, and PD173074 highly selective and highly bioactive FGFR inhibitors. In the development of a novel targeted therapy, we must also recognize the inevitability of acquiring resistance to the drug - either from up-regulation of compensatory pathways or innate mutations rendering the FGFR receptor resistant (Chae Yet al. Oncotarget. 2017).

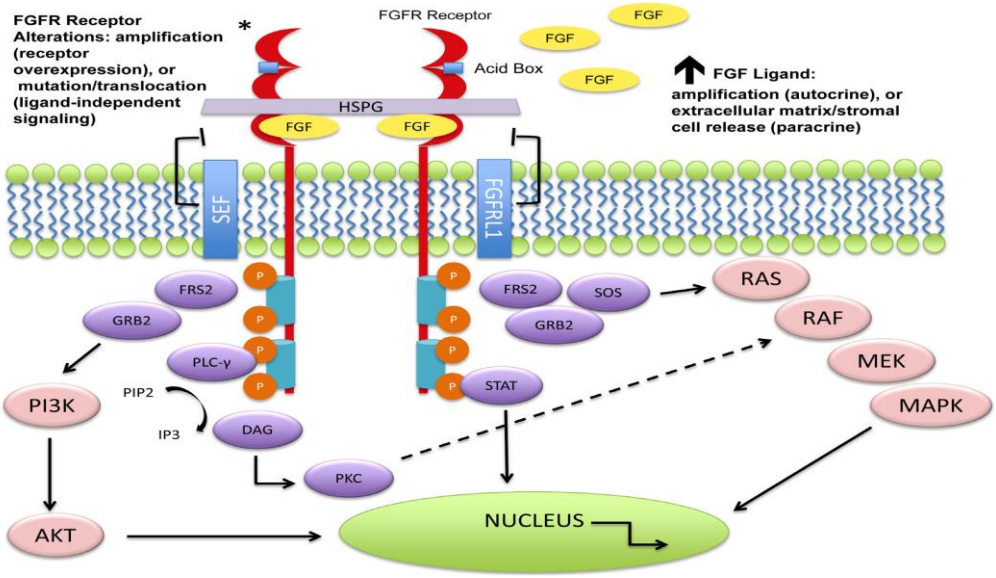


Fig. 2: Molecular aberrations leading to FGFR pathway activation. The FGFRs dimerize upon ligand binding and trigger a downstream cascade of signaling pathways. The FGFR receptors (1-4) can become activated by mutation, translocation, or gene amplification. An increase in circulating FGF ligands can also cause activation. Downstream signaling can trigger the mitogen activated protein kinase (MAPK) pathway, the phosphoinositide-3-kinase (PI3K/Akt) pathway, the phosphorylation of the signal transducer and activator of transcription (STAT), and the PLCγ activation of the DAG-PKC and IP3-Ca2+ cascade resulting in DNA transcription. Negative feedback loops can attenuate the signaling cascade at varying levels. As seen above, the "similar expression to FGF" (SEF) family members can interact with the cytoplasmic domain of FGFRs and inhibit downstream signaling. It is hypothesized that FGFRL1 (atypical receptor/ FGFR5) may serve as a ligand trap, may dimerize with other transmembrane FGFRs and inhibit autophosphorylation, or may increase turnover rates of other FGFRs. No evidence exists for these mechanisms (Chae Yet al. Oncotarget. 2017).

FGFR genomic alterations, including FGFR gene fusions that originate by chromosomal rearrangements, represent a promising therapeutic target. Gene fusions are hybrid genes that originate from the chromosomal rearrangement of two genes, in the form of translocation, insertion, inversion, and deletion. Fusion events, which involve a variety of partner genes, result in the formation of fusion proteins capable of oncogenic transformation and induction of oncogene addiction. The discovery of targetable fusions and the improvement of techniques used for detecting these alterations allowed the development of specific therapies for the treatment of fusion-driven tumors. There are commercially available targeted sequencing kits for fusion detection which are testing DNA or RNA from FFPE or fresh / frozen samples, for FGFR 1- 4, like Hybrid Capture-based, Amplicon-based and Anchored multiplex PCR-based

assays. Increasing evidence suggests that the FGFR system plays an important role in cancer development and progression. However, the results of clinical trials have clearly demonstrated that only tumors carrying genetic alterations of the FGFRs such as mutations or fusions might respond to treatment with FGFR inhibitors, at least when used as single agents (De Luca A et al. International Journal of Molecular Sciences. 2020).
There are more than 250 clinical trials which are testing inhibitor of fibroblast growth factors. In one recent clincal trial, Futibatinib, an oral, irreversible, highly selective fibroblast growth factor receptor (FGFR)1-4 inhibitor with potent preclinical activity against tumors harboring FGFR aberrations was evaluated and its treatment resulted in manageable safety, pharmacodynamic activity, and preliminary responses in patients with advanced solid tumors (Bahleda R et al. Ann Oncol. 2020).
In recent years, single-target drug therapy has led to chemotherapy resistance has been widely reported. The shortcomings of traditional single-target drugs limit the accessibility of these drugs to tumor tissues. Subsequently, higher drug doses are required, leading to non-specific targeting and intolerable cytotoxicity. In recent years, combination therapy or dual-target inhibitors have been used clinically. Dual FGFR inhibitors in clinic trials are underway now, for example Brivanib (FGFR/VEGFR dual inhibitor) is tested for cervical cancer, recurrent ovarian cancer, previously treated advanced hcc, advanced liver cancer. Lucitanib (FGFR/VEGFR/CSF1R inhibitors) is used for solid tumor, extensive-stage small cell lung cancer, advanced/metastatic colorectal cancer, gynecologic cancer. In summary, FGFR inhibitor is a promising targeted therapy for cancer, and dual FGFR inhibitor with good selectivity may be an important direction, as they can overcome drug resistance and avoid off-target effects for its good selectivity to minimize adverse effects (Qi Liang, et al. Eur J Med Chem. 2021 Jan).

After therapy with inhibitors in association with chemotherapeutic drugs or not, the cell lines are evaluated for the viability, proliferation, apoptosis and cytotoxic effect of the therapy. We will present shortly the principle of each of this method.

WST-1 viability assay

The Cell Proliferation Reagent WST-1 is used for the nonradioactive, spectrophotometric quantification of cell proliferation and viability in cell populations using the 96-well-plate format. It is allowing the analysis of cytotoxic and cytostatic compounds, such as anti-cancer drugs and other pharmaceutical compounds.
Principle: the stable tetrazolium salt WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. Cells grown in a 96-well tissue culture plate are incubated with the WST-1 reagent for 0.5 - 4 hours. After this incubation period, the formazan dye formed is quantitated with a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells.
Cell proliferation and viability assays are of particular importance for routine applications in cell biology. Tetrazolium salts (e.g., MTT, XTT, WST-1) are particularly useful for this type of analysis. Tetrazolium salts are cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells.

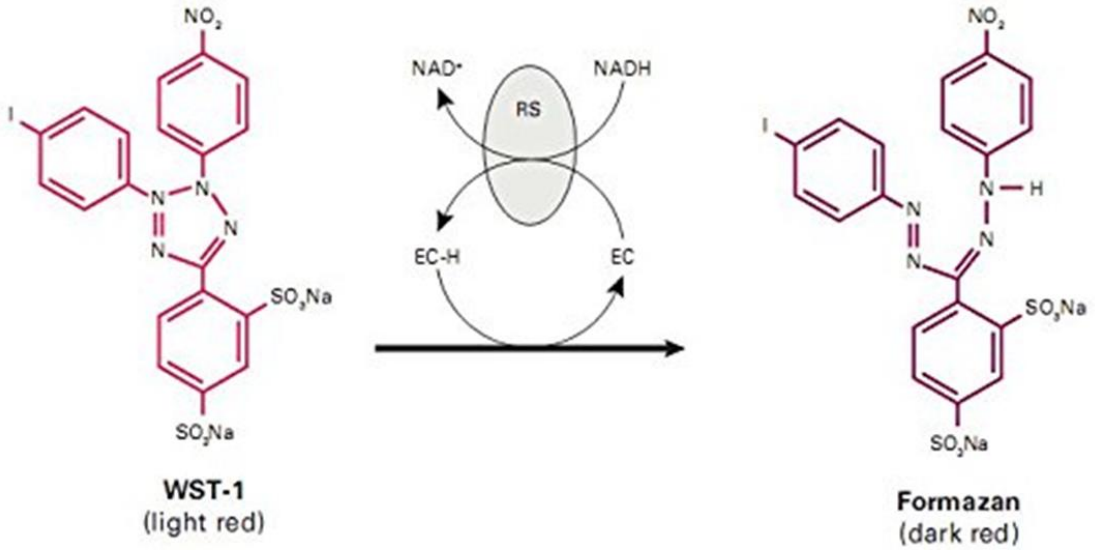


Fig. 3: The principle of viability assay

Features and Benefits**:** WST-1 is convenient, as it is a ready-to-use reagent, it is safe and easy as it eliminates radioactive isotopes, washing steps, and additional reagents. It is accurate, as the absorbance obtained strongly correlates to the cell number. Also is a sensitive method by detecting low cell numbers. And it is a fast assay, allowing processing a large number of samples using a multi-well ELISA reader.
(https://www.sigmaaldrich.com/catalog/product/roche).

IncuCyte Cell Proliferation Assay

Cell proliferation assays are a cornerstone of cancer therapeutic, developmental biology, and drug safety research. Analysis of the sustained signaling pathways that underlie the progression of tumors, for example, accounts for >12,000 manuscripts in PubMed, the majority of which use cell proliferation analysis to evaluate tumor cell growth. Despite this, there has not been a direct, straightforward, scalable method for quantifying cell proliferation as a continuous event. Rather, the traditional approaches are endpoints or at best a series of concatenated endpoints to measure the time-course.

A variety of strategies for kinetic measurement of proliferation are possible using the Incucyte® Live-Cell Analysis System. Selection of label-free or fluorescent assays depends on the specific scientific question being asked and cell models studied. Continuous live cell assays for both adherent and nonadherent cells are possible, as cells stay stationary inside a standard tissue culture incubator while the Incucyte® optics move. There is no sample or stage movement that can cause non-adherent cells to migrate to the edges of microplate wells and negatively impact data accuracy. Incucyte® Live-Cell Imaging and Analysis enables non-invasive, label-free measurements of cell growth based on area (confluence) or cell number (count) metrics, both of which are generated via segmentation (masking) of high quality phase images. To resolve the challenge of quantifying low contrast cells that can be difficult to identify in phase contrast images, Incucyte® Nuclight Reagents for live-cell analysis can be used to fluorescently label nuclei. Fluorescent Incucyte® images can then be acquired over time and analyzed to generate nuclear counts and derive doubling times in either mono- or co-cultures. Additionally, Incucyte® Proliferation Assays can be multiplexed with Incucyte® fluorescent reagents for cell health assessments, including apoptosis (Incucyte® Caspase 3/7 Dye, Incucyte® Annexin V Dye), cytotoxicity (Incucyte® Cytotox Dye), or viability (Incucyte® Nuclight Reagents). Cell boundaries can be identified using the Incucyte® Cellby-Cell Analysis Software Module, and simultaneous assessment of cell death or viability

achieved by measuring the fluorescence intensity originating from within the individual cell boundary (Live-Cell Analysis Handbook, A Guide to Real-Time Live-Cell Imaging and Analysis, Fourth Edition, Sartorius).

Kinetic Apoptosis Assays

Quantitative Assays for Apoptotic Pathway Analysis for Both Drug Discovery

Apoptosis, the biological process by which cells undergo programmed cell death, is required for normal tissue maintenance and development. However, aberrations in apoptotic signaling networks are implicated in numerous human diseases including neurodegeneration and cancer. Apoptotic pathways are initiated by extrinsic factors that result in activation of pro-apoptotic receptors on the cell surface, or intrinsically by many different stimuli such as DNA damage, hypoxia, the absence of growth factors, defective cell cycle control, or other types of cellular stress that result in release of cytochrome C from mitochondria (Cotter TG. Et al. Nat Rev Cancer 2009).

The Incucyte® Caspase-3/7 Green and Incucyte® Caspase 3/7 Red Dyes are an inert, non-fluorescent substrates. When added to tissue culture growth medium, the substrate freely crosses the cell membrane where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and fluorescent labeling of DNA. Incucyte® Annexin V Red, Green, Orange and NIR Dyes are specially formulated, highly-selective cyanine-based fluorescent dyes ideally suited to a simple mix-and-read, real-time quantification of apoptosis in living cells (Daya S,et al. SBS 16th Annual Conference and Exhibition 2010)

The assay provides a full kinetic readout of apoptotic signaling over multiple days, eliminating the need for determining a single, optimum, assay endpoint a-priori which can vary considerably for different cell types and for different compound treatment conditions. Cells can be simultaneously labelled with an Incucyte® apoptosis reagents and Incucyte® Nuclight Reagent for live-cell nuclear labeling to measure apoptotic cell death, cell proliferation and kinetically monitor anti-proliferative effects of compounds. Addition of Incucyte® apoptosis reagents to normal, healthy cells are non-perturbing to cell growth or morphology and yield little to no intrinsic fluorescent signal (Cen H, et al. FASEB J 2008).

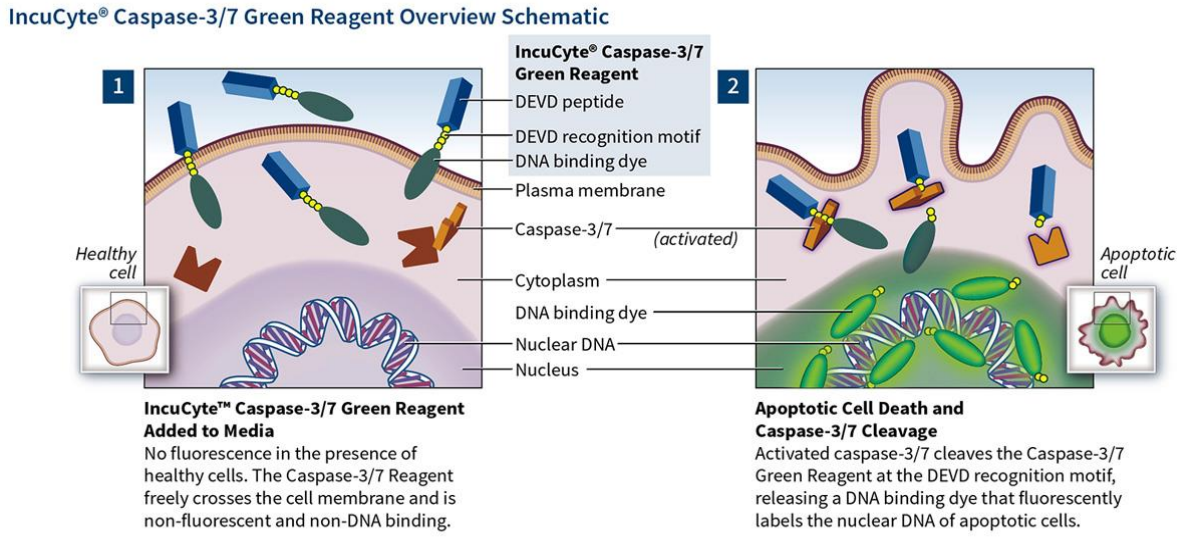


Fig. 4: The principle of apoptosis assay

Kinetic Cytotoxicity Assays

Real-Time, Live-Cell Assay to Quantify and Visualize Cytotoxic Events

The cellular response to cytotoxic exposure is controlled by complex biochemical pathways, such as necrosis or apoptosis, which results in cell death. In apoptosis, morphological changes include pseudopodia retraction, reduction of cellular volume (pyknosis), nuclear fragmentation (karyorrhexis) and eventually loss of plasma membrane integrity (Kepp O, et al. 2011). Morphological changes that characterize necrosis include cytoplasmic swelling and early rupture of plasma membrane (Kroemer G, et al. 2009). Compounds that have cytotoxic effects often compromise cell membrane integrity regardless of the pathway. The Incucyte® Cytotoxicity Assay uses highly sensitive cyanine nucelic acid dyes ideally suited for mix-and-read kinetic measurements of cell membrane integrity overtime. Incucyte® Cytotox Red and Green Dyes are cell impermeant cyanine dimer nucleic acid stains that bind to dsDNA,5 and when added to the culture medium, these reagents fluorescently stain the nuclear DNA of cells that have lost plasma membrane integrity. The assay provides a full kinetic readout of cytotoxicity over multiple days, eliminating the need for determining a single, optimum, assay endpoint a-priori which can vary considerably for different cell types and for different compound treatment conditions. - Addition of Incucyte® Cytotox Dyes to normal, healthy cells are nonperturbing to cell growth and morphology. - Cells can be simultaneously labelled with Incucyte® Cytotox Dyes and Incucyte® Nuclight Reagents for nuclear labeling to measure cytotoxicity and cell proliferation. 96- and 384-well assay format follows a homogeneous "mix-and-read" protocol which can be run over multiple days in full media. No wash or lifting steps required, negating the concern that cells are lost during the experiment or labeling process.

Nuclear staining indicates loss of membrane integrity, a hallmark of cell death. The cell impermeable DNA stain, CytoTox green, stains cell nuclei only when cells have lost membrane integrity following treatment with a cytotoxic compound. Hallmarks of necrotic cell death include cytoplasmic swelling and loss of membrane integrity. Viable cells remain unstained, and their growth unperturbed in the presence of CytoTox green (O'Clair, Lindy et al.,2014). (Fig 5).

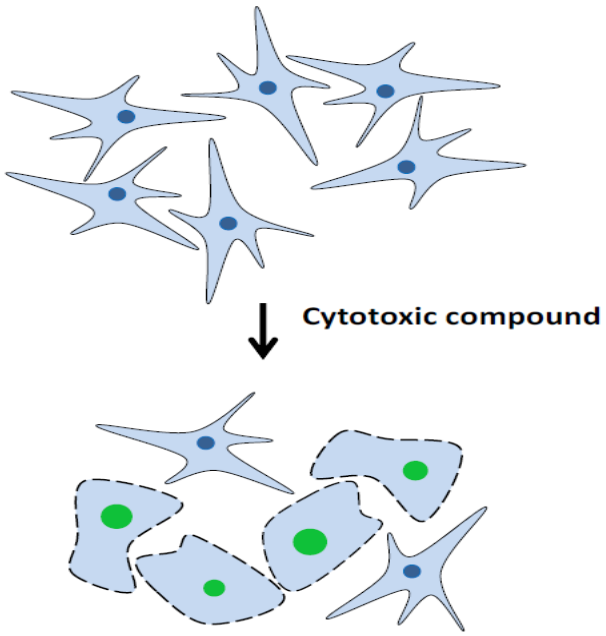


Fig. 5: The principle of cytotoxicity assay

It is more than obvious that there nowdays are important research and discoveries in the field of specific inhibitors developing and of the field of technology for assessing the efficiency of these cell treatment with specific inhibitors.
In the same time, medical specialties develop practices minimally influenced by other specialties. The results obtained from laboratory should be transmitted and applied in clinical practice, for the optimization of the cancer patient follow-up. For example, it would be necessary to know the cytotoxic effect needed for a patient. With selected antibiotics, it is possible determine the lowest dose of antibiotic needed to kill the bacterium. We might do this with tumour target therapy, as for the moment it is not sure if oncologists measure levels of anti-tumoral drugs. The oncologic patients persist with therapy despite moderate side effects. One needs uninterrupted, high levels of drug when using prolonged therapy (antibiotic or likely anticancer); resistance develops with stops/starts or lower doses. For example, patient could tolerate pneumonitis and leg edema from high-dose everolimus. Another important aspect is that the in absorption of oral drugs may be affected by food and also important is to treat the patient with multiple drugs that attack the same target to delay resistance. With antibiotics, we sometimes use two drugs to attack a target, e.g., the cell wall, to delay resistance. I am uncertain if oncologists do this to delay resistance.
Conclusion: Medical specialties might learn from one another (Schmid, 2020).

*Holzhauser S, Lukoseviciute M, Andonova T, **Ursu RG**, Dalianis T, Wickstrom M, Kostopoulou ON. Targeting Fibroblast Growth Factor Receptor (FGFR) and Phosphoinositide 3-kinase (PI3K) Signaling Pathways in Medulloblastoma Cell Lines, Anticancer Res, 2020, 40(1): 53-66. IF=1.994*

Introduction
Cancer is a leading cause of death for children and adolescents around the world and approximately 300,000 children aged 0 to 19 years old are diagnosed with cancer each year (Steliarova-Foucher et al., 2017).

Although leukaemia is the most common cancer in childhood (30% of paediatric malignancies), brain and central nervous system (CNS) tumors are the most frequent among the solid tumors (20% of childhood cancers) (Ward et al., 2014). Medulloblastomas (MBs), which are the main focus of the current study, are among the most common malignant brain tumors, accounting for 16-25% of all CNS tumors in children, and usually arise in the cerebellum (Massimino et al., 2011; Grimmer and Weiss, 2006; Scotting, Walker and Perilongo, 2005; Ivanov et al., 2016). According to recent advances in genomics, gene expression profiles, and DNA methylation analysis, MBs are divided into four subgroups: Wingles/Integrated (WNT), Sonic Hedgehog (SHH), Group 3 and Group 4 (Northcott et al., 2012; Kool et al., 2012; Cho et al., 2011). The largest subgroups are: Group 4 and SHH-activated MB, which account for ~35% and 30% of tumors respectively and they both have intermediate prognosis. Group 3 tumors are found in 25% cases and have the worst prognosis for patients, while WNT comprises 10% of MB tumors and has the best prognosis (Northcott et al., 2012; Gibson et al., 2010; Taylor et al., 2012). The current treatment of MBs consists of removal of the tumor by surgery, radiation therapy (X-rays or protons) and chemotherapy (Millard and De Braganca, 2016). Despite this multipronged approach to therapy, approximately 30% of patients still die from the disease, and survivors suffer from severe long-term side effects, including neurological deficits, endocrine disorders, and secondary cancers

(Dimitrova and Arcaro, 2015). Therefore, novel combination therapies, ideally with fewer side effects, are needed and this is the goal of the current study. This study therefore focuses on the fibroblast growth factor receptor (FGFR) and one of its downstream pathways - the phosphatidylinositol 3-kinase (PI3K), which both could be potential targets for future MB treatment.

FGFRs are a family of receptor tyrosine kinases expressed on the cell membrane, and are crucial during development, as well as in adult cells, and their dysregulation has been implicated in a wide variety of cancers, such as urothelial carcinoma, hepatocellular carcinoma, ovarian cancer and lung adenocarcinoma (Dai et al., 2019). Recently, two cases of germline mutations in *FGFR2* and *FGFR3* respectively, and MB associated with craniosynostosis were reported (Bourdeaut et al., 2013). In addition, in a study investigating the transcriptomic changes in MB, the FGFR pathway was identified as a marker of treatment failure due to poorer outcome in MB patients with a high expression of *FGFR3* (Pomeroy et al., 2002). Furthermore, a study on ependymomas and pilocytic astrocytomas showed an increased FGFR1 and FGFR3 expression in aggressive ependymomas (Lehtinen et al., 2017). Early phase clinical trials have been conducted with FGFR3-directed targeted therapies for glioblastoma multiforme, transitional cell carcinoma, and multiple myeloma carrying mutated FGFR3 (NCT01975701, NCT02278978, NCT02401542, NCT02052778) indicating the potential of such therapies for other cancers. Nevertheless, for childhood cancer, knowledge of FGFR protein family is still limited, and new therapeutic targets would definitely be of help.

The PI3K pathway has been studied in more detail in childhood cancer. Gene amplifications or mutations of the PIK3CA gene have been identified in numerous solid tumors, as well as in MB (Yuan and Cantley, 2008; Samuels and Waldman, 2010; Janku, 2017; Robinson et al., 2012; Kandula et al., 2013; Rosty et al., 2013; Ogino et al., 2013; Shukla et al., 2012), which makes p110α (PI3K catalytic subunit alpha) one of the most frequently mutated kinases in all cancers (Day et al., 2013; Ollikainen et al., 2007). Previous studies on MBs have shown that PI3K inhibitors as single, or combined with chemotherapy could potentially be used for treatment of MB patients (Dimitrova and Arcaro, 2015; Zhao et al., 2017; Chaturvedi et al., 2016).

Notably, we have recently shown that FGFR and PI3K inhibitors and especially in combination efficiently decrease viability and proliferation of childhood neuroblastoma (NB) cell lines (Kostopoulou et al., 2019). To accumulate more information on the sensitivity of MBs to FGFR and PI3K inhibitors could consequently potentially be of importance. In the current study, we have therefore tested a number MB cell lines, mainly without, but some with PI3K mutations, for their sensitivity to FGFR (AZD4547) and PI3K (BEZ235 and BYL719) inhibitors alone, or in combination.

Materials and Methods

Tumor cell lines and culture conditions. MB cell lines DAOY, UW228-3, (group SHH), Med8a, D425 (Group 3) and D283 (in-between Group 3 and 4), were purchased from ATCC, with the exception of D425, Med8a and UW228-3, that were kindly provided by Prof. M. Nistér (Karolinska Institutet). According to gene bank https://depmap.org/portal/, none of the included cell lines had any FGFR3 mutations, while DAOY had an in frame deletion with a non-conserving PIK3R1 mutation. Furthermore, it has been documented that D425 (Langdon et al., 2006) and Med8a (Bigner et al., 1990) have c-Myc amplifications as all being cell line Group 3 members (Ivanov et al., 2016). DAOY was cultured in Minimum Essential Media (MEM) and UW228-3 in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12). D283 and Med8a were cultured in DMEM/F-12 with GlutaMAX, while D425 was cultured in 50% Richer’s improved MEM with zinc and 50% DMEM. Cells were

maintained in media supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. Media and FBS were obtained from Gibco, Waltham, MA, USA.

Cell seeding and drug treatment. For DAOY 2.5 x10^3 and UW228-3 (adherent cells) 5x10^3 cells were seeded in 90 µl medium/well for the viability assay, and for all other assays 5x10^3 cells, from both cell lines, were seeded in 200 µl medium/well in 96-well plates, and the edges were filled with medium to avoid edge effects. For D425, D283 and Med8a (all growing in suspension), 10^4 cells were used for the viability assay, and 2x10^4 cells were used for the proliferation, apoptosis and cytotoxicity assays. Penicillin and streptomycin were excluded in the media in the different assays to avoid any interference with the drugs.

Dactolisib (BEZ235, NVP-BEZ235) and Alpelisib (BYL719) were used as PI3K inhibitors and AZD4547 was used as FGFR inhibitor (all were purchased from Selleckhem Chemicals, Munich, Germany) and introduced 24 hours (h) after cell seeding. The stocks of the drugs were diluted in DMSO, and later diluted further with PBS and then added to the cells to obtain final drug concentrations. The range of concentrations of BYL719 were for BYL719, 0.25 - 100 µM, for BEZ235, 0.25 - 5.0 µM and for AZD4547, 5.0 - 50 µM. Subsequently, the cells were incubated for 24, 48, 72h and in some cases 96h and different assays were applied to investigate cell viability, cytotoxicity, apoptosis and proliferation. For the experiments with the combination of the FGFR and the PI3K inhibitors the concentrations are denoted in Figures 1-5. All the experiments have been repeated at least three times.

WST-1 viability assay. Viability was measured using the WST-1 assay (Roche Diagnostics, Mannheim, Germany) and is based on WST-1 (tetrazolium salt) is cleaved to formazan by cellular enzymes in viable cells as described previously in more detail (Kostopoulou et al., 2019).

Proliferation assay. For the proliferation assay, the cells were seeded in 200 µl medium/well in a 96-well plate. The plates were placed into the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience, Welwyn Garden City, UK) for 24h and the machine was set to scan the plates every 2h. The cells were treated with drugs 24h (or PBS for control measurements) after seeding and the incubation and scanning were continued for 72h. Cell proliferation was observed by analyzing the confluence of cell in the images.

Cytotoxicity assay. The cytotoxicity assay was performed by using the IncuCyte Red Cytotoxicity reagent (Essen Bioscience, Welwyn Garden City, UK). Once cells become unhealthy plasma membrane integrity diminishes, allowing entry of the IncuCyte Cytotox Reagent and yielding a 100-1000-fold increase in fluorescence upon binding to deoxyribonucleic acid (DNA). Briefly, cells were seeded in 200 µl medium/well in a 96-well plate and were incubated for 24h in the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience, Welwyn Garden City, UK). The machine was set to capture the images of the cells every 2h. The IncuCyte Red Cytotoxicity reagent (Essen Bioscience, Welwyn Garden City, UK) was diluted in PBS in order to get final concentration of 250 nM and was then mixed with the medium in a ratio of 1:4000. In plates with the adherent cells, the medium was discarded and replaced with medium containing the cytotoxicity reagent, while in plates with suspension cells, only half the medium was changed very carefully. Subsequently, the PI3K and FGFR inhibitors were added, either alone, or in combination, and the incubation and scanning were continued for 72h. Cells were quantified by the appearance of red nuclei, as dead cells lost their membrane integrity.

Apoptosis assay. The IncuCyte Caspase-3/7 Green Apoptosis assay (Essen Bioscience, Welwyn Garden City, UK) was performed at the same time as the IncuCyte Cytotoxicity assay (Essen Bioscience, Welwyn Garden City, UK) described above using the identical plates. The IncuCyte Caspase-3/7 Greeen Apoptosis Assay Reagent couples the activated caspase-3/7

recognition motif (DEVD) to NucView™488, a DNA intercalating dye to enable quantification of apoptosis over time. When added to culture, this inert, non- fluorescent substrate crosses the cell membrane, where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and green fluorescent staining of nuclear DNA. Subsequently, 24h after seeding the reagent was mixed with medium containing the cytotoxicity reagent (above) in a ratio of 1:1000. The single and combination treatments were added to the cells. Thereafter, the incubation and scanning of the plates were continued for 72h. Apoptotic cells were automatically visualized and quantified by using observed images and IncuCyte analysis software.
Western blot analysis. Cells were lysed with Pierce RIPA buffer (Thermo Fisher Scientific) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (100X) (Thermo Fisher Scientific). DC (Detergent Compatible) - Protein Assay (BioRad) was used to measure the protein concentration and for further details see (Zhao et al., 2017). The primary antibodies against PI3K (from Cell Signaling Technology, 4249S) were diluted 1:1000; against FGFR3 (abcam, ab133644) 1:2000; and against GAPDH (abcam, ab8245)1:10 000.

Statistical analysis. To determine the effects of single or combination treatments, a multiple t-test accompanied by a correction for multiple comparison of the means conferring to the Holm-Sidak method was performed as described in detail previously (Kostopoulou et al., 2019). To evaluate the combined effects of the drugs the effect-based approach 'Highest Single Agent' and dose-effect-based approach 'median-effect method' (based on Loewe Additivity) was used (Foucquier and Guedj, 2015). A combination index (CI) was calculated as described previously and a CI of <1 was considered a positive and a CI of >1 a negative combinational effect (Kostopoulou et al., 2019). Additionally, the combined effects were analyzed using the median-effect method of Chou (Chou-Talalay method) as previoiusly described (Kostopoulou et al., 2019; Chou, 2010). In short, dose-response curves were fitted to a linear model using the median-effect equation, leading to calculation of a median-effect value D (equivalent to IC50) and slope, also described in detail previously (Kostopoulou et al., 2019). Goodness-of-fit was assessed with the linear correlation coefficient, r; and an r>0.85 was required. The degree of drug interaction was ranked using the CI for mutually exclusive drugs, with CI<0.70 defined as synergy, CI<1.45 as antagonism, and values in between as additive effects. One-way ANOVA with the Bonferroni post-hoc test was used to examine differences in means between the single two drugs and the combination treatment, P<0.05 was considered significant.

Results

Single treatments with FGFR and PI3K inhibitors of MB cell lines DAOY, UW228-3, Med8a, D425, and D283

WST-1 viability analysis following treatment of DAOY, UW228-3, Med8a, D425, and D283 MB cell lines with FGFR and PI3K inhibitors independently. To examine the effect of the treatment on cell viability, WST-1 viability assays were performed 24, 48 and 72 h after the treatment of DAOY, UW228, Med8a, D425, and D283 cell lines. Different concentrations of the FGFR inhibitor AZD4547, and the PI3K inhibitors BEZ235 and BYL719 were used. All absorbance values were compared to that of the PBS control.

AZD4547. The FGFR inhibitor AZD4547 induced a dose-dependent effect on cell viability in all investigated cell lines. All MB cell lines exhibited a ≥50% decrease in absorbance (i.e. a decrease in the number of metabolically active cells) following treatment with 50 µM AZD4547 at 24, 48 and 72 h (at least P<0.0001; Figure 1A-E). All cell lines except UW228-3, also exhibited a ≥50% decrease in viability following treatment with 25 µM AZD4547 at 24, 48, and 72 h (Figure 1A-E)This was also the case for DAOY, Med8a, and D425 with 10 µM AZD4547 for all time points, and for D283 at 48 and 72h (at least P<0.05)

(Figure 1A-E). Treatment with 5 µM AZD4547 was less efficient for all cell lines (Figure 1A-E).

BEZ235. The PI3K inhibitor BEZ235 exerted a dose-dependent effect on cell viability in most investigated cell lines. More specifically, as compared to the PBS control, a ≥50% decrease in cell viability was observed in all MB cell lines following treatment with 5 µM BEZ235 after 48 and 72 h (at least $P<0.05$), and for all cell lines except UW228-3, also exhibiting the decrease after 24 h (at least $P<0.05$; Figure 1F-J). All cell lines except UW228-3 also showed a ≥50% decrease in viability with 0.5-1 µM BEZ235 at 48 and 72 h (at least $P<0.05$), as was the case for DAOY and D425 also at 24h, and with 0.25 µM BEZ235 at all time points (at least $P<0.0001$; Figure 1F-J). Nonetheless, treatment with 0.25 µM BEZ235 was generally less efficient (Figure 1F-J).

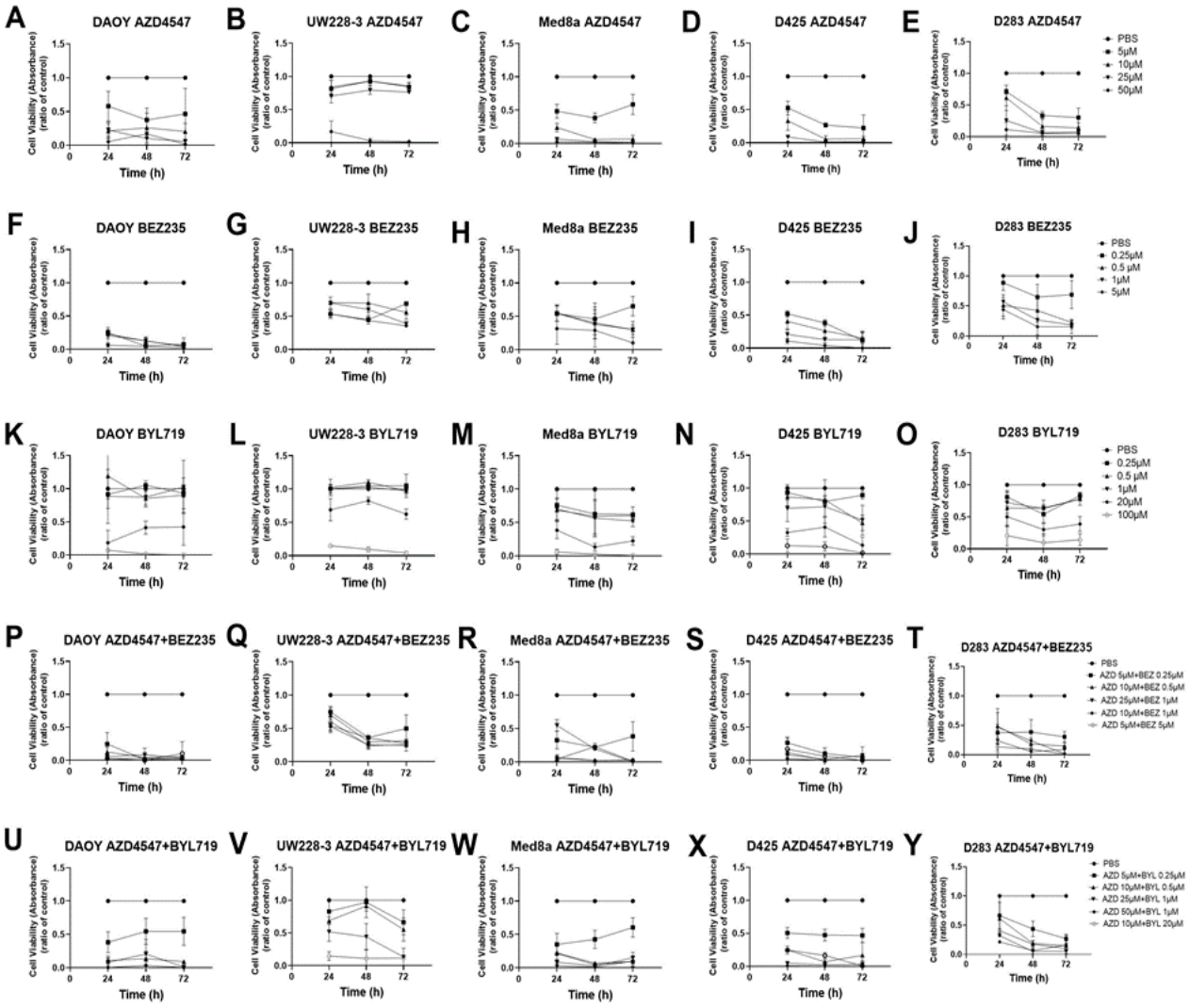


Figure 1. WST-1 viability assays on five MB cell lines. Viability analysis measured as absorbance, after treatment for 24, 48 and 72 h with FGFR inhibitor AZD4547 (A–E) as well as with PI3K inhibitors BEZ235 (F–J) and BYL719 (K–O) on DAOY, UW228-3, Med8a, D425 and D283 respectively. Combined treatment of FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 (P–T) and combined treatment of FGFR inhibitor AZD4547 and PI3K inhibitor BYL719. The graphs represent three experimental runs per cell line and mean with standard deviation (SD) are shown.

BYL719. The PI3K inhibitor BYL719 also exerted a dose-dependent effect in most cell lines, but was relatively less potent than BEZ235 at the concentrations used in this investigation. Treatment with 100 µM BYL719 induced a ≥50% decrease in viability after all time points in all MB cell lines (at least $P<0.01$ for all) (Figure 1K-O). Treatment with 20 µM BYL719 also showed a ≥50% decrease in absorbance for DAOY, Med8 and D425 at all time points, and for D283 at 48h and 72h (at least $P<0.05$ for all), but not for UW228-3 (Figure 1K-O). However, the observed effects tended to decrease with time for many of the cell lines and treatments with 0.25-1 µM BYL719 were in general less or not efficient at all (Figure 1K-O).

IC50. To better evaluate the sensitivity of the different cell lines, IC50 values for all cell lines were calculated and are presented in Table I. The data indicated that D425 was the most sensitive cell line and UW228-3 was the most resistant cell line to treatment with AZD4547, with the other cell lines had intermediate sensitivity. For BEZ235 and BYL719, the IC50 values indicated that UW228-3 cell line was the least sensitive, with all other cell lines having more intermediate sensitivities and BEZ235 being more efficient than BYL719 (Table I).

Cytotoxicity analysis using the IncuCyte Red Cytotoxicity Assay on MB-cell lines DAOY, UW228, Med8a, and D425, following treatment FGFR and PI3K inhibitors independently. The IncuCyte Red Cytotoxicity reagent assay was used to follow cytotoxicity 24 – 72h with FGFR inhibitor AZD4547 and the PI3K inhibitors BEZ235 and BYL719 on MB cell lines DAOY, UW228-3, Med8a, and D425. All cell lines were expressing both PI3K and FGFR3 (Supplementary Figure 1). From the viability test, DAOY, D425 and D283 (one SSH; one Group 3; and one intermediate Group 3 and 4, respectively) were regarded as relatively sensitive lines, and UW228-3 (SHH) as relatively resistant, while Med8a (Group 3) was regarded as sensitive intermediate. No further assays were therefore pursued on the cell line D283 (intermediate Group 3 and 4).

AZD4547. The FGFR inhibitor AZD4547 induced a dose-dependent effect on cell cytotoxicity on DAOY, Med8a and D425, but not on UW228 (Figure 2A-D). Following treatment with 25 µM AZD4547 a substantial significant increase of fluorescent staining, representing cytotoxicity was observed DAOY and D425 throughout the whole observation period, and for Med8a for more than 48 h, but with a drop thereafter (Figure 2A, C, D). A significant cytotoxic dose dependent effect was also observed after treatment with 5 and 10 µM AZD4547 for Med8a and D425, but not for DAOY or UW228-3 (Figure 2A-D).

BEZ235. The PI3K inhibitor BEZ235 did not induce an impressive cytotoxic effect on any of the tested cell lines DAOY, UW228-3, Med8a and D425 as compared to AZD235 (Figure 2E-H). Possibly, for DAOY, Med8a and D425, some minor effect between 48-72h after treatment was observed with the highest dose 5 µM of BEZ235, while for the lower concentrations no significant differences as compared to PBS were observed (Figure 2E-H).

BYL719. The PI3K inhibitor BYL719 did not induce an impressive cytotoxic effect on any of the tested cell lines DAOY, UW228-3, Med8a and D425 either when compared to BEZ235. Similar to BEZ235, marginal effects for the highest dose of 100 µM were possibly observed for DAOY, Med8a and D425 roughly between 48-72h after treatment, while for the lower concentrations no significant differences as compared to PBS were observed (Figure 2I-L).

Table I: WST-1 viability analysis following treatment with the FGFR inhibitor, AZD4547, and PI3K inhibitors, BEZ235 and BYL719 for 24, 48 and 72 h.

			IC50 (μM)		
Cell Lines	**Drugs**		**24h**	**48h**	**72h**
D283	FGFR	AZD4547	11.83	2.773	2.229
	PI3K	BEZ235	2.855	0.404	0.336
		BYL719	7.899	1.494	6.786
D425	FGFR	AZD4547	5.611	3.384	2.742
	PI3K	BEZ235	0.2791	0.1545	<0.250[a]
		BYL719	5.858	6.176	0.961
DAOY	FGFR	AZD4547	5.463	2.177	4.613
	PI3K	BEZ235	<0.250[a]	<0.250[a]	0.001
		BYL719	6.972	10.19	18.56
Med8a	FGFR	AZD4547	4.814	4.303	5.422
	PI3K	BEZ235	0.763	<0.250[a]	0.351
		BYL719	3.620	0.960	0.974
UW228-3	FGFR	AZD4547	30.54	30.48	29.40
	PI3K	BEZ235	0.001	3.815	0.938
		BYL719	32.94	38.25	24.91

The IC50 (inhibitory concentration 50%) for each cell line for each drug was determined from log concentrations-effect curves in GraphPad Prism using non-linear regression analysis. FGFR, fibroblast growth factor receptor; PI3K, phosphatidylinositol 3-kinase. [a] The IC50 value could not be determined; lowest/highest tested concentration closest to the IC50 is reported.

Apoptosis analysis using the IncuCyte Caspase-3/7 Green Apoptosis assay on MB cell lines DAOY, UW228-3, Med8a, and D425, following treatment FGFR and PI3K inhibitors independently.

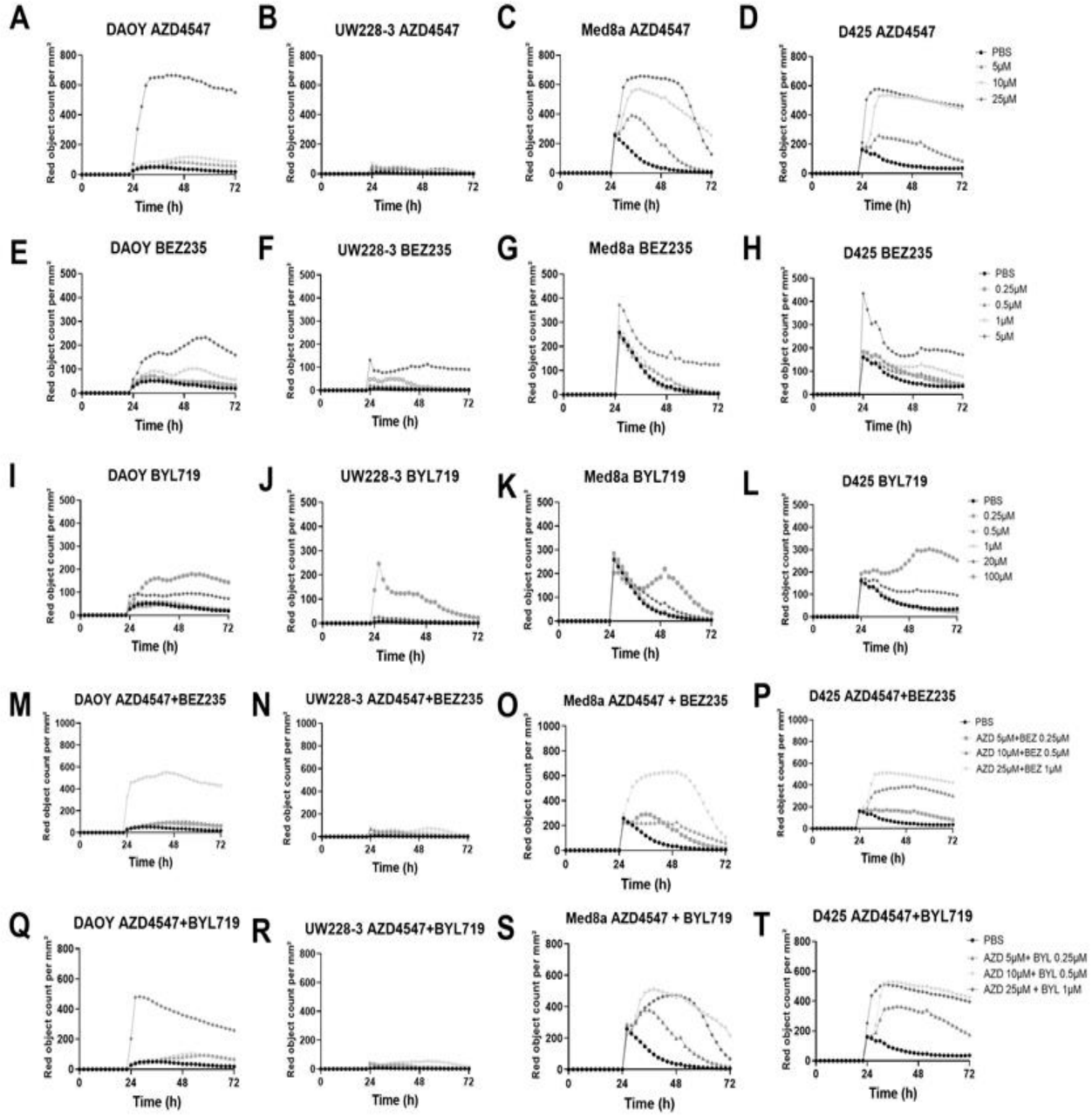


Figure 2. *Cytotoxicity analysis using the IncuCyte Red Cytotoxicity Assay* on MB cell lines DAOY, UW228-3, Med8a, and D425. The cells were treated 24h after seeding and the assay was performed up to 72h with FGFR inhibitor AZD4547 (A–D) and with PI3K inhibitor BEZ235 (E-H) and BYL719 (I-L) on the above-mentioned MB cell lines. Combined treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 on them (M-P) and AZD4547 and BYL719 (Q-T).

AZD4547. The FGFR inhibitor AZD4547 induced an effect on cell apoptosis when used at a concentration of 25 μM on DAOY, Med8a and D425 throughout the whole observation period, while its effect was non-significant on UW228-3 (Figure 3A-D). Treatments with lower AZD4547 concentrations did not induce significant effects.

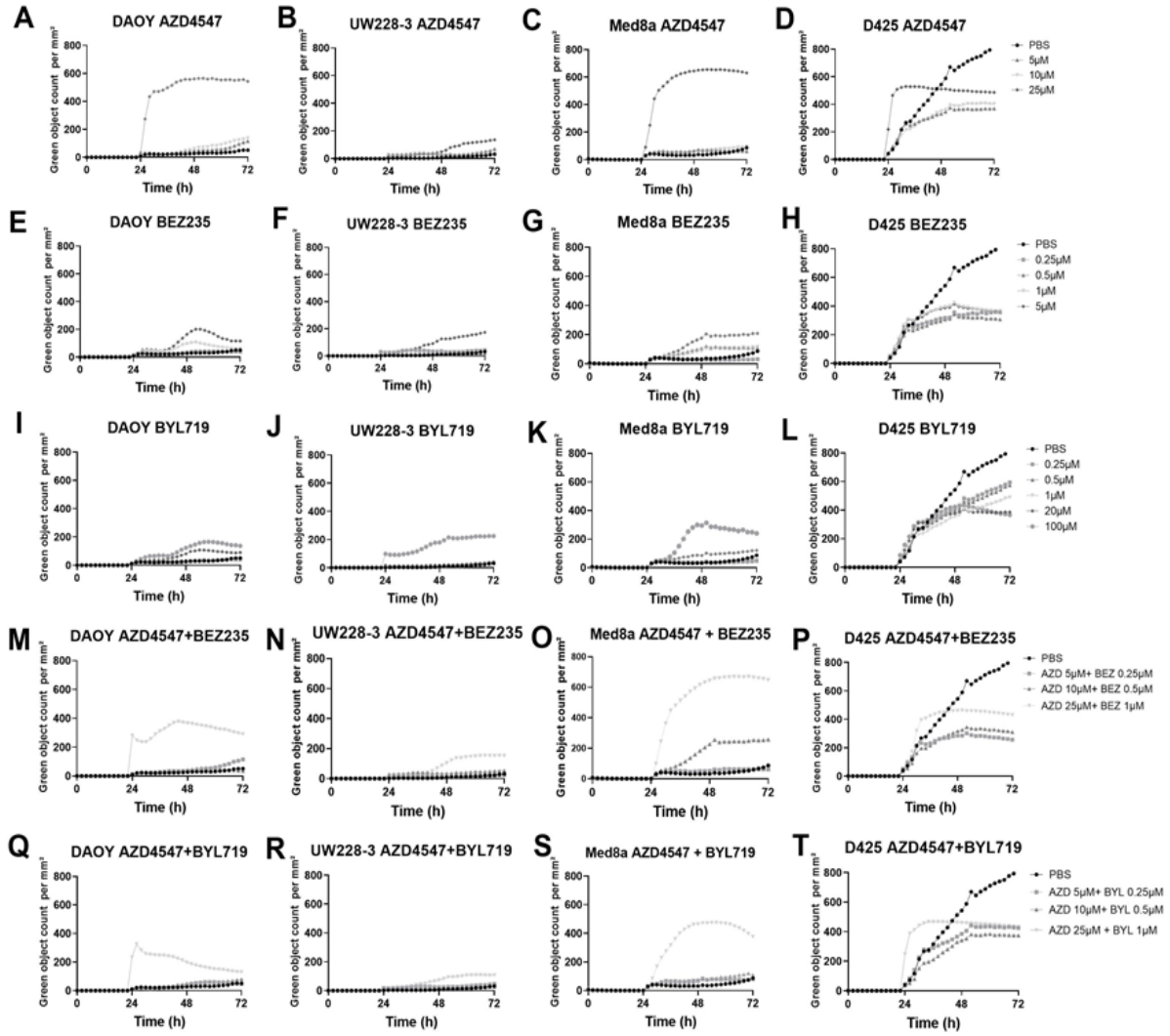


Figure 3. Caspase-3/7 Green Apoptosis assay on cell lines DAOY, UW228-3, Med8a, and D425 with IncuCyte S3 Live-Cell Analysis System. The cells were treated with FGFR inhibitor AZD4547 (A-D), with PI3K inhibitor BEZ235 (E-H) and BYL719 (I-L) 24h after seeding, and the experiment was performed up to 72h, on the four medulloblastoma cell lines. Combined treatment with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 on them (M-P) and AZD4547 and PI3K BYL719 (Q-T).

BEZ235. Treatment with the PI3K inhibitor BEZ235 did not induce significant increases at any of the concentrations used in apoptosis in comparison to AZD4547 in any of the cell lines (Figure 3E-H).

BYL719. Treatment with the PI3K inhibitor BYL719 did not induce significant increases at any of the concentrations used in apoptosis in comparison to AZD4547 in any of the cell lines (Figure 3E-H). However, when 100 μM of BYL719 were used possibly marginal effects were observed for Med8a at 48 h (Figure 3F).

Proliferation assay using the IncuCyte System on MB cell lines DAOY, UW228, Med8a, and D425 after treatment with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BYL719 independently.

AZD4547. All tested MB cell lines MB cell lines DAOY, UW228-3, Med8a, and D425 showed a complete or near complete inhibition of proliferation after treatment with 25 μM

AZD4547 (Figure 4A-D). After treatment with 10 μM AZD4547 inhibition of growth was still observed for Med8a and D425, while this was not the case for the other two lines (Figure 4A-D). Furthermore, D425 was the most sensitive cell line and treatment with 5 μM AZD4547 also inhibited its growth, while for the other cell lines no or only intermediate effects were observed (Figure 4A-D).

BEZ235. After treatment with 5 μM BEZ235 growth inhibition was observed of all four tested MB cell lines. Med8a and D425 were also inhibited in their cell growth after treatment with 1 μM BEZ235 and this was the case for D425 also with 0.5 μM BEZ235, while the effects for the other cell lines were not as prominent. (Figure 4E-H).

BYL719. After treatment with 100 μM BYL719, all MB cell lines were inhibited in their cell growth. This was the case also for 20 μM BYL719, except for DAOY. At all other drug concentrations, the inhibition of proliferation was not as prominent.

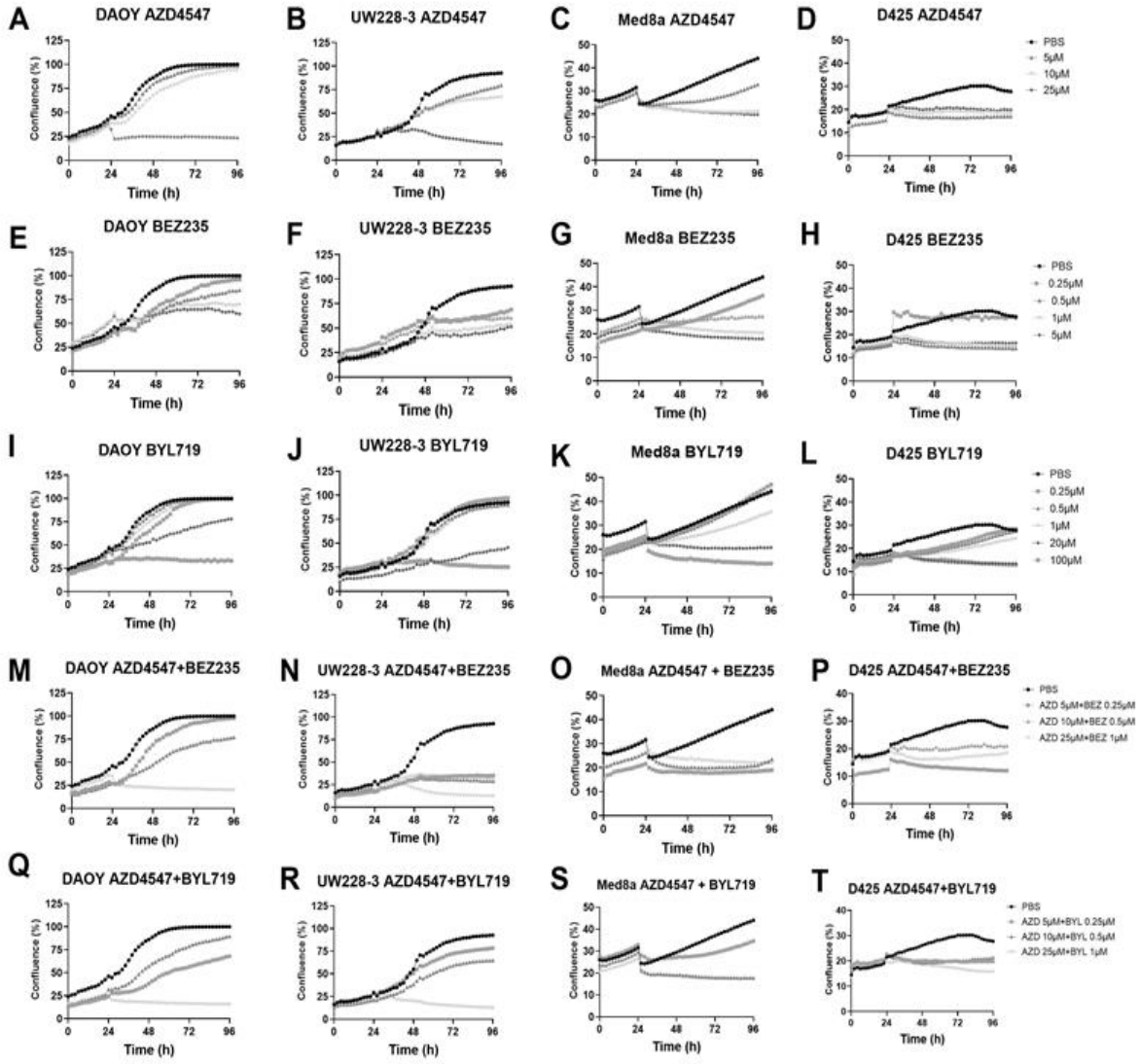


Figure 4. Proliferation assays on cell lines DAOY, UW228-3, Med8a, and D425 Proliferation assays were performed with Incucyte S3 Live-Cell Analysis System. The cells were treated with FGFR inhibitor AZD4547 (A–D) and with PI3K inhibitors BEZ235 (F-H) and BYL719 (I-L) 24h after seeding and the confluency was measured on the above MB cell lines. Combined

treatment with AZD4547 and BEZ235 (M-P) and AZD4547 with BYL719 on them (Q-T). The graphs represent one typical experiment.

Combination treatments with the FGFR inhibitor and the PI3K inhibitors BEZ235 and BYL719 on MB-cell lines DAOY, UW228-3, Med8a, D425, and D283.

General setup. The inhibitory effect on cell viability and proliferation following treatment with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BYL719 has been described above. To further investigate the potential therapeutic role of these drugs and examine whether lower drug concentrations could be used, an analysis was performed combining the two types of drugs. Cell viability was analyzed on all five MB cell lines. Furthermore, cytotoxicity, apoptosis and proliferation were analyzed on all MB cell lines except D283 using the IncuCyte as described above.

WST-1 viability analysis following treatment of on MB-cell lines DAOY, UW228-3, Med8a, D425, and D283 with FGFR inhibitor AZD4547 and PI3K inhibitor BEZ235 or BYL719 combined. All AZD4547 and BEZ235 combinations induced a dramatic decrease in viability for DAOY and D425 with ≥50% decrease in absorbance at all time points after treatment (at least $P<0.01$) (Figure 1 P, S). A decrease in viability was also observed for the three remaining MB cell lines UW228-3, Med8a and D283, especially after 48 and 72 h after treatment with almost all AZD4547 and BEZ235 combinations (at least $P<0.05$; Figure 1 Q, R, T). However, the 5 μM AZD4547 and 0.25 μM BEZ235 combination was slightly less efficient in inducing a ≥50% decrease especially after 72h in the three latter cell lines UW228-3, Med8a and D283, indicating a possible resistance.

Also the AZD4547 and BYL719 combination showed some positive effects, but they were not as prominent at the AZD4547 and BEZ235 combinations. Treatments with 25 μM AZD4547 and 1.0 μM BYL719; and 10 μM AZD4547 and 20 μM BYL719 induced ≥50% decrease in viability for all five MB cell lines, and this was the case for DAOY, Med8, D425 and D283 at all time points (at least $P<0.05$; Figure 1U-Y). For UW228-3 this was also the case at all time points for highest doses, but only after 48 and 72 h for the latter dose (at least $P<0.0001$; Figure 1V). The two remaining combinations also had some effects on all cell lines, but not as prominent and not at all time points and especially not for UW228-3 (Figure 1U-Y).

Analyses of the combinational effect were also performed to further evaluate possible synergism, additive effects or antagonism between the two drug combinations. Two different methods were used: the effect-based Highest Single Agent approach and dose-effect-based median-effect principle. Combination Index (CI) from both approaches assessing combinational effects at 24 h are displayed in Figure 5. The majority the tested combinations were rated as positive by the Highest Single Agent approach (for BYL719: 18 out of 25 and for BEZ235 19 out of 25 tested combinations, Figure 5A, B) and additive/synergistic by the median-effect principle (for BYL719: 18 out of 25 and for BEZ235 13 out of 15 tested combinations that could be evaluated, Figure 5C, D). For the resistant cell line UW228-3 significant improved effect of the combinations of AZD4547 and BEZ235 compared to single drug treatment was observed after 48 h in four out of five tested combinations (at least $P>0.05$; Figure 5E-I).

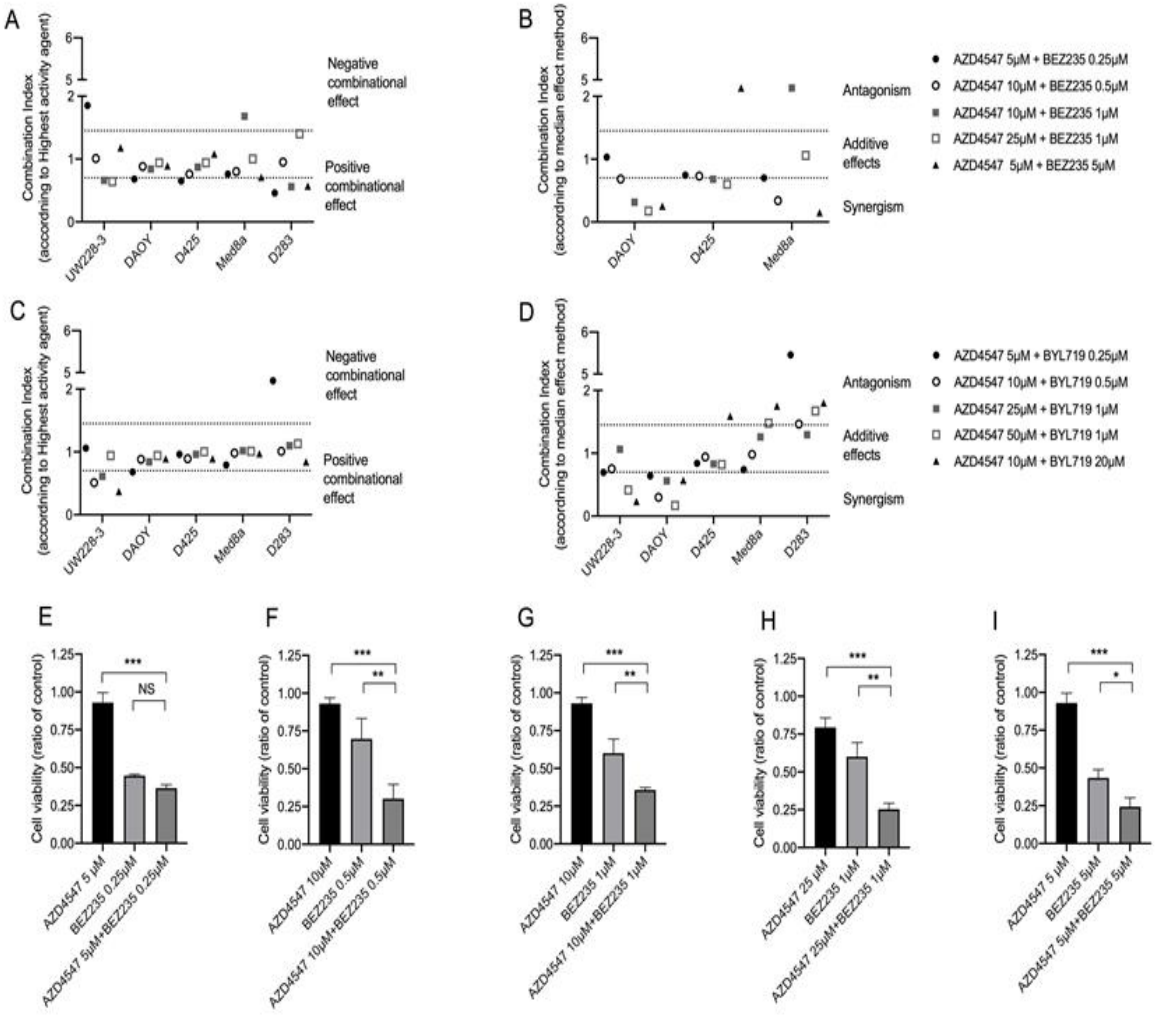


Figure 5. Combinational treatment with AZD4547, BEZ235 and BYL719 in MB cell lines. Combination Index (CI) from analyses of combinational effects from treatments with AZD4547, BEZ235 (A, B) and BYL719 (C, D) for 24 h in MB cell lines. CIs were obtained from Highest Activity Agent method (A, C) where CI<1 indicate a positive combination effect and CI>1 a negative effect, and Median-effect method (B, D), where CI<0.7 suggest synergy, CI>1.45 antagonism and 0.7<CI>1.45 additive combinational effects. CIs were calculated from mean of three experiments, analysed with WST-1. The combination of AZD4547 and BEZ235 could not be analysed with the median-effect method due to poor goodness-of-fit of BEZ235 (r<0.85) in D283 and UW228-3 cell lines and is therefore not presented in (C).

Cytotoxicity analysis using the IncuCyte Red Cytotoxicity Assay on MB cell lines, DAOY, UW228-3, Med8a, and D425 following treatment FGFR and PI3K inhibitors in combination.

AZD4547 and BEZ235. The combined use of 25 µM AZD4547 and 1 µM BEZ235 resulted in a similar increase in fluorescence, i.e. representing cytotoxicity, as the use of 25 µM AZD4547 alone for DAOY, Med8a and D425, while no increase was observed for UW228-3 (Figure 2M-P).

AZD4547 and BYL719. The combined use of 25 µM AZD4547 and 1 µM BYL719 resulted in a similar cytotoxicity as the use of 25 µM AZD4547 alone (Figure 2Q-T). More specifically, an increased fluorescence was observed for DAOY, Med8a and D425, while no increase was observed for UW228-3 (Figure 2Q-T).

Apoptosis analysis using the IncuCyte Caspase-3/7 Green Apoptosis assay on MB cell lines DAOY, UW228-3, Med8a, and D425, following treatment FGFR and PI3K inhibitors in combination.

AZD4547 and BEZ235 in combination resulted in a similar apoptosis pattern as the use of 25 µM AZD4547 alone, however the effects seemed less prominent for DAOY and D425 (Figure 3M-P).

AZD4547 and BYL719. The combined use of 25 µM AZD4547 and 1 µM BYL719 resulted in a similar apoptosis pattern as the use of 25 µM AZD4547 alone, however the effects seemed less prominent for DAOY and Med8a (Figure 3Q-T).

Proliferation assay using the IncuCyte System on MB cell lines DAOY, UW228-3, Med8a, and D425 after treatment with FGFR inhibitor AZD4547 and PI3K inhibitors BEZ235 and BYL719 in combination.

AZD4547 and BEZ235 in combination. Using a combination of 25 µM AZD4547 and 1.0 µM BEZ235 inhibited proliferation of the four tested MB-cell lines (Figure 4M-P). In addition, the combinations 10 µM AZD4547 and 0.5 µM BEZ235, and 10 µM AZD4547 and 0.25 µM BEZ235 were also efficient in inhibiting growth of all cell lines except DAOY (Figure 4M-P).

AZD4547 and BYL719 in combination. Using a combination of 25 µM AZD4547 and 1.0 µM BYL719, inhibited proliferation of the four tested MB-cell lines as would be expected (Figure 4Q-T). In addition, the combination 10 µM AZD4547 and 0.5 µM BYL719, inhibited growth of Med8a and D425 (Figure 4S, T), and this was also the case for D425 with the lowest 5 µM AZD4547 and 0.5 µM BYL719 combination (Figure 4T). For the two latter lower AZD4547 and BYL719, intermediate growth inhibiting effects as compared to PBS were observed for DAOY and UW228-3 (Figure 4Q, R).

Discussion

In this study, FGFR and PI3K inhibitors were tested either alone or in combination for their ability to inhibit the growth of five MB cell lines. Of these, DAOY had an in frame deletion with a non-conserving PIK3R1 mutation (gene bank https://depmap.org/portal/) and D425 and Med8a had MYC amplifications (Ivanov et al., 2016; Langdon et al., 2006; Bigner et al., 1990). All five MB cell lines were more or less sensitive to the FGFR (AZD4547) and PI3K (BEZ235, BYL719) inhibitors alone, as shown by decreases in viability and proliferation, while only the FGFR inhibitor AZD4547 induced pronounced effects on cytotoxicity or apoptosis. Moreover, when combining the two types drugs, the inhibitory effects on viability increased especially of the most resitant cell line UW228-3. Likewise upon combination treatments, inhibition of proliferation was maintained or increased, especially in the most resitant cell line UW228-3, while effects on cytotoxicity and apoptosis were not prominent.

In the viability assays, D425 (Group 3), followed by D283 (intermediate Group 3 and 4) were the most sensitive to the FGFR inhibitor AZD4547, while UW228-3 (SHH group) was the least sensitive, and DAOY (SHH group) and Med8a (Group 3) exhibited intermediate sensitivity. At the concentrations used in these assays, the PI3K inhibitor BEZ235 was in general more efficient compared to BYL719. DAOY, followed by D425 were the most sensitive cell lines to BEZ235, while UW228-3 was the least sensitive, and Med8a and D283 exhibited intermediate sensitivity. All cell lines showed a decrease in viability after treatment with the two highest BYL719 concentrations, with the exception for UW228-3 that was only sensitive to the highest concentration.

Upon combination treatments, an enhanced decrease in viability was observed especially for the UW228-3 cell line, which was the least sensitive cell line, while all other MB cell lines retained the sensitivity they had to the drug they were most sensitive to. Furthermore, at the concentrations used in this report, the dual PI3K inhibitor, BEZ235, was more efficient on single and combination with FGFR inhibitor treatment than the p110a specific PI3K inhibitor, BYL719, in all MB cell lines.

The marked sensitivity of DAOY to PI3K inhibitor BEZ235, in the viability tests could be explained due to that DAOY has an in frame deletion with a non-conserving PIK3R1 mutation.

The overall sensitivity of the D425 and Med8a (Group 3) as well as DAOY and D283, to both types of inhibitors, and especially to PI3K inhibitors is unknown. However, D425 and Med8a (Group 3) have MYC amplifications and DAOY and D283 have overexpression of MYC (Ivanov et al., 2016; Langdon et al., 2006; Bigner et al., 1990). Whether presence of high MYC, similar to the presence of *MYCN* amplifications increases their sensitivity especially to mTOR kinase inhibitors needs to be pursued (Chaturvedi et al., 2018). Nevertheless, it is known that MYC and MYCN have the same phosphorylation sites when tagged for ubiquitination and it has been shown that inhibition of mTOR-kinase destabilizes MYCN and could therefore be a potential therapy for MYCN-dependent tumours (Vaughan et al., 2016). Nonetheless, since the development of resistance against targeted therapy has been reported when using one drug alone, the alternative of using two drugs in combination could be of benefit (Gustafson and Weiss, 2010; Cai, Song and Ai, 2019; Hsu et al., 2018).

In this study, only the FGFR inhibitor (AZD4547), but not the PI3K inhibitor, exhibited observed cytotoxicity and apoptosis. These findings are in line with previous reports by others (Segerström et al., 2011) and us (Kostopoulou et al., 2019).

The PI3K inhibitor (BEZ235) concentrations used here on the MB cell lines were slightly higher than those used in a previous study, but similar to those used by us on neuroblastoma (NB) cell lines (Kostopoulou et al., 2019, Cai, Song and Ai, 2019). The concentrations for the other PI3K inhibitor (BYL719) were however similar to those in a recent study in neuroendocrine tumors (Brands et al., 2017). For the FGFR inhibitor AZD4547, the concentrations which were used were similar to those used by others and by us in similar and different systems (Kostopoulou et al., 2019; Nolting et al., 2017). It is possible that cell lines with FGFR or PI3K mutations may be sensitive to lower drug concentrations, and such cell lines remain to be tested. Nevertheless, it has been reported that PI3K mutations do not always affect drug sensitivity (Munster et al., 2016). However, it has been documented that the sensitivity to FGFR inhibitors is affected in cervical tumors harboring FGFR3-TACC3-fusion genes as compared to those not harboring such genes (Tamura et al., 2018).

There are limitations in this study, since only five MB cell lines were examined. Furthermore, a limited number of drug concentrations were used. Nevertheless, to our knowledge, this is the first time that drugs against FGFR and PI3K were used in combination for treatment of MB cell lines, although PI3K inhibitors have been documented previously for MB cell lines and have shown favorable effects (Zhao et al., 2017; Cai, Song and Ai, 2019).

To conclude, treatment of five MB - cell lines with either FGFR and PI3K inhibitors alone induced a decrease in viability and proliferation in a dose dependent pattern although the sensitivity of the cell lines varied. Some of the variation in sensitivity of the cell lines could possibly be due to an enhanced sensitivity observed in those with c-Myc amplifications or a PI3K variation, but this need to be pursued further. However, upon FGFR and PI3K inhibitor combination treatments the sensitivity of all cell lines increased to some extent, especially in the most resistant cell line, suggesting that further studies on combination treatments may provide a possible therapeutic opportunity for therapy of resistant MBs.

Holzhauser S, Wild N, Zupancic M, Ursu RG, Bersani C, Näsman A, Kostopoulou ON, Dalianis T. Targeted Therapy with PI3K and FGFR Inhibitors on Human Papillomavirus Positive and Negative Tonsillar and Base of Tongue Cancer Lines With and Without Corresponding Mutations. Front Oncol. 2021 May 11;11:640490. IF=6.244

Simple Summary: The incidences of human papillomavirus positive tonsillar and tongue base cancer are increasing, but although a majority of the patients are cured with present treatments, upon recurrence prognosis is poor so new therapies are needed. Since, such tumors often have some specific mutations (PI3K and FGFR3), here, we tested targeted therapy (BYL719 and JNJ-42756493 resp.) directed to such mutations. We also combined the two types of inhibitors and used them with each other or with either cisplatin or docetaxel that are used clinically, as well as tested the cell lines for corresponding mutations. All cell lines, irrespective of having mutations or not, displayed dose dependent responses to the inhibitors and the chemotherapeutic agents, but the sensitivity of the cell lines to the inhibitors and chemotherapeutic agents was not correlated. However, when combining the inhibitors, or the inhibitors with the chemotherapeutic drugs positive effects were often obtained.

Abstract: Human papillomavirus positive (HPV^+) tonsillar and base of tongue squamous cell carcinoma (TSCC/BOTSCC), the major subsites of oropharyngeal squamous cell carcinoma (OPSCC) have favorable outcome, but upon relapse, prognosis is poor and new therapies needed. Since, phosphatidyl-inositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) and fibroblast-growth-factor-receptor-3 (FGFR3) mutations occur in such tumors, here we examined the use of phosphoinositide 3-kinase (PI3K) and FGFR inhibitors in analogous cell lines. The anti-tumor efficacy of the recently food and drug administration (FDA) approved PI3Kα and FGFR inhibitors alpelisib (BYL719) and erdafitinib (JNJ-42756493) were tested alone, or combined, and with/without cisplatin and docetaxel on HPV^+ CU-OP-2, -3, -20 and UPCI-SCC-154 and HPV-negative (HPV^-) CU-OP-17 and UT-SCC-60A. Viability, proliferation, apoptosis and cytotoxicity were analyzed by WST-1 assays and the IncuCyte S3 Live® Cell Analysis System. The cell lines were also tested for PIK3CA/FGFR3 mutations by competitive-allele-specific TaqMan-PCR. HPV^+ CU-OP-2 had a pS249C-FGFR3, and like CU-OP-20, a pE545K-PIK3CA mutation, while no other lines had such mutations. Irrespectively, dose dependent responses to all PI3K/FGFR inhibitors were obtained, and upon combining the inhibitors, positive effects were observed. Cisplatin and docetaxel also induced dose dependent responses, and upon combination with the inhibitors, both positive and neutral effects were found.

Keywords: HPV, tonsillar cancer, base of tongue cancer, oropharyngeal cancer, FGFR, PI3K, targeted therapy

1. Introduction

The incidences of human papillomavirus positive (HPV^+) tonsillar and base of tongue squamous cell carcinoma (TSCC and BOTSCC resp.), the two major subsites of oropharyngeal squamous cell carcinoma (OPSCC), have increased in many countries (Haeggblom et al., 2019; Ernster et al, 2007; Chaturvedi et al, 2011; Garnaes et al., 2015; Rietbergen et al., 2018; Pytynia, Dahlstrom and Sturgis, 2013; Näsman et al., 2020). Usually, HPV^+ TSCC and BOTSCC have a favorable outcome, better than corresponding HPV negative (HPV^-) cancer (Näsman et al., 2020;Näsman et al., 2020; Dahlstrand et al., 2020; Ang et al., 2010; Lassen et

al., 2009; Lindquist et al., 2007; Bersani et al., 2017). Therefore, many patients with HPV^+ TSCC/BOTSCC, do not need the aggressive therapy presently given to head neck squamous cell carcinoma (HNSCC) patients, which has also not improved their overall or disease specific survival (Näsman et al., 2020; Dahlstrand et al., 2020; Ang et al., 2010; Lassen et al., 2009; Lindquist et al., 2007; Bersani et al., 2017). To further develop personalized medicine, and to either de-escalate or find alternative targeted therapies, attempts have been made to find prognostic biomarkers (Bersani et al., 2017; Näsman et al., 2017). High/low CD8+ tumor infiltrating lymphocyte counts, HPV16 E2 mRNA expression, absent/low/high HLA class I, HLA-A*02, CD44, LMP10, LRIG1 and CD98 expression, are such examples (Lindquist et al., 2012; Näsman et al., 2013; Näsman et al., 2013; Näsman et al., 2013; Tertipis et al., 2014; Tertipis et al., 2014; Tertipis et al., 2015; Rietbergen et al., 2014; Näsman et al., 2012; Nordfors et al., 2013; Oguejiofor K et al., 2015; Oguejiofor K et al., 2017; Lindquist D et al., 2014; Lechner et al., 2013). Furthermore, by next generation sequencing, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) and fibroblast growth factor receptor 3 (FGFR3) mutations were revealed to be frequent in HPV^+ TSCC/BOTSCC (Tinhofer et al., 2016; Bersani et al., 2017; Bersani et al., 2018). Moreover, in one study, we found FGFR3 mutations were associated to poorer survival, while in other reports, others and we found FGFR3 overexpressed in TSCC/BOTSCC and OPSCC (Bersani et al., 2018; Koole et al., 2016; Beaty et al., 2020). Likewise, it has just recently been reported that PI3KCA mutations in HPV^+ OPSCC are correlated to worse prognosis (Ghedini et al., 2018). Since targeted therapies, against FGFR1-4 (thereby, including FGFR3) and phosphoinositide 3-kinases (PI3K), (thereby, including PIK3CA) have been initiated for other cancers with such mutations (Arafeh and Samuels, 2019; Holzhauser et al., 2019), we proposed analagous therapies could be useful for TSCC/BOTSCC.

We therefore tested HPV^+ cell lines UM-SCC-47 and UPCI-SCC-154, and the HPV^- cell line UT-SCC-60A for FGFR3 and PIK3CA mutations and for their sensitivity to FGFR inhibitor AZD4547, or PI3K inhibitors BEZ235 and BKM120, alone or combined (André et al., 2019). None of the cell lines displayed the most common FGFR or PIK3CA mutations, when tested by competitive allele-specific TaqMan-PCR (CAST-PCR), but all exhibited dose dependent sensitivity to the drugs used, and upon combination of these drugs, synergy was observed (André et al., 2019). Since then, the Food and Drug Administration (FDA) has approved alpelisib (BYL719, a PI3K inhibitor) and erdafitinib (JNJ-42756493, an FGFR inhibitor) (Bahleda et al., 2019; White et al., 2007). Moreover, we obtained additional cell lines with PIK3CA and FGFR3 mutations (see below).

Here, we have extended our previous study. The effects of FDA approved alpelisib and erdafitinib were examined on HPV^+ UPCI-SCC-154, and HPV^- UT-SCC-60A, as well as on the newly established HPV^+ CU-OP-2, -3, -20 and HPV^- CU-OP-17 lines, of which two here were shown to have FGFR3 and/or PIK3CA mutations. In addition, we also combined the above inhibitors with each other and previously tested inhibitors BEZ235 and AZD4547, as well as cisplatin and docetaxel, the latter, two chemotherapeutic drugs, frequently used clinically for treatment of TSCC/BOTSCC patients.

2. Results

2.1 Detection of FGFR3 and PIK3CA mutations in HPV^+ and HPV^- cell lines

The three most common FGFR3 or PIK3CA mutations were analyzed for by CAST-PCR as described before (Koole et al., 2016; Lange et al., 2008). CU-OP-2 had a pS249C (FGFR3) and a pE545K (PIK3CA) mutation, while CU-OP-20 presented only the latter, while CU-OP-

3 and -17 had no such mutations (data not shown), and this was also the case for HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A (André et al., 2019).

2.2. Effects after single exposure to PI3K and FGFR inhibitors in HPV$^+$ (CU-OP-2, -3, -20, UPCI-SCC-154) and HPV$^-$ (CU-OP-17 and UT-SCC-60A) cell lines measured by WST-1 assays

All cell lines showed dose dependent responses to PI3K inhibitors BYL719 (0.5-20 µM) and BEZ235 (0.25-1 µM) and FGFR inhibitors JNJ-42756493 (0.01-10 µM) and AZD4547 (5-25 µM) compared to PBS when tested by WST-1 assays, evaluating cellular metabolic capacity by absorbance. Data summarizing three experiments per cell line and all inhibitors for 72 h after treatment are shown for HPV$^+$ CU-OP-2, -3 and -20 and HPV$^-$ CU-OP-17 in Figure 1. For HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A data on BYL719 and JNJ-42756493 are presented in Figure 2A-D, while data on BEZ235 and AZD4547 have been published before (André et al., 2019IC_{50} values are presented in Table 1. Briefly, HPV$^+$ CU-OP-20 and HPV$^+$ UPCI-SCC-154 tended to be sensitive lines to all inhibitors, while HPV$^+$ CU-OP-2 and -3 were more resistant, with remaining lines having intermediate sensitivity.

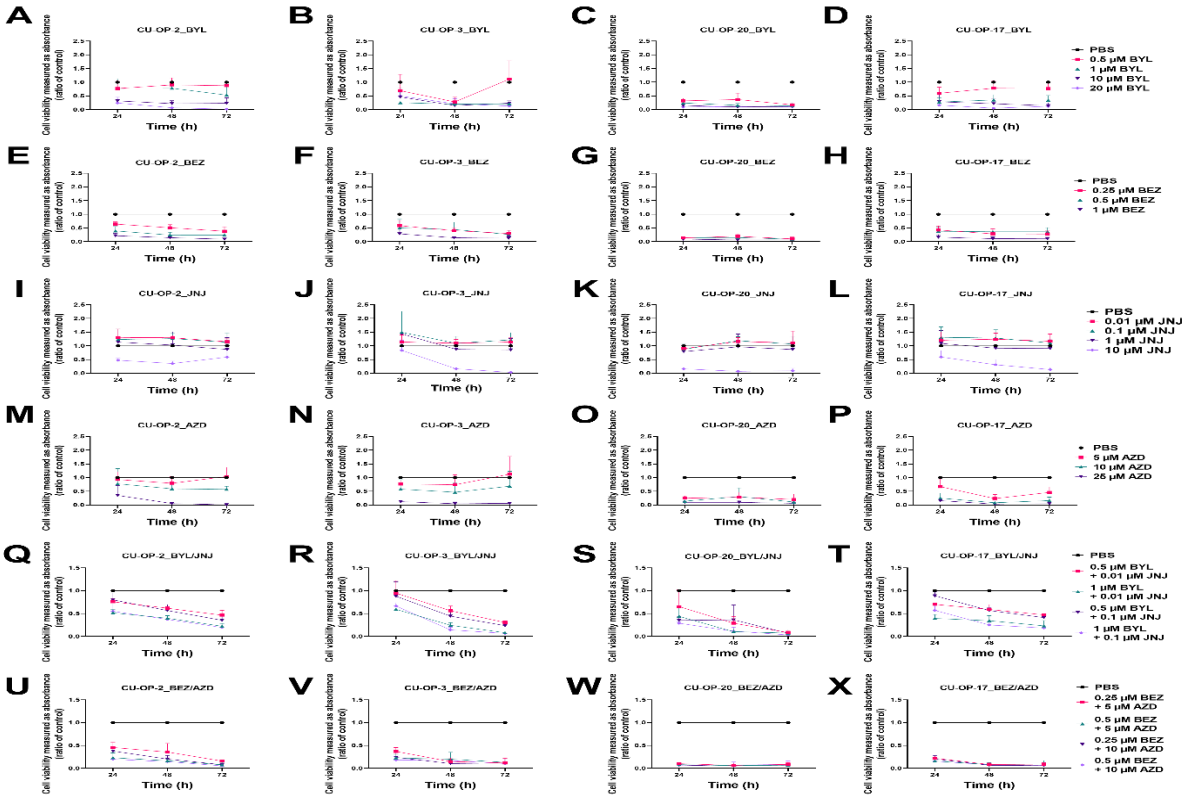


Figure 1. WST-1 viability assays on HPV+ CU-OP-2, -3, -20 and HPV- CU-OP-17 cell lines upon treatment with PI3K inhibitors (BYL719, BEZ235), and FGFR inhibitors (JNJ-42756493, AZD4547). WST-1 viability assays measuring absorbance following treatment for 24, 48 and 72 h of HPV+ CU-OP-2, -3, -20 and HPV- CU-OP-17 with PI3K and FGFR inhibitors, BYL719 (A-D); BEZ235 (E-H); JNJ-42756493 (I-L); and AZD4547 (M-P); and combinational treatments with BYL719 and JNJ-42756493 (Q-T); as well as BEZ235 and AZD4547 (U-X). The graphs represent three experimental runs per cell line and results are presented as the mean ± standard deviation. BYL denotes BYL719; BEZ denotes BEZ235; AZD denotes AZD4547 and JNJ denotes JNJ-42756493.

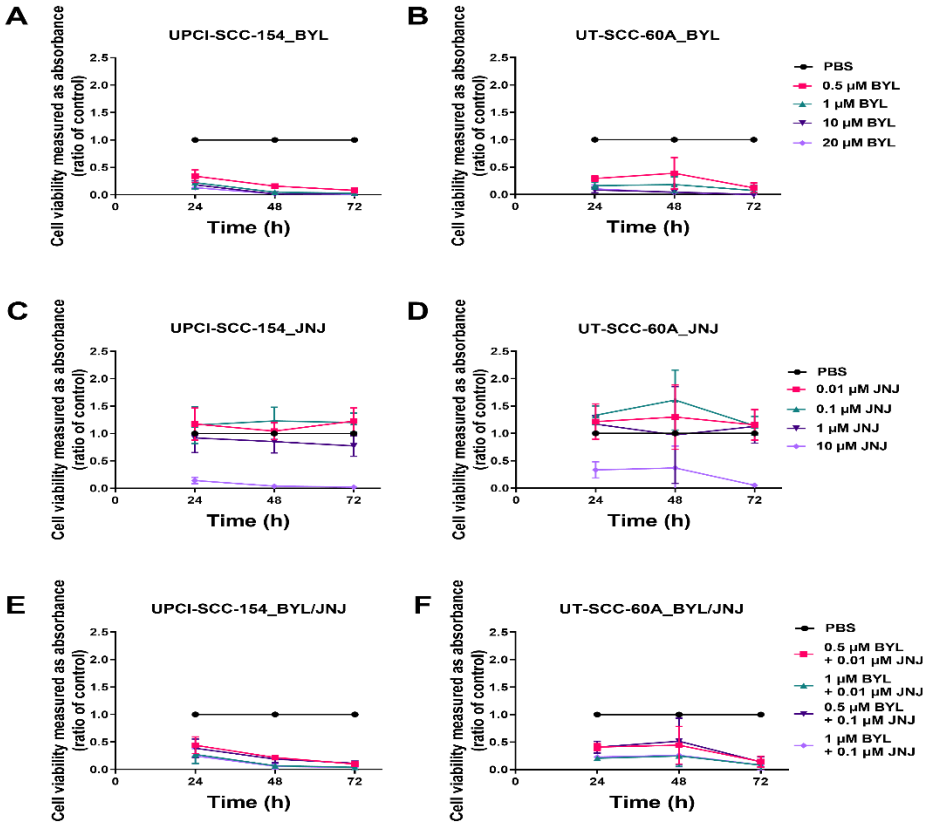


Figure 2. WST-1 viability assays on HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A cell lines upon treatment with PI3K inhibitor BYL719, and FGFR inhibitor JNJ-42756493. WST-1 viability assay measuring absorbance following treatment for 24, 48 and 72 h of HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A with PI3K and FGFR inhibitors, BYL719 (A and B); and JNJ-42756493 (C and D); as well as combinational treatment with BYL719 and JNJ-42756493 (E and F). The graphs represent three experimental runs per cell line and the data are presented with the mean ± standard deviation. BYL denotes BYL719 and JNJ denotes JNJ-42756493.

HPV$^+$ CU-OP-2, -3 and -20 and HPV$^-$ CU-OP-17. Compared to PBS, the highest BYL719 dose (20 µM) decreased viability consistently in all CU-OP lines ($p<0.01$) (Figure 1A-D). Declines in viability were also obtained with 1-10 µM BYL719 for all cell lines at most time points (except CU-OP-2 with 1 µM at all-time points, and CU-OP-3 with 10 µM after 24 h), while with 0.5 µM BYL719 a consistent decrease in viability was only observed for CU-OP-20 (for all, at least $p<0.01$) (Figure 1A-D). BEZ235 at all doses decreased viability in all cell lines the whole 72 h period when compared to PBS, except for CU-OP-2 and CU-OP-3 with 0.5 µM after 24 h (for all others, at least $p<0.05$) (Figure 1.E-H). Notably, only the highest dose JNJ-42756493 reduced viability significantly compared to PBS in all CU-OP lines, at most time points (except CU-OP-2 at 72 h and CU-OP-3 at 24 h) (for all, at least $p<0.05$) (Figure 1I-L). Likewise, only the highest AZD4547 dose (25 µM) decreased viability in all CU-OP lines compared to PBS the whole 72 h period (for all, at least $p<0.01$) (Figure 1M-P), however for HPV$^+$ CU-OP-20 and HPV$^-$ CU-OP-17, this also the case for 5-10 µM AZD4547 at 48 and 72 h (for all, at least $p<0.01$), while CU-OP-2 and -3 tended to be more resistant (Figure 1M-P).

Table 1. Estimation of inhibitory concentration 50 % (IC50) based on WST-1 viability analysis following treatment with the FGFR inhibitor (JNJ-42756493 and AZD4547) , PI3K inhibitors (BYL719 and BEZ235), and the cytostatic drugs cisplatin and docetaxel for 24, 48 and 72 h.

	IC50 per time (h)		
	24 h	***48 h***	***72 h***
CU-OP-2			
BYL[c]	4.09	3.08	1.74
JNJ[c]	11.97[a]	7.6	13.74[a]
BEZ[c]	0.36	0.21	0.15
AZD[c]	22.96	10.2	12.37
Cisplatin[c]	<0.001[b]	8.78[a]	3.75
Docetaxel[d]	2695[a]	39.55[a]	19.5[a]
CU-OP-3			
BYL[c]	6.56	0.24	1.48
JNJ[c]	84.89[a]	3.56	2.81
BEZ[c]	0.42	0.23	0.13
AZD[c]	10.45	7.93	16.29
Cisplatin[c]	<0.001[b]	<0.001[b]	7.78
Docetaxel[d]	<0.001[b]	<0.001[b]	<0.001[b]
CU-OP-20			
BYL[c]	0.28	0.26	0.13
JNJ[c]	2.66	3.58	3.02
BEZ[c]	0.06	0.07	0.03
AZD[c]	1.69	2.73	1.26
Cisplatin[c]	<0.001[b]	24.62[a]	6.71
Docetaxel[d]	<0.001[b]	2.45	0.72
CU-OP-17			
BYL[c]	0.63	0.99	0.93
JNJ[c]	17.25[a]	5.83	3.57
BEZ[c]	0.23	0.15	0.15
AZD[c]	5.85	1.32	3.06
Cisplatin[c]	<0.001[b]	3.71	0.79
Docetaxel[d]	<0.001[b]	2.83	0.39
UPCI-SCC-154			
BYL[c]	0.29	0.08	0.04
JNJ[c]	3.78	2.82	2.34
Cisplatin[c]	<0.001[b]	14.15[a]	2.26
Docetaxel[d]	11.27[a]	1.40	0.26
UT-SCC-60A			
BYL[c]	0.21	0.29	0.07
JNJ[c]	8.43	7.81	4.32
Cisplatin[c]	<0.001[b]	4.44	3.91
Docetaxel[d]	1.40	0.19	0.16

The inhibitory concentration 50 % (IC^{50}) for each cell line for each drug was determined from log concentrations effect curves in GraphPad Prism using nonlinear regression analysis. [a] *Extrapolated IC50 value, i.e., outside the tested concentration range.* [b] *The IC50 value could not be determined; lowest/highest tested concentration closest to the IC50 is reported.* [c] *Micromolar (µM).* [d] *Nanomolar (nM)*

HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A. BYL719 decreased viability compared to PBS in both cell lines at all time points with all doses (at least $p<0.01$) (Figure 2A-B). Also, here, only the highest JNJ-42756493 dose (10 µM) reduced viability significantly compared to PBS at all time points (for all, at least $p<0.05$) (Figure 2C-D).

To summarize, all cell lines showed dose dependent responses to all inhibitors, with IC_{50} values between 0.04-1.74 µM for BYL719 and 2.34-13.74 µM for JNJ-42756493 at 72 h (Table 1). CU-OP-20 and UPCI-SCC-154 were generally more sensitive to all inhibitors, while CU-OP-2 (with both a PIK3CA and FGFR3 mutation) was more resistant, and the remaining cell lines could be sensitive to one, and more resistant to another inhibitor (Table 1).

2.3. Effects upon combined exposure of PI3K and FGFR inhibitors in HPV$^+$ CU-OP-2, -3, -20, UPCI-SCC-154 and HPV$^-$ CU-OP-17 and UT-SCC-60A measured by WST-1 assays and combinational effect analysis

All CU-OP lines were exposed to PI3K inhibitor BYL719 (0.5 and 1 µM) and FGFR1-4 inhibitor JNJ-42756493 (0.01 and 0.1 µM) combinations, and PI3K inhibitor BEZ235 (0.25 and 0.5 µM) and FGFR inhibitor AZD4547 (5 and 10 µM) combinations (excluding the highest resp. dose), and data summarizing three experiments are shown in Figure 1Q-X. For UPCI-SCC-154 and UT-SCC-60A only data on the FDA approved drugs JNJ-42756493 and BYL719 are shown (Figure 2E-F), since data on BEZ235 and AZD4547 have been published (André et al., 2019). Briefly, combining PI3K and FGFR1-4 inhibitors generally resulted in positive effects.

HPV$^+$ CU-OP-2, -3 and -20 and HPV$^-$ CU-OP-17. All BYL719 and JNJ-42756493 combinations consistently reduced viability compared to PBS in all cell lines, except at 24h with the 0.5 µM BYL719 and 0.01 µM JNJ-42756493 combination for CU-OP-3, and -20 and the corresponding 0.5 and 0.1 µM resp. combination for CU-OP-3 and CU-OP-17 (for all remaining, at least $p<0.05$) Figure 1Q-T. Of note, was the increased sensitivity of the resistant CU-OP-2 line (Figure 1Q). All BEZ235 and AZD4547 combinations consistently decreased viability compared to PBS in all CU-OP lines at all time points (for all, at least $p<0.01$) Figure 1U-X.

HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A. All BYL719 and JNJ-42756493 combinations decreased viability compared to PBS in HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A (except 0.5 µM BYL719 and 0.1 µM JNJ-42756493 at 48 h for UT-SCC-60A), for all remaining, at least $p<0.05$ (Figure 2E and F).

Combinational effect analysis. To further dissect the effects of inhibitor combinations, combinational indexes (CIs) of BYL719 (0.5-20 µM) and JNJ-42756493 (0.01-1 µM) combinations were calculated for all cell lines 24-72 h after treatment as done before [36, 39-41]. The CIs according to "highest single agent" approach for the lowest combinational doses after 48 and 72 h are shown in Figure 3 for the CU-OP lines. A positive effect ($CI<1$), with neutral or improved combinational effects on the decrease of viability compared to single drugs was observed at 48 h, especially for CU-OP-2 and CU-OP-17, but not for CU-OP-3, where the treatment effect increased after 72 h (Figure 3). For UPCI-SCC-154 and UT-SCC-60A mainly neutral effects were obtained (data not shown). For BEZ235 (0.25-0.5 µM) and AZD4547 (5-10 µM) a similar analysis disclosed positive effects at 24 and 48 h after treatment for CU-OP-2, -3 and -17, the more resistant cell lines (data not shown), and was reported before for UPCI-SCC-154 and UT-SCC-60A (André et al., 2019).

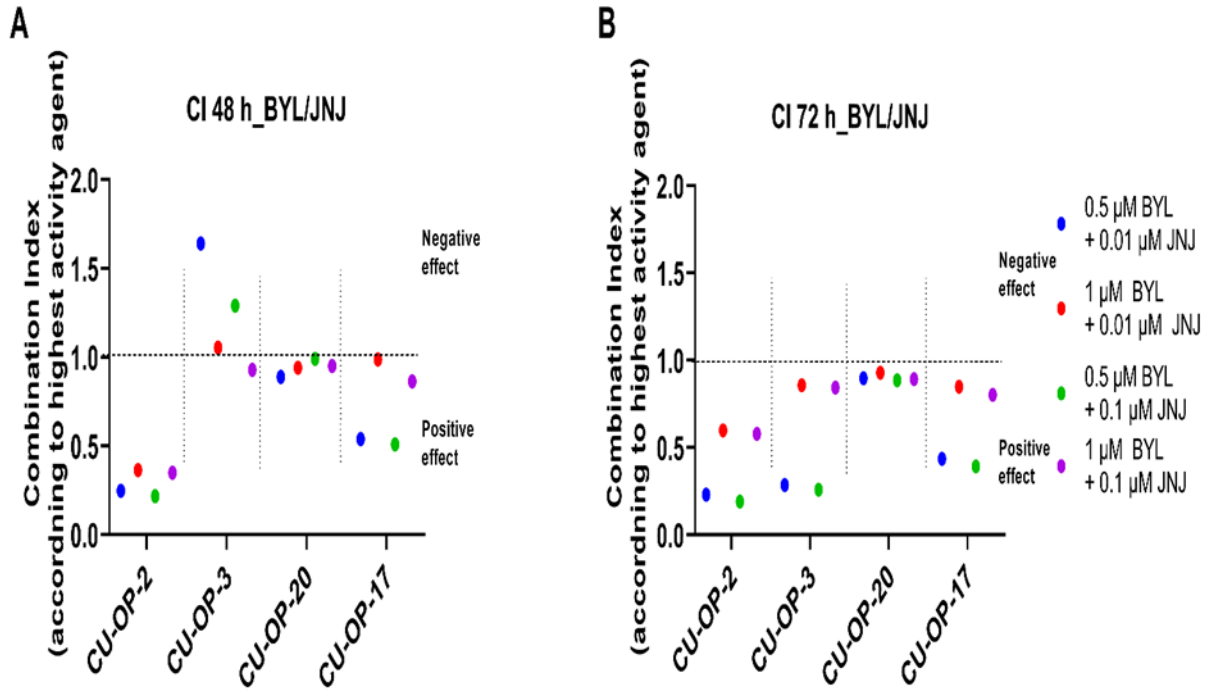


Figure 3. Combinational effects of PI3K inhibitors BYL719 and FGFR inhibitor JNJ-42756493 in HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 cell lines after 48 and 72 h. Combination indexes (CIs) were shown with the highest single agent approach after 48 h (A) and 72 h (B), where CI>1 shows a negative combination effect and CI<1 shows a positive combination effect. CIs were calculated from the mean of three experiments analyzed by WST-1, at 48 and 72 h after treatment. CI denotes combination index; BYL denotes BYL719 and JNJ denotes JNJ-42756493.

To summarize, combining PI3K and FGFR inhibitors, generally resulted in positive combinatory effect for most cell lines.

2.4. Effects after single cisplatin and docetaxel exposure measured in HPV$^+$ CU-OP-2, -3, -20, UPCI-SCC-154 and HPV$^-$ CU-OP-17,UT-SCC-60A by WST-1 assays

All cells lines presented dose dependent responses to single treatments with 2.5-7.5 µM cisplatin or 0.6-6 nM docetaxel as shown with WST-1 assays in Figure 4 and with IC_{50} values in Table 1.

HPV$^+$ CU-OP-2, -3 and -20 and HPV$^-$ CU-OP-17. All CU-OP lines presented dose dependent responses and decreased viability compared to PBS with 2.5-7.5 µM of cisplatin 72 h after treatment, except for CU-OP-20 with 5 µM cisplatin (for all remaining, at least $p<0.05$) (Figure 4A-D). This was also the case, after 48 h, for CU-OP-2 with 7.5 µM cisplatin, and for CU-OP-17 with 5 and 7.5 µM cisplatin (for all, at least $p<0.05$) (Figure 4A and D). All CU-OP lines also had dose dependent responses to docetaxel, but only CU-OP-20 and CU-OP-17 showed decreased viability compared to PBS with all docetaxel doses at 72 h (at least $p<0.05$),

which was also the case for CU-OP-2 ($p<0.05$), but not CU-OP-3, with 6 nM docetaxel (Figure 4G-J).

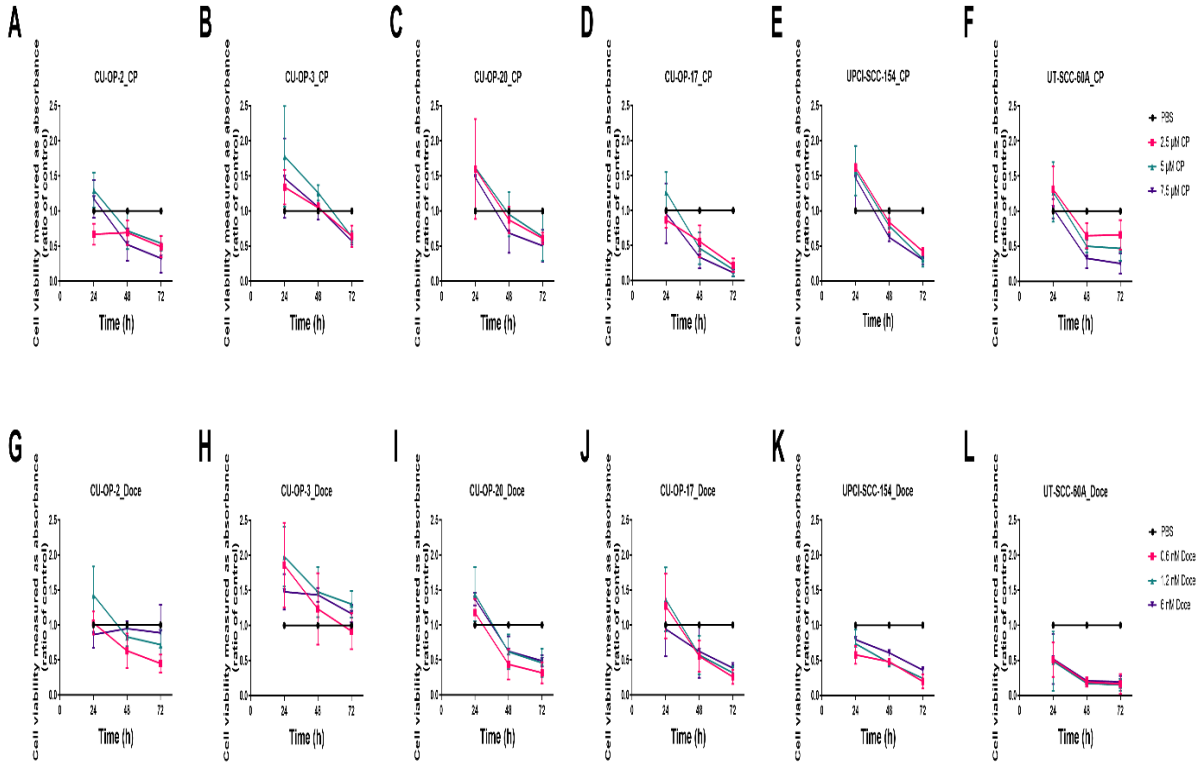


Figure 4. WST-1 viability assays on HPV$^+$ CU-OP-2, -3, -20, and UPCI-SCC-154 and HPV$^-$ CU-OP-17 and UT-SCC-60A cell lines upon treatment with cisplatin and docetaxel. WST-1 viability assays measuring absorbance following treatment for 24, 48 and 72 h of HPV$^+$ CU-OP-2, -3, -20 and UPCI-SCC-154 and HPV- CU-OP-17 and UT-SCC-60A with cisplatin (A-F) and docetaxel (G-L) resp. The graphs represent three experimental runs per cell line and the data are presented with the mean ± standard deviation. CP denotes cisplatin and DOCE denotes docetaxel.

HPV$^+$ UPCI-SCC-154 and HPV$^-$ UT-SCC-60A. Compared to PBS, both lines had dose dependent responses to 2.5-7.5 µM cisplatin, and decreased viability 48 and 72 h after treatment with 7.5 µM, which was also the case with 5 µM for UT-SCC-60A (for all, at least $p<0.05$) (Figure 4E and F). Both also had dose dependent responses to 0.6-6 nM docetaxel and decreased viability compared to PBS at most time points, except at 24 h with 0.6-6 nM treatment (for all, at least $p<0.05$) (Figure 4K and L).

To summarize, all cell lines had dose dependent responses to cisplatin and docetaxel, with IC_{50} values at 72 h between 0.79-7.78 µM for cisplatin and 0.16-19.5 nM for docetaxel (Table 1). UT-SCC-60A and UPCI-SCC-154 were generally more sensitive than the CU-OP lines (Figure 4 and Table 1).

2.5. Effects of BYL719 and JNJ-42756493 and cisplatin and docetaxel combinations in HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 measured by WST-1 assays and combinational effect analysis

FDA approved BYL719 and JNJ-42756493 resp. were combined with either cisplatin or docetaxel and tested for their combinational effects on all cell lines. Data summarizing three WST-1 assays combining BYL719 0.5-10 µM or 0.01-1 µM JNJ-42756493 with 2.5-7.5 µM

of cisplatin or 0.6-6 nM docetaxel are shown for the CU-OP cell lines in Figure 5. Since UPCI-SCC-154 and UT-SCC-60A were, with exception of CU-OP-20, slightly more sensitive to cisplatin and docetaxel than the other CU-OP lines, combination experiments with these lines were not pursued here.

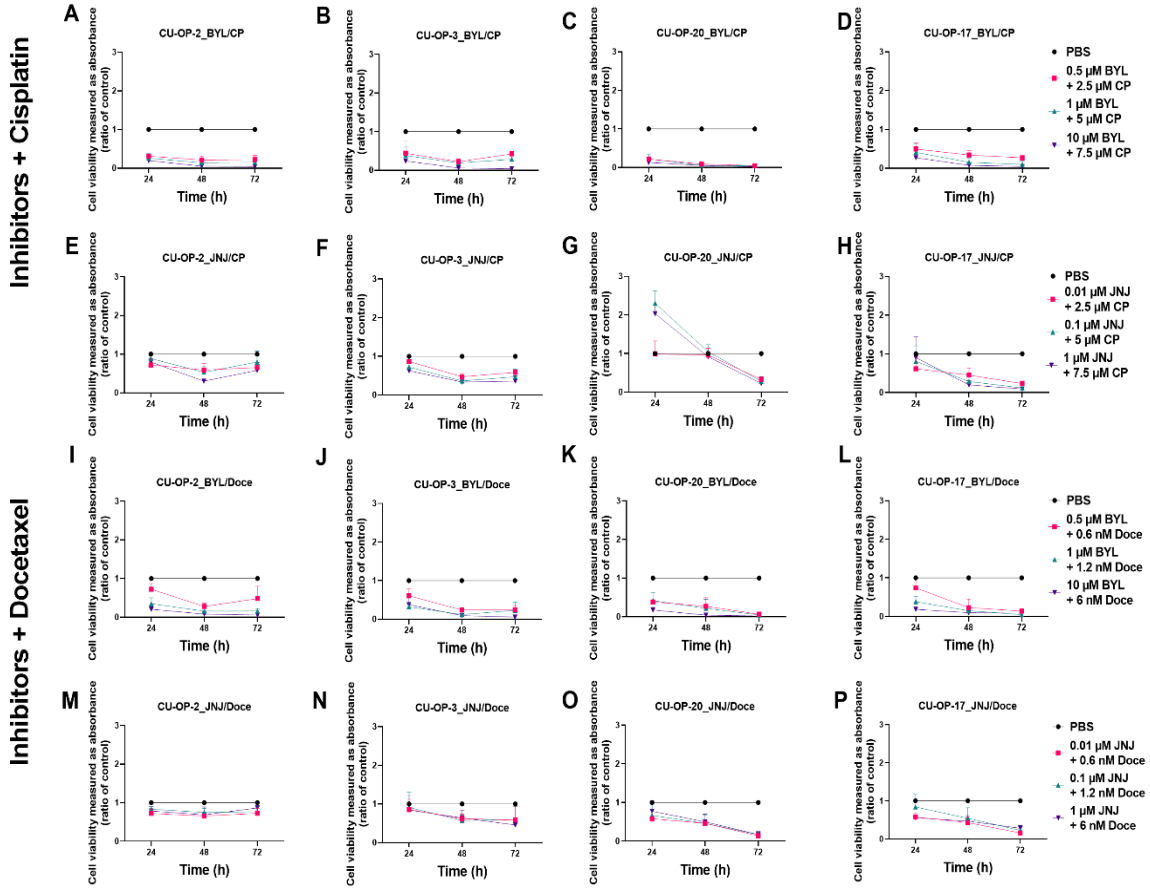


Figure 5. WST-1 viability assays on HPV$^+$ CU-OP-2, -3, -20, and HPV$^-$ CU-OP-17 cell lines upon treatment with BYL719 or JNJ-42756493 with cisplatin and docetaxel. WST-1 viability assays measuring absorbance following treatment for 24, 48 and 72 h of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 with cisplatin together with BYL719 (A-D) or JNJ-42756493 (E-H) resp., as well as docetaxel together with BYL719 (I-L) or JNJ-42756493 (M-P) resp. The graphs represent three experimental runs per cell line and the data are presented with the mean ± standard deviation. CP denotes cisplatin; DOCE denotes docetaxel; BYL denotes BYL719; JNJ denotes JNJ-42756493.

All BYL719-cisplatin and BYL719-docetaxel combinations decreased viability consistently compared to PBS for all cell lines, except the 0.5 µM BYL719 and 0.6 nM docetaxel combination for CU-OP-2 at 24 and 72 h and CU-OP-17 at 24 h, and (for all, at least $p<0.05$) (Figure 5A-D and I-L resp.). This was also the case for all JNJ-42756493 and cisplatin combinations at 72 h after treatment for all cell lines; except for CU-OP-2 with 0.1 µM JNJ-42756493 and 5 µM cisplatin (at least $p<0.05$) (Figure 5E-H). CU-OP-17 was the most sensitive line to the JNJ-42756493 and cisplatin combination with decreased viability compared to PBS with all doses and time points, except at 24 h for all combinations (for all remaining at least $p<0.05$) (Figure 5H). For JNJ-42756493 and docetaxel, CU-OP-20 and CU-OP-17 showed decreased sensitivity compared to PBS 48 and 72 h after treatment, while this

was only occasionally the case for CU-OP-2 and CU-OP-3 then only with the highest dose combinations (for all, at least $p<0.05$) (Figure 5M-P).

Combinational indexes (CIs) of BYL719 (0.5-10 µM) and JNJ-42756493 (0.01-1 µM) combinations with cisplatin (2.5-7.5 µM) and docetaxel (0.6-6 nM) were calculated for all cell lines 48-72 h after treatment (André et al., 2019; Lange et al., 2009; Holzhauser et al., 2020; Pirotte et al., 2018), and the CIs after 48 h are presented for the CU-OP cell lines in Figure 6. Positive (CI<1) or relatively neutral (C~1) effects according to the "highest single agent" approach were dominant for most cell lines, and most prominent for CU-OP-2 and -17 (Figure 6).

To summarize, positive and neutral effects were obtained upon combining BYL719 and JNJ-42756493 resp. with cisplatin and docetaxel on CU-OP lines, with the best effects observed for CU-OP-2 and CU-OP-17.

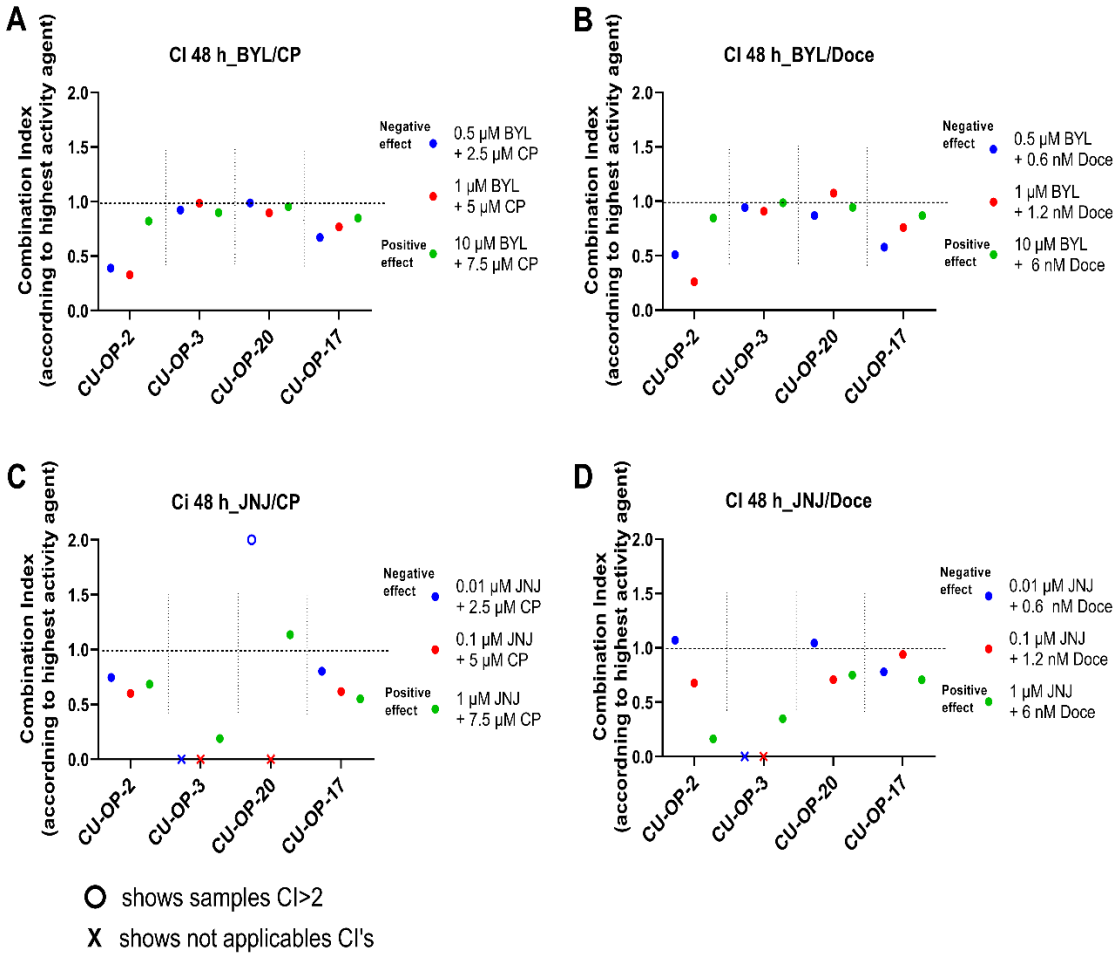


Figure 6. Combinational effects of the PI3K inhibitor, BYL719, and FGFR inhibitor, JNJ-42756493, with cisplatin, and docetaxel on HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 cell lines after 48 h. Combination indexes (CIs) were obtained by the highest single agent approach after treatment with BYL719 or JNJ-42756493 and cisplatin or docetaxel. Combination treatment of BYL with BYL719 or JNJ-42756493 resp. are shown in A and B resp., whereas combination treatment of JNJ-42756493 with the BYL719 or JNJ-42756493 resp. are shown in C and D resp. CIs were calculated from the mean of three experiments, analyzed by WST-1. CI denotes combination index; BYL denotes BYL719; JNJ denotes JNJ-

42756493; CP denotes cisplatin; and DOCE denotes docetaxel. X denotes not applicable to show, so analysis could not proceed; o denotes CI>2, indicating a negative combination effect.

2.6. Proliferation, apoptosis and cytotoxicity after single or combined treatment with PI3K, FGFR inhibitors of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17

Effects of single treatments with BYL719 (0.5-10 µM) and JNJ-42756493 (0.01-10 µM) and combined treatments, on proliferation, apoptosis and cytotoxicity of the CU-OP lines were followed by the IncuCyte S3 Live-Cell Analysis System (Figures 7-9).

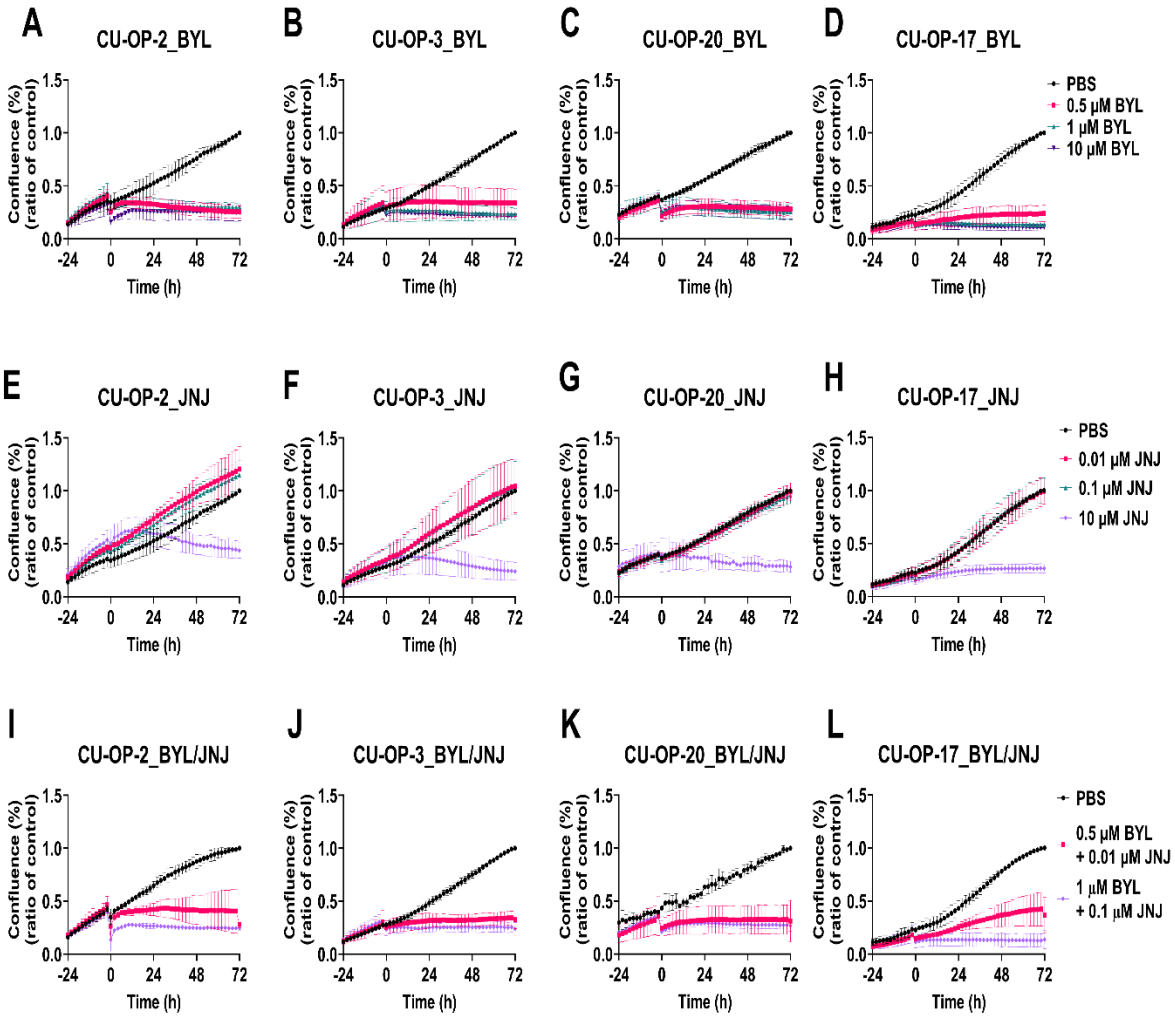


Figure 7. Proliferation response of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 cell lines upon treatment with BYL719 and JNJ-42756493. Proliferation responses of HPV$^+$ CU-OP-2, -3, -20 and HPV- CU-OP-17 after treatment with PI3K and FGFR inhibitors, BYL719 (A-D); and JNJ-42756493 (E-H); and combinational treatment with BYL719 and JNJ-42756493 (I-L). The graphs represent three experimental runs per cell line. Confluence (%) denotes proliferation response; BYL denotes BYL719; JNJ denotes JNJ-42756493; CP denotes cisplatin; and DOCE denotes docetaxel.

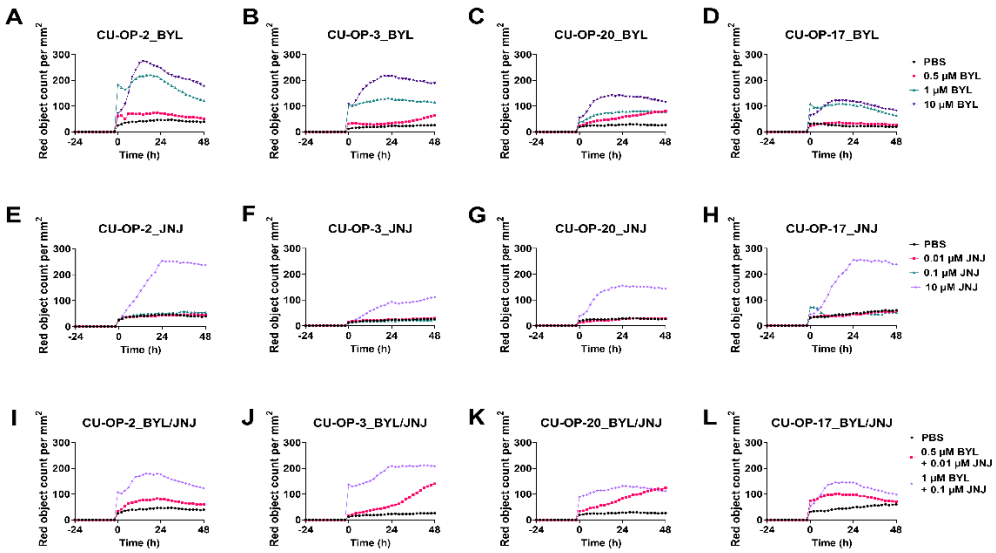


Figure 8. Cytotoxicity response of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 cell lines upon treatment with BYL719 and JNJ-42756493. Cytotoxicity response of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 to PI3K and FGFR inhibitors BYL719 (A-D); and JNJ-42756493 (E-H); and combinational treatment with BYL719 and JNJ-42756493 (I-L). The graphs represent three experimental runs per cell line. Red object count per mm2 denotes cytotoxicity response; BYL denotes BYL719; JNJ denotes JNJ-42756493; CP denotes cisplatin; and DOCE denotes docetaxel.

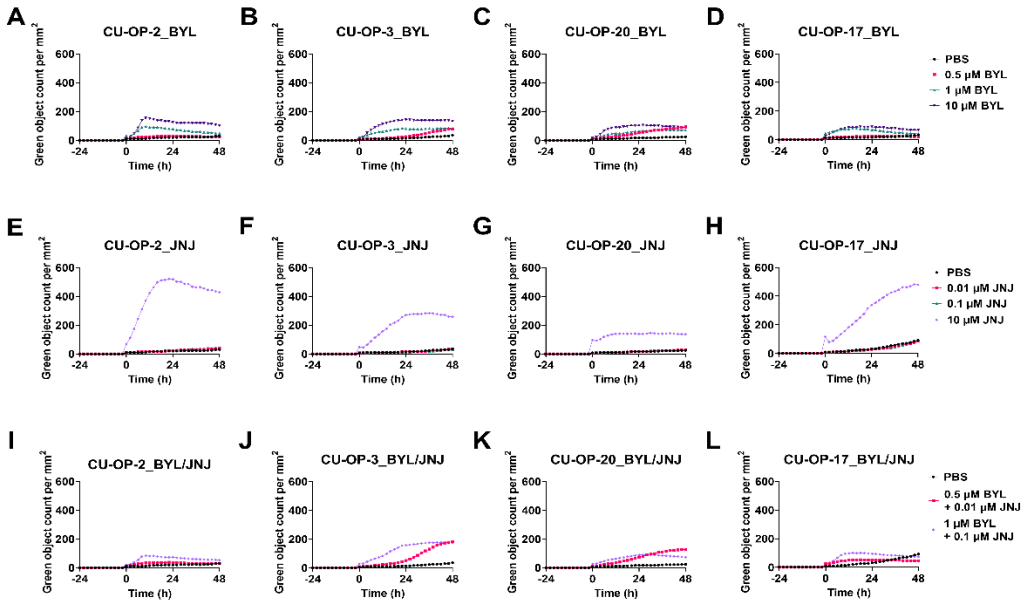


Figure 9. Apoptosis response of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 cell lines upon treatment with BYL719 and JNJ-42756493. Apoptosis response of HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17 to PI3K and FGFR inhibitors; BYL719 (A-D); and JNJ-42756493 (E-H); and combinational treatment with BYL719 and JNJ-42756493 (I-L). The graphs

represent three experimental runs per cell line. Green object count per mm2 denotes apoptosis response; BYL denotes BYL719; JNJ denotes JNJ-42756493; CP denotes cisplatin; and DOCE denotes docetaxel.

All lines showed complete inhibition of proliferation with all BYL719 doses included, while this was only the case for the highest JNJ-42756493 dose, and upon combined exposures excluding the highest doses of both inhibitors, proliferation was inhibited with all combination doses (Figure 7). Cytotoxicity was observed for all CU-OP lines with the two highest BYL719 (1 and 10 µM) and the highest JNJ-42756493 (10 µM) concentrations (Figure 8A-H). Upon combined exposure, cytoxicity was observed in most lines, especially those including 1 µM BYL719 (Figure 8I-L). Marked apoptosis in all CU-OP lines was present only with the highest JNJ-42756493 dose (10 µM) and not prominent in the combination experiments, where the highest JNJ-42756493 dose was excluded (Figure 9).

To summarize, all CU-OP cell lines showed decreased proliferation after single and combined treatments with PI3K and FGFR inhibitors. Cytotoxicity was only observed with the two highest BYL719 and the highest JNJ-42756493 doses, while apoptosis was only observed with the highest JNJ-4256493 concentration.

2.7. Proliferation, apoptosis and cytotoxicity after treatment with single chemotherapeutic agents, or chemotherapeutic agents combined with PI3K and FGFR inhibitors, on HPV$^+$ CU-OP-2, -3, -20 and HPV$^-$ CU-OP-17

The effects of cisplatin (2.5-7.5 µM) and docetaxel (0.6-6 nM) alone or combined with BYL719 (0.5-10 µM) and JNJ-42756493 (0.01-1 µM) on proliferation, apoptosis and cytotoxicity in the CU-OP lines were followed. At the doses used both cisplatin and docetaxel inhibited proliferation completely, while neither induced prominent cytotoxicity or apoptosis (data not shown).

Combining BYL719 (0.5-10 µM) or JNJ-42756493 (0.01-1 µM) with either 7.5 µM cisplatin or 6 nM docetaxel, inhibited proliferation completely (data not shown). Cytotoxicity was observed with the two highest concentrations of BYL719 (1 and 10 µM) and the highest JNJ-42756493 dose (10 µM) with cisplatin or docetaxel for CU-OP-2 and -3, while CU-OP-20 and CU-OP-17 were generally less sensitive especially when docetaxel was present with JNJ-42756493 (data not shown). Prominent apoptosis was only observed upon combinations of the chemotherapeutic agents with the highest JNJ-42756493 dose (10 µM) (data not shown).

3. Discussion

In the current study, the FDA approved drugs alpelisib (PI3K inhibitor) and erdafitinib (FGFR inhibitor) were demonstrated to have dose dependent effects with decreased viability and proliferation on the HPV$^+$ CU-OP-2, -3, -20 and UPCI-SCC-154; the HPV$^-$ CU-OP-17 and UT-SCC-60A TSCC/BOTSCC cell lines. Notably, this was irrespective of whether the cells had specific FGFR3 or PIK3CA mutations. Furthermore, when combining these inhibitors mainly positive or neutral effects were disclosed with comparable decreases in viability and proliferation using lower doses of the inhibitors. All tested TSCC/BOTSCC cell lines above were also observed to have dose-dependent effects with diminished viability and proliferation when treated with clinically often used chemotherapeutic drugs cisplatin and docetaxel, although e.g. CU-OP-2 and CU-OP-3 were relatively more resistant. Subsequently, only the CU-OP cell lines, were followed for their responses to targeted inhibitor-chemotherapeutic drug combinations, and here also positive and neutral effects were disclosed.

The data on the FDA approved drugs alpelisib and erdafitinib paralleled the inhibitory effects on viability and proliferation, as well as the effects on cytotoxicity and apoptosis of recently tested inhibitors BEZ235 (PI3K inhibitor) and AZD4547 (FGFR inhibitor) on UPCI-SCC-154 and UT-SCC-60A (André et al., 2019), suggesting on-target effects of alpelisib and erdafitinib. Alpelisib and erdafitinib may therefore be of interest for future clinical evaluation in patients with recurrent TSCC/BOTSCC, or for patients with TSCC/BOTSCC, with an

estimated risk for a very poor prognosis, due to their tumors disclosing several poor prognostic markers (Bersani et al., 2017, Näsman et al., 2017). The data presented here suggest that patients, with tumors both with and without PIK3CA or FGFR3 mutations could respond to the above inhibitors, and that it is not possible upfront to expect that patients with tumors exhibiting PIK3CA or FGFR3 mutations, will respond better or worse to the resp. corresponding inhibitor.

That the cell lines above had drug dependent dose decreases in viability and proliferation, to FDA approved inhibitors and that effects were enhanced upon combining the two was anticipated, since UPCI-SCC-154 and UT-SCC-60A had responded in an analogous way, to similar inhibitors BEZ235 and AZD4547 (André et al., 2019). Moreover, here we could also show that the CU-OP cell lines also reacted similarly to BEZ235 and AZD4547. In addition, we were able to disclose that HPV^+ CU-OP-20 and UPCI-SCC-154 had a tendency to be the most sensitive cell lines to both the older and newer PI3K and FGFR inhibitors, while CU-OP-2 and CU-OP-3 often were less affected. However, upon combination treatments with BYL719 and JNJ-42756493, all TSCC/BOTSCC lines were sensitive.

The enhanced efficacy on inhibition of viability and proliferation, upon combining BYL719 and JNJ-42756493 was also in line with previous reports in other cell types with similar or corresponding inhibitors, suggesting that PI3K and FGFR inhibitors indeed can be used together and have synergistic effects (André et al., 2019, Lange et al., 2009, Holzhauser et al., 2020, Kostopoulou ON et al., 2019). Furthermore, apart from the positive effect of combining PI3K and FGFR inhibitors, it could allow for the use of lower doses of the drugs, thereby potentially decreasing the numbers of side effects as well as decrease the risk for development of resistance, when using only one inhibitor.

That also BYL719, at the doses used, and not only JNJ-42756493 showed effects on cytotoxicity, was slightly unexpected, and not completely in line with our previous report, where the AZD4547 (FGFR inhibitor) was superior to the included PI3K inhibitors with regard to inducing cytotoxicity (André et al., 2019, Lange et al., 2009, Holzhauser et al., 2020). Nevertheless, with regard to effects on apoptosis, our data were analogous to our previous reports, with JNJ-42756493, an FGFR inhibitor being superior to BYL719, a PI3K inhibitor (André et al., 2019).

Nonetheless, joining all data, they definitely support that BYL719 and JNJ-42756493 could be of potential clinical interest for treatment of recurrent TSCC/BOTSCC.

Interestingly, here we demonstrated that TSCC/BOTSCC cell lines, both with and without FGFR or PI3K mutations, were sensitive to PI3K and FGFR inhibitors and those with such mutations were not necessarily more sensitive to the inhibitors. This has been reported before, by others and us, in other tumor types (André et al., 2019; Lange et al., 2009; Holzhauser et al., 2020; Kostopoulou et al., 2019; Holzhauser et al., 2020; Foucquier and Guedj, 2015; Tertipis et al., 2015; Wang et al., 2017; Wang et al., 2015; Herrera-Abreu et al., 2013; Brands et al, 2017). More specifically, it was disclosed that different tumors and tumor lines could respond to PI3K and FGFR inhibitors, despite not having PI3K and/or FGFR mutations or chromosomal rearrangements (André et al., 2019; Lange et al., 2009; Holzhauser et al., 2020; Kostopoulou et al., 2019; Holzhauser et al., 2020; Foucquier and Guedj, 2015; Tertipis et al., 2015; Wang et al., 2017; Wang et al., 2015; Herrera-Abreu et al., 2013; Brands et al, 2017). Furthermore, some but not all studies showed having mutations resulted in enhanced sensitivity (White et al., 2007; Lange et al., 2009).

So far we have no explanation to why the TSCC/BOTSCC lines tested here differed in their sensitivity to the included PI3K and FGFR1-4 inhibitors, e.g. despite CU-OP-2 having both a PIK3CA and FGFR3 mutation, CU-OP-20 having a PIK3CA mutation, and CU-OP-3 having neither. However, it is of note that the two PI3K and FGFR inhibitor most resistant lines

CU-OP-2 and -3 were previously demonstrated to be fairly radioresistant, while the more sensitive CU-OP-20, was radiosensitive (Singleton et al., 2015).

The TSCC and BOTSCC cell lines HPV^+ CU-OP-2, -3, -20 and UPCI-SCC-154 and the HPV^- CU-OP-17 and UT-SCC-60A were also examined for their responses in viability to single cisplatin and docetaxel therapy. Notably, also here CU-OP-2 and -3 were the most resistant cell lines to the effects of cisplatin and docetaxel on viability, while the remaining cell lines tended to be more sensitive. This pattern was not reflected upon by the effects of cisplatin and docetaxel on proliferation, where proliferation was largely inhibited in a similar way for all cell lines. At the doses used however, none of the chemotherapeutic agents disclosed prominent effects on cytotoxicity or apoptosis, but better effects may have been obtained upon using higher concentrations or repeated treatments. However, in these experiments, we aimed to use as low doses as possible, in order to disclose combinationtional effects upon combining the chemotherapeutic agents with the inhibitors.

Upon examining potential combinational effects using cisplatin and docetaxel together with BYL719 or JNJ-42756493, mainly neutral and positive, with occasional negative effects were disclosed. Of note were however some positive combinations with BYL719 and cisplatin or docetaxel especially on CU-OP-2, but also with the other cell lines, and the data certainly suggest it would be worthwhile pursuing the effects of such combinations.

To our knowledge, the pursuit of potential positive effcts of alpesilib and erdafitinib upon combination with cisplatin and docetaxel have not been examined before in TSCC/BOTSCC, and there are only limited studies on other cell lines, comprising some of the drug-inhibitor combinations included in this report. One study on nasopharyngeal cancer cell lines, showed neutral or limited positive effects upon combining BYL719 and cisplatin, while another report on a non-small lung cancer cell, showed more positive effects (Cai, Song and Ai, 2019, Munster et al., 2016).

However, when using corresponding combinations in other cell lines, we obtained not only positive and neutral, but also negative effects [personal communication]. The latter can possibly partially explained by that BYL719 may induce G_0/G_1 arrest, and thereby may have inhibited some of the cytotstatic effects of e.g. cisplatin (Cai, Song and Ai, 2019; Tamura et al., 2018).

This report has some limitations. Despite six TSCC/BOTSCC cell lines were included here, more cell lines could have been examined. Nonetheless, UPCI-SCC-154 and UT-SCC-60A have extensively been used by the scientific community in the head and neck field and the CU-OP cell lines, have the benefit of allowing the studies of cell lines having PIK3CA and FGFR3 mutations (Singleton et al., 2015; Wong et al., 2015; Keam et al., 2015; Ma et al., 2014).

Nevertheless, and of note, the data do suggest that drug-drug interactions of PI3K and FGFR inhibitors with chemotherapeutic drugs could most likely be used for treatment of TSCC/BOTSCC. However, further studies, with the use of wider dose ranges, the establishment of which incubation times are optimal, and the acquisition of information on in which sequence the drugs should be administered would be useful, to establish ways of obtaining the best anti-tumor efficacy. Nonetheless, the chemotherapeutic drug and inhibitor doses tested here are in line with those tested during standard conditions by others and should allow for comparisons (Bahleda et al., 2019; Herrera-Abreu et al., 2013; Cai, Song and Ai, 2019; Munster et al., 2016; Tamura et al., 2018; Zhu et al., 2020). Future studies will be needed to explore the mechanisms involved and possibly provide better rationale of how to better proceed with these possible combinations clinically.

To conclude, this study supports the potential of further exploring the combined use of FDA approved drugs alpelisib and erdafitinib for the treatment of recurrent TSCC and BOTSCC especially when other options e.g. check point inhibitors are not useful.

4. Materials and Methods

4.1. Cell lines and culture conditions

HPV^+ UPCI-SCC-154 and HPV^- UT-SCC-60A squamous cell carcinomas of the tongue and tonsil resp. were provided by Susanne Gollin, University of Pittsburgh USA, and Reidar Grénman, University of Turku, Finland resp. and their culture conditions have been described before (Wong et al., 2015; Keam et al., 2015). HPV^+ CU-OP-2, CU-OP-3, CU-OP-20 and HPV^- CU-OP-17, derived from the tonsil, were provided by Ned Powell, Cardiff University UK, and grown in GMEM with 10 % FBS, and additional supplements, on 60 Gy irradiated 3T3 fibroblasts as feeder layers as described before (Singleton et al., 2015; Ma et al., 2014).

4.2. Competitive allele-specific TaqMan PCR (CAST-PCR)

Competitive Allele-Specific TaqMan® PCR technology (Thermo Fischer Scientific, Waltham, MA, USA) exposing the reference FGFR3 gene and its variants p.R248C, p.S249C and p.K650Q and the reference PIK3CA gene and its variants p.E542K, p.E545K and p.H1047R was used (Koole et al., 2016, André et al., 2019, Lange et al., 2009).

4.3. Cell seeding and treatments

Seeding. UPCI-SCC-154 and UT-SCC-60A, with 5 000 cells/well, and CU-OP lines (without feeders), with 7 500 cells/well, were seeded in 96-well plates in 90 µl to 200 µl, and incubated for 24-96 h according to the assays, and outer wells of the plates were filled with medium to avoid edge effects.

Inhibitors. BYL719, BEZ235, AZD4547 and JNJ-42756493 were purchased from Selleckhem Chemicals (Munich, Germany). Stock solutions of the drugs were diluted in DMSO, and then further diluted for the intended concentrations, which were: AZD4547 5-25 µM; JNJ-42756493 0.01-10 µM; BEZ235 0.25-1 µM; and for BYL719 0.5-20 µM.

Chemotherapeutic agents. Cisplatin (Accord Healthcare Limited, Middlesex, UK) was diluted in PBS, and used at 2.5-7.5 µM. Docetaxel (Actavis, Hafnarfjordur, Island) was diluted in PBS and used at 0.6-6 nM.

4.4. WST-1 viability assay

Relative viability was quantified using the WST-1 assay (Roche Diagnostics, Mannheim, Germany) and followed for 72 h according to the instructions of the manufacturer and before (André et al., 2019; Lange et al., 2009).

4.5. Cell proliferation, apoptosis and cytotoxicity assays

Proliferation. Cells in 96-well plates, were placed into the IncuCyte S3 Live® Cell Analysis System (Essen Bioscience, Welwyn Garden City, UK) and images taken every 2 h for 96 h (for details see Lange et al., 2009).

Apoptosis and cell cytotoxicity assays. Apoptosis and cytotoxicity were examined in parallel to proliferation in the 96-well plates above using the IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent and the Incucyte™ Cytotox Red Reagent resp. (both Essen Bioscience, Germany), with images taken and analyzed every 2 h as described before (Lange et al., 2009).

4.6. Statistical analysis

To verify the effects of single or combination treatments compared to the negative control, a multiple t test accompanied by a correction for multiple comparison of the means conferring to the Holm Sidak method was done as described in detail before (André et al., 2019; Lange et al., 2009; Holzhauser et al., 2020). Additionally, the combined effects were evaluated applying the effect-based approach 'Highest Single Agent' (Pirotte et al., 2018). Details of the 'Highest Single Agent' were presented previously (André et al., 2019; Lange et al., 2009; Holzhauser et al., 2020).

5. Conclusions

HPV^+ TSCC/BOTSCC is increasing epidemically, but has a favorable outcome, however upon recurrence, prognosis is poor and additional therapeutic approaches are urgently needed. Since, PIK3CA and FGFR3 mutations are frequent in such tumors, PI3Kα and FGFR inhibitors alpelisib (BYL719) and erdafitinib (JNJ-42756493) were tested alone, or combined, and with/without cisplatin and docetaxel on analogous cell lines.

HPV^+ CU-OP-2, -3, -20 and UPCI-SCC-154 and HPV^- CU-OP-17 and UT-SCC-60A TSCC/ BOTSCC cell lines were used and when tested for PIK3CA/FGFR3 mutations, HPV^+ CU-OP-2 had a pS249C-FGFR3, and as CU-OP-20, a pE545K-PIK3CA mutation, while none of the others had such mutations. Dose dependent responses to BYL719 and JNJ-42756493 were obtained irrespective of PIK3CA/FGFR3 mutation status, and upon combining the inhibitors, positive effect was found. Cisplatin and docetaxel also induced dose dependent responses, and upon combination with the inhibitors, both positive and neutral effects were observed.

The data suggest that the combined use of FDA approved drugs alpelisib and erdafitinib for treatment of recurrent TSCC/BOTSCC should be further explored, for use when other options are not functional.

Section III. Future directions in professional, academic and scientific

III.1. Developments in academic and professional activity

Teaching Microbiology in a medical university requires a permanent training as well as the continuous acquisition of new knowledge. Professional development is essential for the teacher in order to provide an image of the contemporary scientific world and to learn effective methods of interacting with students.

Perspectives, objectives in academic field

- the continuous professional and didactic self-improvement, through permanent individual study, participation in national and international courses, congresses, conferences;
- a constant upgrade of teaching materials and courses, integration of novelties along with the classic, fundamental and essential elements of medical training;
- the permanent updation of the teaching materials uploaded on the university platform;
- improving the evaluation methods, with the elaboration of tests and suggestive images for bacteria;
- refining the teaching act, both during courses and internships, based on the personality and skills of each recipient (student/resident physician);
- increasing the enthusiasm for postgraduate education, by involving as many residents as possible in practical and research activities and by increasing the quality of the courses in accordance with the novelties in the field.

III.2. Developments in research activity

I plan to continue with the research direction that I have settled in the microbiology field after I completed my Ph.D. thesis but, also, to start new ones, as follows:

i. Assessing the role of oncogenic viruses in selected tumours

The International Agency for Research on Cancer (IARC) estimated that 15-20% of cancers are associated with infectious agents. Around 12% of cancers are causally linked to 7 viruses: Epstein-Barr virus (EBV), hepatitis B virus (HBV), human papillomavirus (HPV), human T-cell lymphotropic virus (HTLV), hepatitis C virus (HCV), Kaposi's sarcoma herpesvirus (KSHV), and Merkel cell polyomavirus (MCPyV). The above-mentioned viruses belong to *Polyomaviridae, Papillomaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae* families and represent the research subject for many authors, mainly regarding their life-cycle into the host cell and the risk factors which lead to tumor transformation.

a. The estimated number of new cases in 2020, Romania, both genders, all ages, for kidney cancer (C64 – C65) is 2750, with 7.7 age-standardised rate, being situated in the second half as incidence from all the European countries. Renal cell carcinoma (RCC) is the most common form of kidney cancer, around 90 percent of all kidney cancers being attributed to RCC. The grading system for RCC was reviewed and validated lately by International Society of Urological Pathology (ISUP) and World Health Organization (WHO). Tumor pathology is a critical direction research in the medicine of XXI century. As part of tumor pathology, the *knowledge on RCC development, pointing out the genic and molecular sequences of carcinogenic mechanism, represents an important issue with a direct impact on scientific achievements* (contributing to the current state of the art in carcinogenesis), *technological*

advances (by developing and/or improving tools for performant investigations) and *socio-economic effects* (mirrored by a possible reduction of the global burden of this malignancy in general population through a better monitoring and new targeted treatment options). Recent scientific data are sustaining oncogenic role of some viruses in carcinogenesis of RCC. The recent scientific literature presents the results of studies realized for a single oncogenic virus or only by PCR or by IHC analysis. *This proves the difficulty elements in the research of this issue focusing on the possible involvement of viruses in the renal carcinogetic mechanism, and the limitations of current approaches.*

The originality and innovation of this future research direction is that we intend ***to develop a comprehensive and integrated analysis of at least 3 oncogenic viruses*** (e.g., BK, EBV, HCV) in order ***to confirm their role in RCC carcinogenesis***. For this purpose, we intend to use a multiplex genotyping and an IHC exam on RCC samples (FFPE and of fresh tumoral samples).

b. The recent IARC publication (September 2018) - the WHO Classification of Skin Tumors contains data regarding diagnostic criteria, pathological features, genetic and other associated molecular alterations, prognosis, and protective factors for each of the tumor types covered. Skin tumors have risk factors (e.g., sun susceptibility and/or exposure). The TNM classification of skin tumors includes certain types which are viral correlated, e.g., Merkel cell carcinoma of skin, skin carcinoma of the head and neck, and EBV-positive mucocutaneous ulcer. GLOBOCAN rates Romania on 7th place in Central and Eastern Europe regarding incidence of all non-melanoma skin cancer and Kaposi sarcoma (male incidence 274.9 versus 81.5 female incidence, per 100 000 all ages). The percentage of melanoma cases in 2012, attributable to ultraviolet (UV) radiation exposure was 68.8 for men and 44.3 for women, all ages (30+ years).

Oncogenic viruses and their potential role in skin cancer:

- Polyomaviruses: recent sensitive molecular assays, like next generation sequencing, have led to the discovery of 10 human polyomaviruses (HPyVs): BKV, KIV, JCV, MCV, WUV, TSV, HPyV6, HPyV7, HPyV9, and SV40. Merkel cell carcinoma (MCC), produced by MCV (Merkel cell polyomavirus) is known as a rare skin tumor which can have an aggressive clinical evolution with an unfavorable prognosis.
- Herpesviruses: Two members of the herpesvirus family, Epstein-Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV) have been shown to be, at least partially, responsible for cancer in humans.
- Cutaneous beta papilloma viruses'types: the beta HPV types (HPV 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, and 96) are originally isolated in non-melanoma skin cancer (NMSC) of individuals with a rare genetic disorder called Epidermodysplasia Verruciformis. Immunosuppressed organ transplant recipients have a 50-100-fold increased risk of developing NMSC compared to the general population.

The results of these studies (testing RCC and skin cancers for oncogenic viruses) will help to understand the mechanism of these cancers, and if the viral etiology could be confirmed minimum as co-factor. As consequence, our results can contribute to the development of new therapeutic strategies. A tumour induced virus could be efficiently prevented by vaccination, or treated by oncolytic viral therapy and, or by targeted therapy, in parallel with preventive measures for other co-factors.

Testing oncogenic viruses would be possible if we will be able to achieve, thanks to a PCE 2021 project submitted, a new equipment, LX200, a device which supports up to 100-plex assays. The newly launched device has the possibility to run dual reporter assays, effectively allowing to get two signals from one bead. This could potentially be a cost saver on consumable costs. Dr. Ursu has worked already with a similar equipment at IARC, Lyon, and then she continued working at Karolinska Institute, with a similar equipment. The LX200 is having the

advantage that once implemented the method and validated, became cost and time eficient and more important, reliable, in comparison with almost all the available viral genotyping assays on market.

ii. Targeted cell lines therapy

It is obvious that I would like to implement here, in our university, the similar technology like the ones from IARC or Karolinska Institute, regarding cell lines targeted therapy. For achieving this goal, we would need to apply for other funding of research projects, to allow me to achieve the Incucyte® SX5 HD/3CLR System, and to prepare specific research laboratories.

Beside cell lines therapy, it is more than evident that there nowdays are important research and discoveries in the field of specific inhibitors developing and of the field of technology for assessing the efficiency of these cell treatment with specific inhibitors.

In the same time, medical specialties develop practices minimally influenced by other specialties. The results obtained from laboratory should be transmitted and applied in clinical practice, for the optimization of the cancer patient follow-up. For example, it would be necessary to know the cytotoxic effect needed for a patient. With selected antibiotics, it is possible determine the lowest dose of antibiotic needed to kill the bacterium. We might do this with tumour target therapy, as for the moment it is not sure if oncologists measure levels of anti-tumoral drugs. The oncologic patients persist with therapy despite moderate side effects. One needs uninterrupted, high levels of drug when using prolonged therapy (antibiotic or likely anticancer); resistance develops with stops/starts or lower doses. For example, patient could tolerate pneumonitis and leg edema from high dose everolimus. Another important aspect is that the in absorption of oral drugs may be affected by food and important is to treat the patient with multiple drugs that attack the same target to delay resistance. With antibiotics, we sometimes use two drugs to attack a target, e.g., the cell wall, to delay resistance. Similar procedure would be useful for oncological therapy, to delay resistance.

iii. Future directions in bacteriology research

My PhD thesis and post doc activity are mainly in Virology fields. Even so, as by education I am a microbiologist, I performed a few research studies with bacteria also. Recently, I received proposals of collaboration for testing the therapeutical activity of some nanoparticles against selected bacterial species. Also, another direction could be testing the sensitivity of *Helicobacter pylori* to antibiotics, by INNO LiPA assay.

SECTION IV. REFERENCES

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