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Human Polyomaviruses Are Not Frequently Present in Cancer of the Salivary Glands

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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 (1). 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 (2). 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 (3).

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 (4, 5). Most of these viruses were isolated from nasal samples, skin, serum or stool samples (6). 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 (7).

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 (8). 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 (9). 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 (10). 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 (11-14). Due to their established or suspected oncogenic potential, there have been numerous studies on the occurrence

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of human PyVs in different tumor types, mostly without any viral association being established (10, 15-18).

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*. (19) and modified as in Franzén *et al*. (20). 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*. (19). To evaluate the presence of cellular DNA, β-globin was also assayed with primers as presented in Ramqvist *et al*. (15).

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 (19, 20). 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 (21). 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.

Table I. *Patient and tumor characteristics*.

	Acinic cell carcinoma	Adeno-carcinoma NOS	Adenoid cystic carcinoma	Basal cell adeno-carcinoma	Epithelial-myoepi-thelial carcinoma	Mucoepi-dermoid carcinoma	Myoepi thelial carcinoma	Oncocytic carcinoma	Poorly differentiated carcinoma	Salivary duct carcinoma	Secretory carcinoma	Squamous cell carcinoma	Total
Total, n	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	10	11	3	2	18	1	1	1	3	1	3	73
N1	0	3	0	0	0	0	0	0	1	1	0	1	6
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, years													
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	1 4 - 96
Analyzed metastasis	0	4	0	0	0	0	0	0	0	2	0	2	8
MCPyV-positive	0	1	1	0	0	1	0	0	0	0	0	0	3
% MCPyV-positive	0%	6%	9%	0%	0%	6%	0%	0%	0%	0%	0%	0%	3%

NOS: Not otherwise specified; MCPyV: Merkel cell polyomavirus; dx: right side; sin: left side.

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 (22). 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 (23, 24). 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 (25). 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.

Conflicts of Interest

The Authors declare no conflicts of interest.

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