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Overexpression of FGFR3 in HPV-positive Tonsillar and Base of Tongue Cancer Is Correlated to Outcome

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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 further analyzed. 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-free 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 and wild-type FGFR3, overexpression of FGFR3 was correlated with better DFS.*

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 of the other head and neck squamous cell carcinomas (HNSCC) (1-6). Also, in many countries the incidences of TSCC/BOTSCC, the oropharyngeal squamous cell carcinoma (OPSCC) subtypes where HPV is most common, have increased (5-14). 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 be of benefit (5, 15, 16). To better tailor therapy, attempts have been made to find prognostic markers (17-25). In HPV$^+$ TSCC/BOTSCC, examples of such markers are 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 (16-25). Mathematical models, combining some of the above markers, can identify 40-56% of patients with high probability for a 3-year DFS (26, 27). However, to recognize more patients that can respond to therapy and to introduce de-escalated or targeted therapy more markers are needed (5, 16).

To find new prognostic biomarkers, next-generation sequencing (NGS) of hot spot mutations was performed in 50 cancer related genes and found discrepancies between HPV$^+$ and HPV$^-$ TSCC/BOTSCC (28). HPV$^+$ TSCC/BOTSCC more frequently had *PIK3CA* and *FGFR3* mutations, while *TP53* mutations dominated in HPV$^-$ cancer, similar to data by Tinhofer *et al.* (29). Also HPV$^+$ TSCC/BOTSCC with *FGFR3* mutations was correlated with worse prognosis (28, 29).

Here, FGFR3 expression and mutation status were examined and correlated to outcome. FGFR3 expression was tested by immunohistochemistry (IHC). A Competitive Allele-Specific Taqman PCR (CAST-PCR) was used to detect the three most common *FGFR3* mutations in samples previously not sequenced by NGS, and the two methods were compared.

Patients and Methods

Patients and tumor characteristics. Patients diagnosed from 2000 to 2011 at Karolinska University Hospital, with TSCC (ICD-10 code

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C09.0-9) or BOTSCC (ICD-10 code C01.9), treated with curative intent, were analyzed for FGFR3 by IHC (Table I). HPV+ TSCC/BOTSCC was defined as having an HPV DNA positive tumor with 16^{INK4A} (p16+) overexpression, or expression HPV16 E7 mRNA (30). Totally, 115 HPV+ TSCC/BOTSCC fulfilling these criteria, according to our previous studies (10, 14, 23, 28), were analyzed for FGFR3 expression with IHC and 98 of them had already been tested for their FGFR3 status (28).

To obtain the FGFR3 status of the remaining samples in the IHC analysis, and to compare two methods for detecting *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 Iasu, 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 (23, 31). p16 had been tested previously using the monoclonal antibody (mAb) clone JC8 (Santa Cruz Biotech, Santa Cruz, California, USA), or the E6H4™ mouse mAb clone (CINtec®, Ventana, Tucson, Arizona, USA) (23, 31).

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 PCR amplification using the Ion AMpliSeq Cancer Hotspot Panel v2 (CHPv2 - Thermo Fisher Scientific), and of these 98 were included in the FGFR IHC analysis (28).

Competitive allele-specific Taqman PCR (CAST-PCR). Detection of *FGFR3* mutations was performed by Competitive Allele-Specific Taqman® PCR technology (Thermo Fischer Scientific, Waltham, MA, USA). The analysis was performed in 384-well plates, in 10 μl comprising 5 μl 2X Taqman Genotyping Mastermix (Thermo Fischer Scientific, Waltham, MA, USA), 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: 95˚C, 10 min followed by 5 cycles at 92˚C, 15 sec and 58˚C, 1 min and 40 cycles at 92˚C for 15 sec and 60˚C for 1 min. The PCR result was analyzed with the SDS 2.3 software program and Mutation Detector Software 2.0 (Thermo Fischer Scientific, Waltham, MA, USA). 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 (21, 23). Staining was blindly evaluated by two researchers, including one pathologist. 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 I. Of the HPV+ TSCC/BOTSCC, 98 had been analyzed for mutations by targeted amplification using the Ion AMpliSeq Cancer Hotspot Panel v2, and of these 10 had mutations within the FGFR3 region (28). 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 (28)) 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 (28), for details see Table II. 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 (28) (Table II).

Furthermore, when 15 HPV+ TSCC/BOTSCC samples, determined as having *FGFR3* mutations when tested by NGS sequencing (28), were analyzed also by CAST-PCR, 13/15, (87%) of these *FGFR3* mutations were confirmed. In addition, 16/16 (100%) of the previously NGS sequenced HPV+ TSCC/BOTSCC samples, determined as having wild-type *FGFR3*, were also classified as having wild-type *FGFR3* when analyzed by CAST-PCR (data not shown).

Table I. *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)	60		61		58		59.6	
Median (years)		60		60		59		59.5	
Range (years)		42-78		30-90		39-78		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%
Gender	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	I	4	14%	4	10%	18	38%	26	23%
(AJCC 8th Edition)	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%

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 of mutated *FGFR3* (1/46, 2.1%) among HPV$^-$ samples (28).

FGFR3 expression in HPV$^+$ TSCC/BOTSCC analyzed by immunohistochemistry. FGFR3 staining was evaluated as a percentage of tumor cells per intensity group (defined as absent/low, medium or strong expression) (Table I 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

Table II. *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%

their highest intensity staining to create three intensity groups (absent/low, medium and strong). In total, 48 samples were defined as 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).

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 a 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 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).

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 (28). After analyzing the 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.* (32) 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. Nevertheless, FGFR3 expression was not correlated to DFS or OS irrespective of whether OPSCC was separated into HPV$^+$ and HPV$^-$ cases (32). 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 wild-type or mutated (32). Nonetheless, the authors concluded, similar to us in our sequencing report,

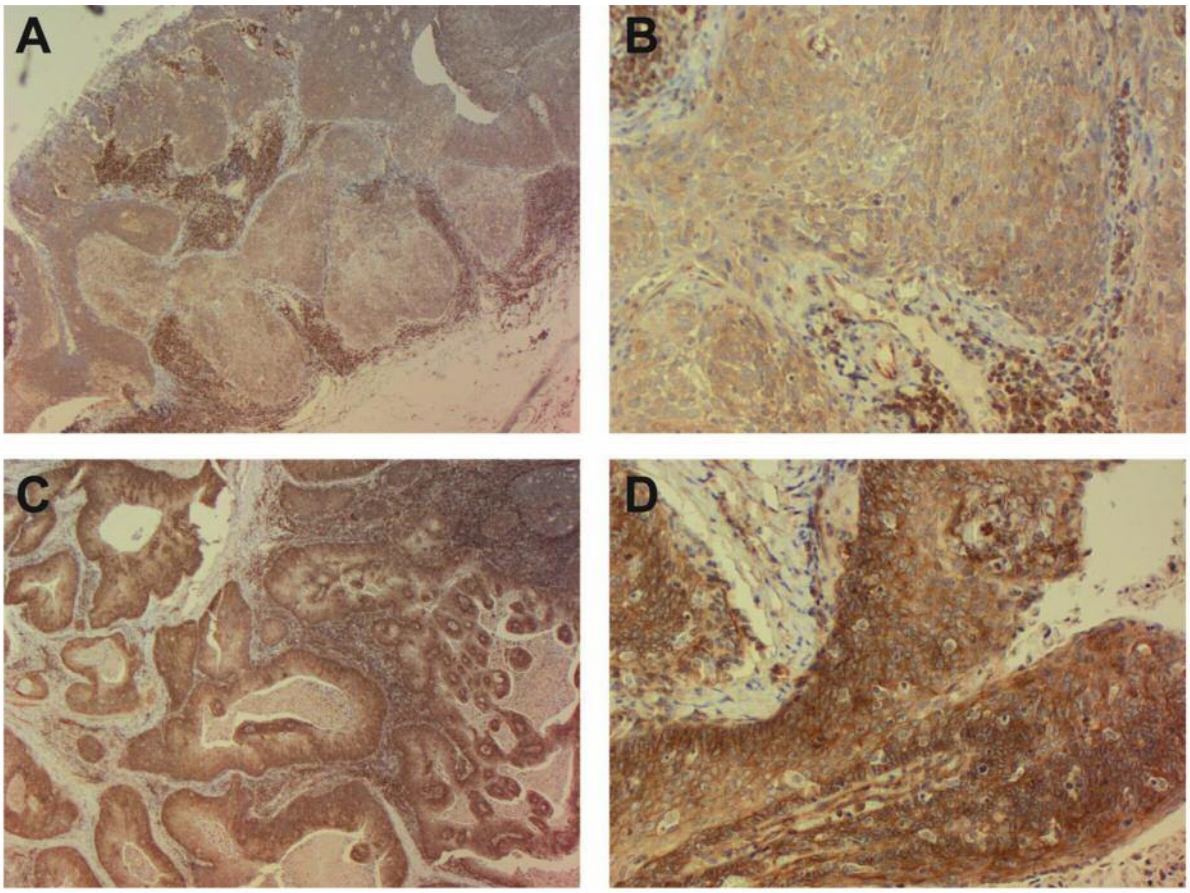

Figure 1. *Immunohistochemistry of FGFR3. A and B: High FGFR3 expression in HPV+ TSCC, 4× and 20× respectively; C and D: Low FGFR3 expression in HPV+ TSCC, 4× and 20× respectively.*

that FGFR3 could serve as a therapeutic target for FGFR3-directed therapies (28, 32).

FGFR3 expression has been investigated in more detail in other tumors, especially in *e.g.* bladder cancer (33-35). Bertz *et al*. (33) examined the role of angiogenesis and FGFR3 protein expression in bladder cancer and showed that high FGFR3 expression was correlated to better survival in a multivariate analysis. The authors suggested that FGFR3 could be a potential therapeutic target also from the angiogenesis perspective (33).

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 (36). 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 (37).

Here, using CAST-PCR, 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 was evaluated (28). In this new cohort we could show that 8.6% of the tumors exhibited FGFR3 mutations. This was insignificantly higher than that in our previous study (5.4%) and similar to that (10%) recently published by Mirghani *et al*. (28, 38). The method discovered 87% of the mutations detected before 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 an 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

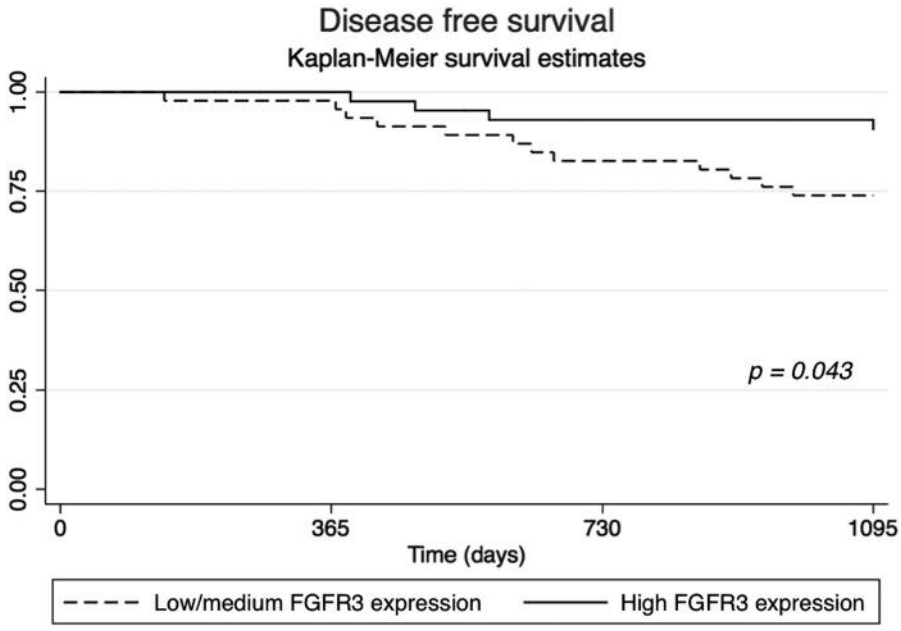

Figure 2. *Disease-free survival (DSF) for patients with HPV+ TSCC/BOTSCC stratified for low/medium and high FGFR3 expression. Cumulative DSF for HPV+ TSCC/BOTSCC stratified for medium/low FGFR3 and high expression. Differences in survival were analyzed with the log-rank test.*

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 to obtain different results. 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, caution is required, 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.

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