Anna Ivanova

ORONASOPHARYNGEAL AND SALIVARY GLAND TUMORS CLINICAL AND MORPHOLOGICAL INVESTIGATION, THE ROLE OF THE HERPES GROUP VIRUSES IN THE MALIGNANT PROCESS

Summary of the Doctoral Thesis

Speciality – Oral and Maxillofacial Surgery

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The present Doctoral study has been conducted at the Riga East University Hospital Oncology Centre of Latvia in collaboration with Institute of Anatomy and Anthropology and August Kirchenstein Institute of Microbiology and Virology of Rīga Stradiņš University. It has been approved by the Ethics Committee of RSU 23th September 2010.

Scientific supervisors:

*Dr. habil. med.*, Associate Professor **Valerija Groma**,
RSU Institute of Anatomy and Anthropology

*Dr. habil. med.*, Associate Professor **Egils Kornevs**,
RSU Department of Oral and Maxillofacial Surgery, Riga East University Hospital the Oncology Centre of Latvia

Approved reviewers:

*Dr. habil. med.*, Professor **Ingrida Cema**, RSU

*Dr. habil. med.*, Professor **Jekaterina Erenpreisa**, Latvian Biomedical Research and Study Centre

*Dr. habil. med.*, Professor **Juris Berzins**, LU Institute of Experimental and Clinical Medicine

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Secretary of Doctoral Council:

*Dr. habil. med.*, Professor **Ingrida Cema**
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TOPICALITY OF THE RESEARCH

During the last 50 years there is a tendency of decreasing of the lip cancer incidence in Latvia, reflecting the decrease in use of smoking appliance [1]. By contrast, the incidence of the other types of the oral and oropharyngeal cancers increases in recent decades [1,2]. Carcinoma of the floor of the mouth occur in 35% of the cases, retromolar cancers and tumors of the buccal mucosa occur in 10 - 20%, tongue - 25%, lips - 10%, gingival neoplasms - 1 - 5%, palate cancers occur in 2 - 7% of the cases [1]. The oral squamous cell carcinoma (SCC) are 3.5-5.5 times more common in men between the age 40 and 60, with highest rate in 55-65 years. The survival rates have not improved for decades and alarming rises in incidence in younger subjects amongst both men and women have been reported [1,2,3]. There are eight types of Herpes viruses that infect human tissue: herpes simplex virus 1, herpes simplex virus 2, varicella zoster virus, Epstein-Barr virus (EBV), cytomegalovirus, human herpes virus 6, 7 un 8 [5-8]. The herpes simplex virus type 2, linked to cervical carcinoma, human cytomegalovirus associated with cervical carcinoma, adenocarcinomas of the prostate and colon, and Kaposi’s sarcoma; human herpesvirus 6,7 and 8 (HHV6,7,8) associated with lymphoproliferative disorders and Kaposi’s sarcoma-associated herpesvirus. EBV is a causative factor for infectious mononucleosis and found to be associated with nasopharyngeal carcinoma (NPC), human lymphoid and epithelial cancers, such as gastric carcinoma, breast, prostate and colon cancer as well [9-15]. Caselli et al. (2007) [11] reported that the salivary glands have been described as an in vivo reservoir for HHV-6 infection. Sada et al. (1996) [14] suggested that HHV-7 could develop a productive infection in the salivary glands and also latent persist in periodontal tissue it can be associated with lymphoproliferative disorders, but HHV-6 develop the latent infection with the low replication rates.
It is also investigated whether HHV-6 can transform human cells to establish its relationship to human malignancies [13,14].

Interaction of adhesion molecules on EBV-loaded memory B cells and heparan sulphate of CD44v3 on the surface epithelial cell has been demonstrated to be essential for the initiation of EBV infection of epithelial cells [16,17]. There are only few reports on prediction of clinical behavior of benign salivary tumors [18,19]. Ki-67 proliferation marker has been absent or had low positivity in pleomorphic adenomas (PA) indicating that these mixed tumors have low proliferative rate and good prognosis [19]. The tumor cell proliferation in benign salivary glands tumors affecting the Latvian population, using Ki-67 as a marker has not been conducted until now. In this study, we analyzed the Ki-67 expression in various types of benign salivary gland tumors and its association with clinical course of tumor. The appearance of tumors of the parotid gland deep lobe is a parapharyngeal location. The neoplasms of the parapharyngeal space (PPS) could be subdivided into: salivary gland tumors, neurogenic tumors and miscellaneous tumors. Besides salivary gland primary tumors a direct extension from the deep lobe of parotid gland occurs as well as metastases from elsewhere [20-22]. Although PA is a benign tumor of the parotid gland, it has potential to become malignant. The incidence of malignant transformation increases with the duration of the tumor.
1. AIM OF THE RESEARCH, HYPOTHESIS, OBJECTIVES, NOVELTY OF RESEARCH

1.1 Aim of the research

To investigate the clinical course and etiopathogenesis of the oronasopharyngeal and salivary gland tumors using clinical, morphological and molecular virusology methods; deciphering possible connection of herpes virus infection with these neoplasms.

1.2 Hypothesis of research

There is a connection between oronasopharyngeal tumors, salivary gland tumors and herpes virus infection. For improvement of tumour treatment strategy, it is important to recognize their relationship.

1.3 Objectives of research

1. To determine the relative frequency and distribution of the various types of benign salivary gland tumors in a Latvian population.
2. To analyze surgical interventions for PPS neoplasm removal and revise complications arising in postoperative period in various types of PPS tumors.
3. Using a quantitative immunohistochemical study estimate the expression of the proliferation marker Ki-67 in benign salivary gland tumors for understanding of proliferative peculiarities and clinical behaviour of the primary and recurrent neoplasms.
4. To determine the occurrence and possible involvement of CD44 surface receptor for hyaluronate in development and progression of
EBV infection in patients with oral squamous cell carcinoma (SCC) and NPC.

5. To evaluate EBV infection in nonendemic conditions estimating serum Epstein-Barr virus (EBV) antibodies titers, correlating these with tissue LMP-1 oncoprotein expression in patients with benign and malignant salivary gland tumors, oral SCC, and comparing these indices with NPC.

6. Using the electron microscopy to estimate herpes virus verification possibilities in oronasopharyngeal and salivary gland tumors.

7. Using PCR to detect and to characterize EBV, HHV6 and HHV7 DNA in oronasopharyngeal and salivary gland tumors.

8. Using the quantitative and semiquantitative statistical technique find out the possible correlation between oronasopharyngeal tumors and herpes virus infection.

**Novelty of research**

The assessment of oronasopharyngeal and salivary gland tumors in context of herpes virus infection using clinical, morphological and molecular virusology methods might provide a novel approach of tumor behavior prognostication and is a promising strategy of cancer treatment that might be particularly useful in combination therapy for unresectable cancers or as an adjuvant therapy for resectable tumors.
2. MATERIAL AND METHODS

2.1. Oronasopharyngeal cancer patient group

The study group consisted of 14 adult subjects. The patients attending the Department of Head and Neck surgery, Riga East University Hospital, the Oncology Center of Latvia between 2009 and 2011, were eligible for the study. All patients were evaluated by clinical history and physical examination. Oral SCC and NPC tissues archived at the Pathology Department at Riga East University Hospital between 2009 and 2011 were examined using light microscopy. Further, hematoxylin and eosin (H&E) stained tissue sections of neoplasms were histologically evaluated. The morphologic classification of SCC by degree of differentiation was used in the description of the histopathologic specimen. The histological grading of well (grade 1), moderately (grade 2) and poorly differentiated (grade 3) tissue was given according to the World Health Organization (1969-1981). Histologically, there was squamous cell carcinoma grade 2 in five cases. Data on tumor localization and size, invasion of cervical nodules, distant metastasis, and Tumor, Nodes and Metastasis (TNM) stage were gathered from the patients’ medical records. The examination group included four patients with locally advanced oral SCC, according to WHO T_3-T_4, N_1-N_2 stage III-IV, and one patient with carcinoma of the floor of the mouth with T_2N_0M_0 stage II. The tumors occurred at the base of the tongue (one case) and at the floor of the mouth (four cases). The age range was 39 - 77 years, one female and four males. One patient with NPC was a 25-year-old male, and he underwent radical radiotherapy. One patient had undergone tumor radical resection due to the oral SCC, and we conducted an incisional biopsy of the oral mucosa from/near the border of the oral SCC, which was affected by inflammation. The second incisional biopsy used for comparison was from clinically normal buccal mucosa obtained from a patient.
with carcinoma of the floor of the mouth. Additionally, non-neoplastic salivary gland tissue samples obtained from a normal salivary gland tissue and a benign lymphoepithelial lesion were used for comparison.

2.2. Salivary gland tumors patient group

322 patients with the histologically confirmed diagnosis of salivary gland tumor treated between 1996 and 2007 at the Oncology Center of Latvia were used in this study. In total 212 female and 110 male patients were recorded. The clinical data of patients were obtained with respect to age, gender, duration and type of the tumor at the time of presentation, clinical features, anatomic location, and course of the tumor.

2.3. Parapharyngeal space (PPS) tumors patient group

32 PPS tumors removed during surgeries as well as associated biopsies performed at Riga East University Hospital Oncology Center of Latvia at the Department of Head and Neck Surgery from 2001 till 2006 were included in this study. Patients were divided into four groups according to the type of PPS pathology: group A (n=22) – benign salivary gland tumors; group B (n=5) – malignant salivary gland tumors; group C (n=4) – neurogenic tumors; group D (n=1) – miscellaneous tumors. Patients were followed-up for three years in order to evaluate the impact of surgical complications on later outcomes. Results were summarized using descriptive statistics methods, and the data on types of PPS tumors, rates and types of postoperative complications were systematized using Microsoft Excel data processing program.
2.4. Immunohistochemical detection of proliferation marker in salivary gland pleomorphic adenoma

An immunohistochemical detection of tumor cell proliferation in the benign parotid, submandibular and palatinal salivary gland tissue using Ki-67 as a marker was performed. Control tissues were taken along the surgical removal, from histologically intact salivary gland tissue areas. For conventional light microscopy and immunohistochemistry tissues were fixed in 10% formalin, processed through absolute ethanol and xylene, and embedded in Paraplast Plus wax (58°C). For diagnostic purposes, sections (5µm) were stained routinely with hematoxylin and eosin. The clone MIB-1 anti Ki-67 monoclonal antibody (DAKO A/S, Glostrup, Denmark). Sections were dewaxed in xylene, immersed in absolute ethanol and then traditionally in graded alcohols, transferred to a methanol/0.3% hydrogen peroxide solution for 20 min in order to abolish endogenous peroxidase activity. After quenching of endogenous peroxidase activity sections were washed three times in double distilled water, immersed in 0.01 M phosphate-buffered saline (PBS), pH 7.2-7.4, for 10 min and then incubated with anti-mouse Ig biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) 1:500 dilution for 30 min and streptavidin-biotin-peroxidase preformed complex (BioGenex Laboratories, San Ramon, CA, USA) 1:250 dilution for 30 min. The immunological reaction was developed with 3, 3'-diaminobenzidine tetra hydrochloride (50 mg in 100 ml of PBS with 0.03% v/v hydrogen peroxide). Sections were counterstained with Harries hematoxylin and mounted in Kaiser’s glycerol gelatin. Cells labeled by the antibody displayed a brown nuclear staining pattern. Lymph node sections were used as positive controls for the reaction with MIB-I. We determined Ki-67 positive cells and compared these data with the total cell number appearing within the same field. Additionally, the cell proliferation index was calculated as the number of Ki-67-positive nuclei per 1000 cellular
nuclei. The means, standard error means and confidence intervals of the major variables were determined. Statistical analysis was performed using the SPSS system (release 17.0 software) and the difference was considered at the 0.05 significance level.

2.5. Immunohistochemical detection of CD44, immunocompetent cell CD19, CD68 and S100 expression, its semiquantitative estimation and statistical analysis

Histological sections of 4-5 µm were cut from formalin-fixed, paraffin-embedded tissues and mounted on slides. Consecutive sections were used as negative controls of the immunohistochemical reactions and for hematoxylin and eosin (H&E) staining to confirm the diagnosis. Immunohistochemistry was performed manually using sections collected on SuperFrost Plus slides (Gerhard Menzel GmbH, Germany). Sections were deparaffinized in xylene, immersed in absolute ethanol and then, traditionally, in graded alcohols, and endogenous peroxidase activity was blocked with 0.1% H₂O₂ in methanol for 20 minutes. After quenching of endogenous peroxidase activity sections were washed three times in double distilled water. Heat-induced antigen retrieval was accomplished with the sections placed in 10mM citrate buffer for 30 minutes in a vapor lock. After antigen retrieval, specimens were allowed to cool for 20 minutes. Non-specific binding was blocked with 1% bovine serum albumin/5% normal goat serum in phosphate buffered saline. Thereafter, consecutive sections were successively incubated at 4°C overnight with the following primary antibodies: [65]; monoclonal mouse anti-human CD44 (DakoCytomation, Glostrup, Denmark, 1:50 dilution, clone DF1485), raised against all forms of CD44, and appearing to play a role in cell proliferation and lymphocyte homing, as well as correlating with depth of invasion in oral tumors [43]; monoclonal mouse anti-human CD19 (Dako Corporation,
Glostrup, Denmark, 1:50 dilution, clone LE-CD19), a marker for normal and neoplastic B cells; monoclonal mouse anti-human CD68 (DakoCytomation, Glostrup, Denmark, 1:100 dilution, clone PG-M1), which labels monocytes and macrophages and polyclonal rabbit anti-bovine brain S100 (Dako Corporation, Glostrup, Denmark, ready-to-use), a calcium-binding protein found in dendritic antigen-presenting cells and demonstrated in normal and altered oral mucosa and salivary glands. The staining procedure was achieved by the EnVision technique using Dako ready-to-use, peroxidase-conjugated, rabbit/mouse EnVision reagents and peroxidase substrate solution (diaminobenzidine-H₂O₂). Finally, sections were washed with distilled water, and counterstained with Mayer’s hematoxylin, washed with tap water, mounted, and covered with coverslips. Immunohistochemical controls included omission of the primary antibody or substitution of it with non-immune IgG or phosphate buffered saline solution (pH 7.4). Sections from a tonsil, colon, and lymph node tissue were used as positive controls for CD44, CD68, S100 and B19, respectively. The sections were photographed by a Leitz DMRB brightfield microscope using a digital camera DC 300F. Immunostaining for CD44 was identified by brown stain confined to the cell membrane. The levels of immunopositivity for CD44, CD68, CD19 and S100 were defined semiquantitatively and graded into three groups: minimal: few cells stained (<10%); moderate: a moderate number of cells (<50%) stained; diffuse: majority of cells (>50%) stained, as described previously. Statistical analysis was performed using the SPSS system (version 19.0). The Spearman’s rank correlation coefficient was used to estimate relationships between immunostaining patterns of the antibodies used in this study. Correlation between antigen expression and clinical data was studied by χ² statistics. P values of <0.05 were considered significant.
2.6. Immunohistochemical detection of LMP1 and HHV-6

Monoclonal mouse anti-EBV, LMP (DakoCytomation, Glostrup, Denmark, 1:100 dilution, clones CS.1-4), which recognizes the EBV LMP-1 oncoprotein appearing in the oral epithelial mucosal and neoplastic - SCC and NPC cells and contributing to oncogenesis and tumor maintenance was used. Sections from a case of Hodgkin’s disease were used as positive controls for EBV LMP. Anti-HHV-6 from Santa Cruz Biotech was used in this study. The staining procedure was achieved by the EnVision technique using Dako ready-to-use, peroxidase-conjugated, rabbit/mouse EnVision reagents and peroxidase substrate solution (diaminobenzidine-H$_2$O$_2$). Finally, sections were washed with distilled water, and counterstained with Mayer’s hematoxylin, washed with tap water, mounted, and covered with coverslips. Immunostaining of HHV-6 was identified by brown stain confined to the cell cytoplasm. The average percentages of the LMP-1 positive cell were evaluated from 5-45 areas of the tissue sections under light microscope. The following semiquantitative scale of scores was used: 0 - staining was not observed, 1 is 1 – 10%, 2 is 11 – 30%, 3 is 31 – 75% and 4 is 75 – 100% LMP-positive cells observed, respectively.

2.7. Patients selected for detection of EBV antibodies

Three groups of patients treated from January 2009 to January 2011 were enrolled in this study. The first group included ten patients selected from surgically resected cases of the parotid salivary gland tumors. The second group included four cases of oral SCC and one case of NPC. The third group included two controls subjects. One control subject had no personal history of any type of cancer, and another one had already developed skin basal cell carcinoma and has been operated and irradiated two years ago and had no clinical recurrence.
2.8. Nested polymerase chain reaction

PCR was used for the detection of viral sequences in DNA isolated from peripheral blood leukocytes, tissue and plasma. Total DNA was isolated from 0.5 ml of fresh whole blood and approximately 40mg tissue by phenol-chloroform extraction. For DNA purification from 200 µl of cell free blood plasma, QIAamp Blood Kit (QIAGEN, Germany) was used. Plasma samples were treated with deoxyribonuclease I before DNA purification. To assure the quality of the PBL DNA and tissue DNA and to exclude contamination of plasma DNA by cellular DNA, a β-globin PCR was performed. PCR amplification for the viruses was carried out in the presence of 1 µg of PBL DNA, of tissue DNA and 10 µl of plasma DNA (corresponding to 100 µl of plasma. Positive (virus genomic DNA) and negative (DNA without virus-specific sequences) as well as water controls were included in each experiment. The results were visualized using 1.7% ethidium bromide electrophoreses gel.

2.9. Electron microscopy

For transmission electron microscopy tissue samples were fixed in 2.5 % glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated through graded ethanol series and embedded in epoxy resin (Sigma-Aldrich). 1 mkm thick were made prior the fine sections and stained with toluidine blue. Ultrathin sections of thickness 70–80 nm were cut with LBR ultramicrotome, collected on formvar-coated grids, double stained with uranyl acetate and lead citrate, and examined with a JEM 1011 electron microscope (JEOL, Japan). Specimens were examined at magnification x2000 – x90000.
3. RESULTS

3.1. Oronasopharyngeal tumors and herpes virus DNA detection and analysis

Clinical, histopathological and molecular virusology results are presented in the Tables 3.1 and 3.2. Using PCR EBV DNA was detected in oral and pharyngeal neoplastic epithelium (in 5/6 cases), one of them was carcinoma of the base of the tongue, three - carcinoma of the floor of the mouth and the last one - NPC. In one case of stage II carcinoma of the floor of the mouth with moderate differentiation grade EBV DNA result was negative.

Table 3.1.

Results of EBV testing PCR on patient samples

<table>
<thead>
<tr>
<th>Type of the sample</th>
<th>EBV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>whole blood</td>
</tr>
<tr>
<td>Ca base of the tongue</td>
<td>pos</td>
</tr>
<tr>
<td>Ca floor of the mouth</td>
<td>pos</td>
</tr>
<tr>
<td>Ca floor of the mouth</td>
<td>pos</td>
</tr>
<tr>
<td>Ca floor of the mouth</td>
<td>neg</td>
</tr>
<tr>
<td>Ca reg.retromolaris</td>
<td>pos</td>
</tr>
<tr>
<td>NPC</td>
<td>pos</td>
</tr>
<tr>
<td>BLEL</td>
<td>pos</td>
</tr>
<tr>
<td>Border-line inflammation</td>
<td>neg</td>
</tr>
<tr>
<td>Normal salivary gland</td>
<td>pos</td>
</tr>
<tr>
<td>Normal buccal mucosa</td>
<td>neg</td>
</tr>
</tbody>
</table>

Ca – carcinoma, NPC – nasopharyngeal carcinoma, BLEL – salivary gland benign lymphoepithelial lesion

Positive EBV DNA results were obtained in all cases of moderate differentiation grade of oral squamous cell carcinoma in advanced stage (III and IV). However, no EBV DNA was found within the tumor tissue revealing
grade 2 oral SCC of the floor of the mouth in a stage II. Furthermore, EBV DNA was also identified in a case describing border-line lesion of the oral SCC in advanced stage and in benign salivary gland lymphoepithelial lesion (BLEL). On the contrary, EBV DNA was not evident in normal salivary gland tissue and in clinically healthy buccal mucosa. In total, EBV DNA has been detected in 7 from 8 cases (87.5%) of oral malignant, benign and other lesions screened in this study. Comparing the above mentioned data with the results of EBV testing PCR on patients’ blood samples we have found that in all patients studied plasma EBV DNA concentrations were at undetectable level clearly indicating an absence of active EBV infection.

Table 3.2

Patients characteristics: clinical, morphological and nested PCR results for EBV

<table>
<thead>
<tr>
<th>Patients nr.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td>Number of investigated localizations</td>
<td>1</td>
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<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Gender M/F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Age, years</td>
<td>25</td>
<td>39</td>
<td>50</td>
<td>60</td>
<td>61</td>
<td>63</td>
<td>66</td>
<td>77</td>
</tr>
<tr>
<td>Ca base of the tongue</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca floor of the mouth</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca retromolaris</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td>T₄</td>
<td>T₂</td>
<td>T₄</td>
<td>T₃</td>
<td>T₂</td>
<td>T₄</td>
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<td>Nₐ</td>
<td>N₀</td>
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<td>TNM</td>
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<td>IV</td>
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<tr>
<td>Grade</td>
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<td>Gr2</td>
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<td>Gr2</td>
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</tr>
<tr>
<td>NPC</td>
<td>X</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BLEL</td>
<td>X</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Border-line inflammation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Normal salivary gland</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal buccal mucosae</td>
<td>X</td>
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<td></td>
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<td></td>
<td></td>
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<td>EBV PCR</td>
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<td>pos</td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>pos</td>
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<td>neg</td>
</tr>
</tbody>
</table>

NPC – nasopharyngeal carcinoma, BLEL – salivary gland benign lymphoepithelial lesion
Three negative results were obtained analyzing whole blood samples – a case of border-line lesion, normal buccal mucosa, and, surprisingly, a single case of small SCC of the floor of the mouth with a grade 2 and stage II, whereas, patient with normal salivary gland tissue had positive whole blood EBV DNA. A total negativity – both blood and tissue EBV DNA was obtained in one patient who provided to types of samples – a sample of normal buccal mucosa and small-sized SCC of the floor of the mouth. A total negativity – both blood and tissue EBV DNA was obtained in the above mentioned patient. HHV6, HHV7 un EBV DNA in oral squamous cell carcinoma and in NPC cells. In case of carcinoma localized at the base of the tongue we found latent HHV-6 and HHV-7 infection. There were no signs of any virus DNA in normal buccal mucosa.

Latent EBV infection was detected in carcinoma of the floor of the mouth, in retromolar carcinoma and in NPC. We found the active HHV-6 and HHV-7 infection in case of carcinoma of retromolar region. The active HHV-6 infection and latent HHV-7 infection was detected in case of NPC. Furthermore, in both cases of the carcinoma of the floor of the mouth the active HHV7 infection has been determined.

3.2. Immunohistochemical detection of CD44, immunocompetent cells markers and EBV-related oncoprotein expression in oronasopharyngeal and salivary gland tumors

Table 3.3 summarizes the results regarding CD44, LMP, CD19, CD68 and S100 expression levels estimated for malignant oral and pharyngeal tumors, oral borderline tissue affected by inflammation, benign lesion and normal salivary gland, as well as normal buccal mucosa. Cells labeled by the anti-LMP antibody for EBV oncoprotein displayed a brown cytoplasmic staining pattern. We did not notice any LMP expression in tumor infiltrating
lymphocytes. Expression of LMP in neoplastic cells was demonstrated in EBV-positive oral SCC and NPC enrolled for the study. Levels of this EBV-related oncoprotein expression were varying from absence and minimal to moderate – 50.3, 43.6, 6.0% and 91.1, 6.7, 2.2% for SCC and NPC, respectively. LMP-positive neoplastic cells demonstrated both a diffuse and clustered pattern of distribution in case of SCC. Some SCC cases demonstrated a moderate level of LMP immunopositivity chiefly along a former basal aspect of tumor cord, close to stromal interface. Minimal and moderate oncoprotein expression was found in giant cells appearing in one case of oral SCC. We noticed absence of LMP expression in EBV-positive cases of benign salivary gland lesion and inflammation. Immunostaining for EBV-encoded LMP oncoprotein was lacking in EBV-negative normal salivary gland and normal buccal mucosa. We found that CD19-positive B lymphocytes were intermixed with neoplastic cells, and often heavily infiltrated the tumor. Regarding the presence of S100-positive dendritic Langerhans cells in SCC and NPC patients, we found them in the stromal compartment of NPC. Very few of them were between the epithelial cells, demonstrating a pavemented arrangement of tumor. In the oral mucosa, S100-positive dendritic cells appeared much more frequently within the epithelial layer, and were diffusely distributed between the cells of the basal and spinous layer, and diffusely impregnated the tumor mass. Oral malignancy cases revealed the interrelationship between the expression patterns of immune cells markers CD19 and S100 (r=0.151; p<0.03). Immunohistochemical assessment of CD68-positive cells revealed their presence in tumor, and basically being associated with vascular beds. Comparing the levels of CD19, CD68 and S100 expression reflecting the presence of immunocompetent cells in both types of epithelial malignancies studied, we found statistically significant differences for CD19 and S100 but not for CD68 immunolabeling (CD19: $\chi^2=14.255; \ df=2; \ p<0.001$; S100: $\chi^2=19.887; \ df=2; \ p<0.001$). Simultaneously, both malignant tumors revealed that the levels of CD19, CD68
and S100 expression were statistically different as compared with the levels obtained in benign lesions (CD19: $\chi^2=26.162$; df=2; $p<0.002$; S100: $\chi^2=12.244$; df=2; $p<0.002$; CD68: $\chi^2=18.274$; df=2; $p<0.001$ and CD19: $\chi^2=40.303$; df=2; $p<0.001$; S100: $\chi^2=27.644$; df=2; $p<0.001$ for SCC and NPC, respectively). In case of SCC and NPC, we found CD44-positive membranous staining of tumor epithelial cells, lymphocytes, stromal macrophages, and staining in basal and suprabasal cells of the normal oral mucosa epithelium. In the neoplastic cells and oral mucosa, epithelium immunostaining was restricted to the cell membrane, whereas stromal macrophages appeared to be demonstrating membranous and cytoplasmic pattern. CD44-positive immunostaining of neoplastic cells represented delicate outlines as compared with heavily impregnated epithelial cell contours of the normal oral mucosa. These microscopic findings were in accordance with the levels of CD44 expression found in malignancies and significantly deviated from the levels revealed in normal buccal mucosae. Distribution of CD44 levels in oral SCC and PNC revealed statistically significant differences ($\chi^2=10.806$; df=2, $p<0.005$). Comparing CD44 levels in malignant and benign tumors, normal salivary gland and benign salivary gland lesions, we found $\chi^2=19.099$; df=2; $p<0.001$ (SCC vs. benign salivary gland lesion) and $\chi^2=24292$; df=2; $p<0.001$ (normal salivary gland vs. benign salivary gland lesion), respectively. Regarding the relationship between the expression of CD44 and EBV-encoded oncoprotein in the tissue samples studied, CD44 expression was correlated with the LMP in oral SCC ($r=0.482$; $p<0.001$). In case of SCC and NPC, we found CD44-positive membranous staining of tumor epithelial cells, lymphocytes, stromal macrophages, and staining in basal and suprabasal cells of the normal oral mucosa epithelium. In the neoplastic cells and oral mucosa, epithelium immunostaining was restricted to the cell membrane, whereas stromal macrophages appeared to be demonstrating membranous and cytoplasmic pattern. CD44-positive immunostaining of neoplastic cells represented delicate
outlines as compared with heavily impregnated epithelial cell contours of the normal oral mucosa. These microscopic findings were in accordance with the levels of CD44 expression found in malignancies and significantly deviated from the levels revealed in normal buccal mucosae. Distribution of CD44 levels in oral SCC and PNC revealed statistically significant differences ($\chi^2 = 10,806; \text{df}=2, p<0.005$). Comparing CD44 levels in malignant and benign tumors, normal salivary gland and benign salivary gland lesions, we found $\chi^2 = 19.099; \text{df}=2; p<0.001$ (SCC vs. benign salivary gland lesion) and $\chi^2 = 24292; \text{df}=2; p<0.001$ (normal salivary gland vs. benign salivary gland lesion), respectively. A major portion of lymphocytes associated with tumor mass appeared to be heavily labeled with this antibody showing a membranous pattern of staining. Within infiltrates invading tumor, the proportion of CD44-negative lymphocytes was very low. CD44 expression was correlated with the LMP in oral SCC ($r=0.482; p<0.001$). Using anti-HHV6 antibodies the intensive expression was determined in oral squamous cell carcinoma tissues (Fig. 3.3). We didn’t find any anti-HHV expression in the stroma of tumor, the expression was detected only in malignant keratinocytes.

### 3.3. Evaluation of the EBV antibody titres in oronasopharyngeal and salivary gland tumors

Significantly higher values of VCA IgG and EBNA IgG antibodies were determined in all patients with histologically verified oral SCC stage II and IV and in all epidermoid salivary gland carcinomas compared with the control group of healthy adults and bening salivary gland tumors (Table 3.4). Elevation of anti-EBV antibodies titers determining the latent infection was observed in case of NPC. Immunohistochemically detected LMP-1 expression correlated with elevated titers of VCA IgG anti-EBV antibodies demonstrated in the first and the second group of patients. Expression of LMP-1 was determined in all
carcinomas of the floor of the mouth. We found that IgG EBV antibodies titers were elevated in the cases of epidermoid carcinoma either oral SCC or salivary gland tumors. Basically, VCA IgG titers were markedly elevated in cases of epidermoid carcinomas of parotid salivary gland and parotid LEC (Table 3.4).

Table 3.4

Results of EBV testing PCR, EBV titers and LMP1 expression in benign and malignant salivary glands

<table>
<thead>
<tr>
<th>Salivary gland tumors/ EBV A/b titres</th>
<th>IgM EA</th>
<th>IgM VCA</th>
<th>IgG VCA</th>
<th>IgG EBNA</th>
<th>LMP1 semiquntitative scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermoid carcinoma</td>
<td>neg</td>
<td>neg</td>
<td>&gt;200</td>
<td>118</td>
<td>3</td>
</tr>
<tr>
<td>Pleomorphic adenoma</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>91</td>
<td>0</td>
</tr>
<tr>
<td>Carcinoma ex pleomorphic adenoma</td>
<td>neg</td>
<td>neg</td>
<td>136</td>
<td>neg</td>
<td>0</td>
</tr>
<tr>
<td>Epidermoid carcinoma</td>
<td>neg</td>
<td>neg</td>
<td>253</td>
<td>neg</td>
<td>0</td>
</tr>
<tr>
<td>Ductal Carcinoma</td>
<td>neg</td>
<td>neg</td>
<td>25</td>
<td>neg</td>
<td>0</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>86</td>
<td>no data</td>
</tr>
<tr>
<td>Recurrent pleomorphic adenoma</td>
<td>neg</td>
<td>neg</td>
<td>156</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Epidermoid carcinoma</td>
<td>neg</td>
<td>neg</td>
<td>136</td>
<td>55</td>
<td>no data</td>
</tr>
<tr>
<td>Pleomorphic adenoma</td>
<td>neg</td>
<td>neg</td>
<td>158</td>
<td>neg</td>
<td>no data</td>
</tr>
<tr>
<td>Lymphoepithelial carcinoma LEC</td>
<td>neg</td>
<td>neg</td>
<td>259</td>
<td>neg</td>
<td>no data</td>
</tr>
</tbody>
</table>

The absence of elevation of any EBV antibodies in ductal parotid carcinoma and adenocarcinoma of parotid gland. We have determined the absence of IgA antibodies that suggests an absence of active infection or reinfection (table 3.5). Normal parotid salivary gland tissue and submaxillary gland lymphoepithelial lesion placed in the second group revealed the presence of EBV DNA. EBV DNA was determined in all oral SCC grade 2, 3 and advanced clinical stage (stage III and IV). In the second group of patients presented with carcinoma of the floor of the mouth the elevated anti-EBV antibody titers correlated with the presence of EBV DNA sequence in tumor tissues.
### Distribution of CD44, LMP, CD19, CD68 and S100 expression levels in oronasopharyngeal carcinoma and oral borderline tissue affected by inflammation, benign lesion and normal salivary gland, as well as normal buccal mucosa

<table>
<thead>
<tr>
<th>Type of the sample/diagnosis</th>
<th>CD19 min</th>
<th>CD19 vid</th>
<th>CD19 dif</th>
<th>CD68 min</th>
<th>CD68 vid</th>
<th>CD68 dif</th>
<th>S100 min</th>
<th>S100 vid</th>
<th>S100 dif</th>
<th>CD44 min</th>
<th>CD44 vid</th>
<th>CD44 dif</th>
<th>LMP1 0 min</th>
<th>LMP1 0 vid</th>
<th>LMP1 0 dif</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoma of the floor of the mouth</td>
<td>131 (69.7%)</td>
<td>32 (17.0%)</td>
<td>25 (13.3%)</td>
<td>43 (22.4%)</td>
<td>80 (41.7%)</td>
<td>69 (35.9%)</td>
<td>218 (70.8%)</td>
<td>73 (23.7%)</td>
<td>17 (5.5%)</td>
<td>42 (15.5%)</td>
<td>82 (30.3%)</td>
<td>147 (54.2%)</td>
<td>217 (50.4%)</td>
<td>188 (43.6%)</td>
<td>26 (6.0%)</td>
</tr>
<tr>
<td>NPC</td>
<td>35 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>6 (11.5%)</td>
<td>22 (42.3%)</td>
<td>24 (46.2%)</td>
<td>107 (90.7%)</td>
<td>11 (9.3%)</td>
<td>0 (0%)</td>
<td>15 (16.7%)</td>
<td>43 (47.8%)</td>
<td>32 (35.5%)</td>
<td>123 (91.1%)</td>
<td>9 (6.7%)</td>
<td>3 (2.2%)</td>
</tr>
<tr>
<td>BLEL</td>
<td>20 (33.3%)</td>
<td>19 (31.7%)</td>
<td>21 (35.0%)</td>
<td>0 (0%)</td>
<td>26 (43.3%)</td>
<td>34 (56.7%)</td>
<td>42 (58.3%)</td>
<td>30 (41.7%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>11 (17%)</td>
<td>51 (82.3%)</td>
<td>41 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Oral borderline tissue affected by inflammation</td>
<td>7 (46.7%)</td>
<td>8 (53.3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>5 (41.7%)</td>
<td>7 (58.3%)</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
<td>0 (0%)</td>
<td>2 (12.5%)</td>
<td>10 (62.5%)</td>
<td>4 (25.0%)</td>
<td>9 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Normal salivary gland tissue</td>
<td>54 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>28 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>54 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>12 (22.2%)</td>
<td>19 (35.2%)</td>
<td>23 (42.6%)</td>
<td>35 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Normal oral mucosa</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>8 (100%)</td>
<td>0 (0%)</td>
<td>14 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

min – minimal, mod – moderate, dif – diffuse
Fig. 3.1. Oral SCC revealing CD44-positive membranous staining; the antibody also labeled the surface of lymphocytes and surface and cytoplasm of stromal macrophages adjacent to tumor mass. Original magnification 400×.

Fig. 3.2. Carcinoma of the floor of the mouth. EBV LMP-1 oncoprotein expression in the tumor cell cytoplasm. x 400. Original magnification 400×.

Fig. 3.3. Carcinoma of the floor of the mouth. Expression of HHV-6 in the tumor cells, and its absense in stroma x 400. Original magnification 400×.

Fig. 3.4. Ductal carcinoma of the parotid gland. Intensive HHV-6 expression in the tumor cells. x 250. Original magnification 250×.
Table 3.5

**Results of EBV testing PCR, EBV titers and LMP1 expression in oral SSC and NPC**

<table>
<thead>
<tr>
<th>Diagnosis and stage</th>
<th>EBV antibody titres</th>
<th>LMP -1</th>
<th>EBV DNS in tumor tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM EA</td>
<td>IgM VCA</td>
<td>IgG VCA</td>
</tr>
<tr>
<td>Carcinoma of the floor of the mouth T2N0M0 st II</td>
<td>neg</td>
<td>neg</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Carcinoma of the floor of the mouth T2N2M0 st IV</td>
<td>neg</td>
<td>neg</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Carcinoma of the floor of the mouth T4N0M0 st IV</td>
<td>neg</td>
<td>neg</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Carcinoma anguli labiorum T3N0M0 st III</td>
<td>neg</td>
<td>neg</td>
<td>77</td>
</tr>
<tr>
<td>NPC stIV</td>
<td>neg</td>
<td>neg</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Basalioma</td>
<td>neg</td>
<td>neg</td>
<td>88</td>
</tr>
<tr>
<td>Healthy adult</td>
<td>neg</td>
<td>neg</td>
<td>74</td>
</tr>
</tbody>
</table>

3.4. Epidemiology, peculiarities of the clinical course and pathomorphological features of tumors of the salivary gland

Benign salivary gland tumors occurred in the major (parotid and submandibular) and the minor (palatinal) salivary glands. The parotid gland accounted for 274 (85%) of all benign salivary gland tumors. There were variations in proportions of benign tumor lesions at different lobes of the parotid gland reflecting a predominant involvement of the superficial lobe of this gland. The submandibular and minor salivary glands revealed much less frequent involvement compared with the parotid gland - 35 (11%) and 13
Pleomorphic adenoma was the most common benign salivary gland neoplasm and accounted for 242 (75%). The majority of these tumors were in the parotid gland 198 (82%) and the remainder in the submandibular 27 (11%) and the minor palatinal 17 (7%) salivary glands. The second most common benign tumor was adenolymphoma which accounted for 45 (14%), whereas monomorphic adenoma was the third most common benign tumor - 29 (9%). The parotid gland was the exclusive site of involvement in case of adenolymphoma and monomorphic adenoma. Less common benign tumors including oncocytoma 2 (1%), myoepithelioma 2 (1%), and vascular tumors 2 (1%) were also reported. The age range was 17 - 86 years. Peak occurrence in female patients was in the fifth decade, whereas in male patients – in the seventh decade (fig.3.5). Females were more commonly affected than males in the vast majority of decades. In the fifth decade a number of affected female patients was significantly higher (p=0.05) than a number of males, whereas, in the seventh decade a number of affected male patients was significantly higher (p=0.05) than a number of females. The patient records showed no correlations between the duration of neoplasm, the tumor size, and the fact of recurrence. In a majority of cases pleomorphic adenoma tumors were small, well-circumscribed, encapsulated nodules measuring from 1 up to 10 cm. The size of most tumors varied from about 1-3 cm (47%), followed by 3-5cm (38%). Some reported cases showed much larger size 5-10cm (15%). We studied parotid, submandibular and palatinal pleomorphic adenomas with the tumor history 1-3 years (55.5% of the cases), 3-5 years (19.8%), 5-10 years (9%) and more than 10 years (15.5%). Duration and size of these tumors had no association with pathological type of pleomorphic adenoma. Histologically, the epithelial component of pleomorphic adenoma often formed the bulk of the tumor and showed a wide variety of cell types including cuboidal, basaloid, spindle and clear cell.
Fig. 3.5. Age and gender of the benign salivary gland tumor patients

The stromal component was mucoid/myxoid, cartilaginous or osseoid. In stromal type or equally stromal and epithelial, the mesenchymal-like component constituted the bulk of the tumor. Wartin’s tumors showed prominent lymphoid tissue typically covered by epithelial cells.

3.5. Surgical complications of the parapharyngeal space tumors

Group A constituted a majority of tumors 68.7% (22 cases) presented with benign salivary gland neoplasms. From them, eighty percent were pleomorphic adenomas. In group B constituting 18.5% (5 cases) mucoepidermoid carcinoma was the most common malignant tumor followed by carcinoma ex pleomorphic adenoma. Parapharyngeal neurogenic tumors included in the group C constituted 12.5% (4 cases). These commonly presented with a poststyloid mass, and were subdivided into paragangliomas, carotid body tumors, and vagal paragangliomas or schwannomas. Miscellaneous tumors were rare and heterogeneous neoplasms – 0.3% (1 case).
The case appeared under the scope of this study was lymphoproliferative disease. The transoral approach was used in 1% of the cases for removal of small, benign neoplasms that originate in the prestyloid PPS and present with an oropharyngeal mass. Limitations of this approach were the restricted access, inability to visualize great vessels, increased risk of facial nerve injury and tumor rupture. The transoral approach may be combined with an external approach to fix lesions with significant oropharyngeal component. Transcervical – submandibular approach was used in 56% of surgeries. Transparotid - cervical approach was used in 25% of the cases. This approach combined a parotidectomy approach and visualization of the main trunk of the facial nerve and its lower or all branches with a transcervical approach. Extended approaches with the mandibulotomy, which gave an excellent exposure to the PPS but was associated with certain morbidity and, therefore, was applied for extensive vascular tumors or recurrent pleomorphic adenoma with multiple large nodules was used in 17% of cases. Various locations for osteotomy have been used including mandibular body, angle, ramus, and parasymphseal. For lesions localized at the base of the skull an infratemporal fossa approach was needed in 1%. The highest rate constituting 45% was demonstrated for B group patients, whereas, the lowest 5% – for A group patients. The spectra and rates of greatly varying postoperative complications occurring in surgical interventions in PPS are summarized at the 3.6 figure. A common complication in 30% of the cases was so-called “first bite syndrome”. The temporary injury of the facial nerve from traction affection and permanent due to inadvertently sacrificing or affected manipulation was appearing in 4 and 1% of surgical interventions, accordingly. Cranial nerve palsies were demonstrated in 20% of the cases resulted from removal of poststyloid PPS lesions. Injury of the spinal accessories nerve occurred in 15% of the cases and manifested with weakness of the trapezius muscle, winging of the scapula, and adhesive capsulitis.
Tracheostomy was required for airway protection in 0.5% of the cases when multiple cranial nerve deficits appeared from resection. Palatal weakness, vocal cord paralysis and fistula were fixed very rarely (0.5%). A cerebrospinal fluid leak was detected after removal of tumors with jugular foramen or intracranial extension in 1% of the cases. The prevalence of intraoperative vascular injury and of perioperative stroke has been detected at 9% for poststyloid lesions. Injury to the lingual and hypoglossal nerves developed from the transcervical approach was noticed (5%). The vagus nerve was the most commonly affected nerve. Complications of mandibulotomy included infection, temporomandibular joint dysfunction, nonunion, plate extrusion, and tooth loss in 5% of the cases. The recurrence rate of benign PPS neoplasms following surgical extirpation constituted 9%. 

Figure 3.6. Spectra of postoperative complications in PPS surgery.
3.6. Detection of the proliferation marker in benign salivary gland tumors

Ki-67 expression was detected in stromal and epithelial components of salivary gland pleomorphic adenoma. The greatest cellularity and the number of Ki-67-positive cells per visual microscopic field was observed in recurrent pleomorphic adenomas, where the mean number of Ki-67-positive cells per visual microscopic field constituted \( 2.14 \pm 1.60 \) \(^1\) (95% CI \(^2\) 1.47-2.47) comparing with 1.43 (95% CI 0.97-1.55) revealed in primary tumors when the whole material was taken into consideration. Still when the morphological peculiarities of the tumor type were estimated along with evaluation of the expression of proliferation marker, a wide range of expression became visible. The number of the Ki-67-positive cell varied from 0.07 ± 0.03 (95% CI 0.01 – 0.14) to 4.81± 0.60 (95% CI 3.61 – 6.02), and from 0 to 0.79 ± 0.11 (95% CI 0.57 – 1.00), respectively. A great Ki-67 expression was observed in the epithelial tumor variants. Much lower expression of the proliferation marker was detected in the stromal type of the tumor. In the chondroid stroma the Ki-67 expression was almost nil, whereas, a richly developed mucoid stroma revealed higher cell proliferation marker expression. No significant differences were found between the mean number of the Ki-67-positive cells per visual microscopic field in the principally stromal variant of the tumor and a control tissue - 0.14 ± 0.04 (95% CI 0.05 – 0.22) and 0.04 ± 0.03 (95% CI 0 – 0.09), respectively. The patients included in this study have had two and three previous surgeries due to the tumor, and with the last tumor developed in 1 year. All of the recurrences were developed in the parotid salivary gland. The estimated recurrence of the mixed tumor was about 4%, and recurrence rates were higher in previously recurrent tumors. Subsequent recurrence after an

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\(^1\) Standard error mean
\(^2\) Confidence interval
initial recurrence occurred at a rate of 25%. The next recurrence was reported at a shorter interval in cases with the history of recurrence. Re- recurrent tumors revealed a high Ki-67 expression. In the re- recurrent pleomorphic adenoma tumors cell proliferation demonstrated using Ki-67 as a marker was revealed in both – epithelial and stromal component, and the estimated cell proliferation index was 7.15, comparing with 3.27 revealed in the non-recurrent tumors. The predominantly epithelial and extremely epithelial types of re- recurrent tumor compared with the first recurrent tumor showed the mean value of Ki-67 equal to 1.87 (range 0.23 – 4.81) and 1.59 (range 1.33 – 1.86), respectively; whereas, the stromal types - 0.32 (range 0 – 0.79) and 0.24 (range 0.22 – 0.27), respectively. Moreover, there was a significant increase in the Ki-67 expression within the above mentioned stromal component in all recurrent pleomorphic adenomas comparing with this in the non-recurrent cases and a normal salivary gland tissue, and the mean number of the Ki-67-positive cells constituted 0.79 ± 0.11 (95% CI 0.57 – 1.00); 0.14 ± 0.04 (95% CI 0.05 – 0.22), and 0.04 ± 0.03 (95% CI 0 – 0.09), respectively. We haven't got an evidence of higher Ki-67 expression in Wartin’s tumors and other types of monomorphic adenoma comparing with the pleomorphic adenoma.
4. DISCUSSION

Our evidence regarding the contribution of EBV infection to the pathogenesis of oral SCC is based on a small number of cases, and the situation appears to be very similar to that in analyses published in the available literature [23,24]. Tumors used in the present study were moderately differentiated neoplasms, and we were not able to suggest a necessity of low differentiation of tumor for the existence of stable EBV infection. A clear prevalence of certain oral localizations of EBV-positive SCC. The tumors analyzed in the present study were localized in close vicinity to the tonsils – lingual, pharyngeal, palatinal, and there are some publications demonstrating a restriction of EBV receptor/CD21 mRNA to tonsil epithelium [26]. An interesting finding of our study is the presence of EBV DNA in a borderline mucosa lesion localized near SCC. Awareness regarding surgical tactics, as well as the amount of incised tissue, appears from the present study. In our borderline case with histologically confirmed inflammation, the high number of immunocompetent cells distributed within the inflammation area could be a possible explanation for EBV DNA positivity in this region.

The literature data appear to vary widely regarding a number of oral SCC cases, revealing EBV DNA positivity an acceptable explanation for such variability was provided by Gonzalez-Moles et al. (2002), [23,24] and some of the causes were as follows: inability of discrimination for the origin of the amplified viral DNA (tumor cells, lymphocytes, saliva), and heterogeneity of tissue sample types and ways of their collection where some samples were present as brush biopsies, oral smears, and, finally, surgically removed but stored frozen samples. Currently, there is no data regarding double sampling collection. We included a single SCC case and performed double sampling – from a neoplasm affected and control area obtained from the same patient. Unfortunately, this case was EVB-negative.
A number of LMP-positive neoplastic cells revealed in the present study appeared to vary widely. This varied from absence and minimal to moderate – 50.3, 43.6, 6.0% and 91.1, 6.7, 2.2% for SCC and NPC, respectively, conducted by Horiuchi et al. (1995) [26]. Moreover, some EBV-infected tumor cells were diffusely distributed within the neoplasm, still the others revealed a strong clustering pattern. Some of our SCC demonstrated LMP-positive cells with a focal but linear pattern of distribution tending to appear along the former basal aspect of tumor cord, and in close vicinity to the stromal compartment. Estimating samples obtained in a case of Hodgkin’s disease, Murray et al. (1992) explained this finding by phosphorylation of the protein and its binding to the cytoskeleton [27]. Other investigators have shown correlations between stromal invasion, tumor grade and immunohistochemical expression of CD44 in salivary gland lesions Franci et al. (2001) [28]. The level of CD44 immunostaining in neoplastic cells was significantly lower as compared with normal oral mucosa, and the expression of CD44 was correlated with the LMP in oral SCC (r=0.482; p<0.001). The CD44 splice-variant isoforms are differentially expressed in some tumors [16], reported by Assimakopoulos et al. (2002) was shown to be down-regulated in squamous cell carcinomas of the head and neck [29]. The results reported in head and neck cancer by Reategui et al. (2006) [30] showed that tumor tissues express CD44v3 levels that are elevated 4.5 times more than normal tissues, while Wang and Bourguignon (2011) [31] reported an implication of CD44v3, -v6 and v10 containing isoforms in progression of SCC of the head and neck. The results obtained by Oliveira et al. (2011) [32] showed that the CD44-positive immunophenotype reveals significant differences between the overall survival curves, and that it is an independent factor of poor prognosis in multivariate analysis. Recent CD44 transcriptomics studies conducted by Rajarajan A. et al. (2012) [33] demonstrated that although CD44 has been shown to be highly expressed in oral and pharyngeal cancers in comparison to other cancer types. EBV infection
is initiated by the interaction of the major viral envelope glycoprotein, gp350, with the complement receptor, CD21, on the surface of B lymphocyte. Shannon-Lowe and Rowe (2011) [16] found that this process is followed by the interaction of CD11b on the B lymphocyte with heparan sulphate of CD44v3 on the cultured epithelial cell. Besides, as it emerges from the same study, the extracellular matrix represents a barrier to epithelial cell infection by EBV virus, and interaction of lymphocyte with fibronectin facilitates migration of lymphocytes to the epithelium. Migration of EBV-infected B lymphocytes into mucosal epithelium is a rare event (Shannon-Lowe et al., 2011) [16], whereas, EBV-infected monocytes may serve as a vehicle for virus transmission between the blood compartment and oral epithelium. Macrophages and dendritic cells examined in this study were found to be adjacent to the mucosal epithelium and LMP-positive tumor mass displaying CD44 immunolabeling, and could be assumed to be possible candidates mediating EBV infection of epithelial cell. Based on our results confirming the presence of CD44, a cell surface receptor for hyaluronate, on the malignant oropharyngeal epithelial cells, lymphocytes and stromal macrophages in sustained EBV infection in patients with SCC and NPC, we provide a new insight regarding the CD44-mediated signaling in EBV infection and pathogenesis of oral and pharyngeal tumors. The increasing number of diseases that are linked to EBV infection underlies the long-term importance either of developing an effective vaccine that can protect against disease or, for the virus-associated malignancies, and of developing novel antiviral agents that can target the virus-carrying cells.

EBV, HHV6 and HHV7 was found in retromolar carcinoma, NPC and in salivary gland BLEL, were the lymphoidal tumor component was also present and was prominent. Therefore, healthy oral mucosa and salivary gland tissue were free from any of the viruses. Latent EBV infection were present in all immunosuppressed cases due to malignant disease. We suppose that HHV6 infect the oral tumor tissue coming from salivary gland where its persists
chronically [34]. In cases of the carcinoma of the floor of the mouth the HHV7 and EBV reservoir was periodontal tissue [34]. Oncolytic viruses can be genetically engineered to induce cell lyses through virus replication and cytotoxic protein expression. Arming herpes virus with therapeutic genes merits further investigation for potential clinical application (Ottolino-Perry et al., 2010) [35].

Caselli et al (2007) reported that HHV-6 suppressed all three lineages of hematopoiesis, i.e., erythroid, granulocyte/macrophage, and megakaryocyte, whereas HHV-7 did not have any suppressive effect. We suggest, that the treatment of EBV, HHV6 and HHV7 infection must ultimately meet three different objectives: inhibition of active viral replication; cure of latent viral infection; and interruption of EBV-induced or may be HHV6 or HHV7-induced cellular proliferation and transformation. An immunohistochemical staining using anti-HHV-6 antibody was used to detect HHV-6 antigen in salivary gland tumor and normal tissues. Our results demonstrated the presence of HHV-6 in salivary gland ductal carcinoma, therefore, HHV-6 possibly play a role in the pathogenesis of this tumor.

In our study we have retrospectively summarized also the clinico-pathological data on benign salivary gland tumors treated between 1996 and 2007 at the head and neck department of Oncology Center of Latvia. Pleomorphic adenoma with a parotid gland involvement was the most common salivary gland tumor affecting the Latvian population and accounted for 75% of all benign salivary neoplasms. These statistical data on a large series of salivary gland tumors in a European and American population confirm relatively closed figures [36-38]. A double higher number of females affected by benign salivary gland tumor were demonstrated in the present study, with a ratio of 1:1.9. The present study showed that benign salivary gland tumors had the higher incidence in the fifth and seventh decade in female and male patients, respectively. These results are similar to that of most other studies [36,38]. Ki-
67 immunoreactivity has been reported to be a prognostic factor in numerous human cancers and also in some salivary gland carcinomas. From these, Skalova et al. (1994) [39] showed that none of the patients with low MIB-1 indices developed salivary gland acinic cell carcinoma recurrence during a long follow-up period. Some papers specify the Ki-67 expression in particular sites of involvement. Alves et al. (2004) [40] showed that in case of pleomorphic adenoma less than 5% of Ki-67-positive cells were present, counting at least 1000 cells. The others reported that the Ki-67 value was significantly higher in large salivary gland tumors and in cases with treatment failure. In study published by Luka et al. (2006) [41] the proliferative capacity of salivary gland tumor as measured by the volume corrected index of Ki-67 corresponding to Ki-67 /mm² of tumor tissue has been shown to be one of the most powerful indicators of tumor behavior. The results of the present study allow us to suggest that appearance and elevation of stromal proliferative activity in the recurrent salivary gland pleomorphic adenoma may be responsible for more aggressive clinical behavior. Varying architectural and cellular composition of pleomorphic adenoma was characterized by a marked diversity in the cell proliferation reflected by Ki-67 expression levels. The increase of tumor cell proliferation in the stromal component may be responsible for more aggressive clinical course. Primary parapharyngeal tumors are rare and these are located in a complex anatomical region. Clinical presentation of these tumors can be subtle [42]. The transoral is not the approach of choice for most lesions of the PPS. The “first bite syndrome” is the most common complication arising from the damage of the sympathetic innervation of the parotid gland. It has been shown very recently in the paper published by Costa et al. (2012) [43] that botulin toxin type A has been suggested as a treatment. A vagal injury combined with injury of n.glossopharyngeus and n.hypoglossus, may result in significant problems with swallowing and aspiration. Patients should be
carefully evaluated prior to the institution of oral feedings, and an alternate method of feeding (nasogastric tube, gastric tube) should be instituted if necessary Khafif et al. (2005) [20].

Since primary parapharyngeal tumors are exceedingly rare, only very few large cohort studies are available. Shahab et al. (2005) [44], reviewed 114 parapharyngeal tumors. Boedeker et al., (2005) [22], paragangliomas recur in approximately 5% of cases, and, since 10% are multicentric, the risk of developing a second tumor remains. Patients with a familial paraganglioma syndrome have a 35% risk of multicentricity. In addition, patients with paragangliomas who are being treated nonoperatively must be alerted to the risk of malignant degeneration, which constitutes approximately 10% and is usually associated with rapid growth. Malignant tumors of the PPS have a much higher rate of recurrence - 25-77%, depending on histology, extent of resection, and duration of follow-up [22]. Postoperative radiation therapy for PPS malignancies is recommended to prevent recurrences. For malignancies the 5 year survival was 93%, but fall to 57% at 10 years. While surgery is the mainstay of the treatment for parapharyngeal tumor, radiation therapy should be considered in elderly patients with paragangliomas. Mandibulotomy can be recommended for vascular tumors extending into the superior PPS [44]. solid tumors that are confined to the superior aspect of the PPS and malignant tumors invading the skull base [22,44]. Embolization is recommended for vascular lesions greater than 3 cm in which obvious feeding vessels can be identified on angiography [22]. The arguments in favor of observation are that paragangliomas grow very little per year in the order of 1 mm, and they are almost always benign tumors, therefore, morbidity and mortality is low if tumor left untreated [22].
5. CONCLUSION

- The benign salivary gland tumors in Latvia are characterized by the incidence that is comparable with that in the other European populations; moderate tendency for female predominance; the incidence of the advanced stage oral malignancy is higher.
- The elevation of stromal cell proliferative activity Ki-67 in the recurrent salivary gland pleomorphic adenoma may be responsible for more aggressive clinical behavior.
- Surgery of PPS tumors is the mainstay of treatment still radiation therapy should be considered in patients at a high risk.
- The CD44 surface receptor for hyaluronate on the malignant oropharyngeal epithelial cells, lymphocytes and stromal macrophages in sustained EBV infection in patients with SCC and NPC suggests the CD44-mediated signaling in EBV infection and pathogenesis of oropharyngeal tumors.
- EBV infection is a neoplasm co-morbid pathology is evidenced by elevated titers of antibodies. Identification of LMP1 expression and EBV DNA at advanced tumor stage. There is a predominance of the EBV-positive tumors among oral SCC.
- The betaherpesviruses and EBV co-infections seems to play a significant role in pathogenesis of oronasopharyngeal tumors. Necessity to perform a double check biopsy - from a neoplasm affected and control area obtained from the same patient arises from the present study.
- Electron microscopy used in combination with other methods provides additional information about oronasopharyngeal neoplastic tissue architecture under exposure of sustained viral infection.
6. REFERENCES


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7. PUBLICATIONS ON THE RESEARCH TOPIC


7.1. Abstracts on the research topic


7.2. Oral presentations at congresses and conferences on the research topic


**7.3. Poster presentations at congresses and conferences on the research topic**

