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ORIGINAL ARTICLE

Cytomegalovirus induces Interleukin-6 mediated inflammatory response in salivary gland cancer

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Summary

Purpose: *The purpose of this study was to examine whether cytomegalovirus (CMV) is present in different histological types of salivary gland cancer (SGC) by detecting CMV immediate-early (IE) and early gene products, and to determine the presence of its association with the overexpression of interleukin (IL)-6.*

Methods: *Immunohistochemical analysis of 92 cases of different histological types of SGC was performed to determine the presence of IL-6 and CMV antigen and its intensity in tumor tissue. Twenty samples of normal salivary gland tissue obtained during autopsy served as healthy controls.*

Results: *CMV antigens were not found in healthy acinar tissue of salivary glands, but were expressed in epithelium of salivary gland ducts. Negative expression of CMV antigens was also found in salivary gland tissue surrounding tumors. On the other hand, CMV was detected in 65/92 SGC cases (70.6%). Higher expression of IL-6 was found in SGC (70.7%) than in normal tissue (20%). There was a high association of CMV antigen presence with the presence of IL-6, and with the IL-6 expression intensity.*

Conclusions: *Positive expression of CMV antigens in a high percentage of SGC cells suggests that it might play an important role in carcinogenesis by increasing IL-6 production and leading to inhibition of apoptosis and tumor development.*

Key words: *CMV, IL-6, immunohistochemistry, salivary gland cancer*

Introduction

SGCs are rare tumors characterized by an enormous morphological diversity between different subtypes going along with diverse clinical courses [1]. Its incidence is about 2.5–3.0 per 100 000 per year [2]. Besides morphological variations among individual tumors, the morphology can also vary greatly within the same tumor mass, making the diagnosis and classification of salivary gland tumors a major challenge [3]. The etiology of SGC is still unknown, although a correlation has been shown between its occurrence and some environmental factors, such as ionizing radiation or direct contact with asbestos [4].

Even though environmental carcinogens and inborn genetic mutations are in the focus of most oncological research, only a very small percentage of malignancies develop as a direct result of these factors. However, there is growing evidence that infectious agents are frequently associated with human cancer. In the last several decades there is increased evidence that infectious agents can directly lead to the development of cancer, such as

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hepatoma, gastric cancer or cervical cancer [5]. In malignancies that currently cannot be attributed to infectious agents, it has been shown that long lasting inflammation can lead to the transition from neoplastic precursors to malignancy, even though it has no oncogenic effect on normal cells [6, 7]. During various infections, one of the most commonly present cytokines is interleukin-6 (IL-6), that has a very effective role in fighting off infectious agents. However, it has been shown that IL-6 has a very important protective role on the cells from the harmful inflammation products, including reactive oxygen species (ROS) and free radicals. This protective effect is also present in cells with abnormal cell cycle regulatory pathways [8]. IL-6 has been shown to induce cell proliferation, cell survival and inhibit apoptotic signals. Besides this, IL-6 shows an effect on the general metabolism and can affect many cells of other tissues and organ systems. In various cells IL-6 can activate different protein kinase cascades such as mitogen activated protein kinase (MAPK) and phosphatidylinositol-triphosphate kinase (PI-3 kinase) [9].

Human CMV is a ubiquitous β-herpesvirus and, depending on the socioeconomic and geographical status, 50-100% of the adult population is seropositive [10]. Although CMV has not been proven as a causative agent of human cancer, recently, growing evidence shows that active CMV infection is associated with various malignancies, such as tumors of the breast, brain, colon, prostate, and lung [11-17]. In these cancers CMV is specifically detected at low levels of expression, which might imply that chronic infection with this pathogen induces an inflammatory response and modulates the cellular environment, which could lead to the development of cancer [18,19].

After primary exposure and infection, CMV establishes a latent infection and a lifelong persistence. During reactivation, in order to achieve a productive viral cycle, the first viral genes that are expressed are the major immediate-early (MIE) genes (e.g. IE1, IE2). It is still uncertain exactly what triggers the reactivation of CMV from its latent form; however, it is known that the MIE gene expression is regulated by various cis-acting elements that enable CMV reactivation only in certain phases of the cell cycle and differentiation [20].

The salivary gland (SG) is a particularly interesting tissue for CMV research since this virus shows tropism for the SG ductal epithelium in both the active and latent form [21]. The aim of the study was to examine whether CMV is present in SGC by detecting CMV immediate-early (IE) and early gene products, and to determine the importance of its association with the overexpression of IL-6.

Methods

Study subjects

This retrospective study included 92 patients diagnosed with salivary gland carcinoma (SGC), surgically treated at the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia from 2004 to 2013. The inclusion criterion was the diagnosis of any malignant head and neck salivary gland tumor and the exclusion criterion was the presence of recurrences. Healthy salivary gland tissue from 20 autopsy cases with no malignancies and salivary gland pathology served as controls. All procedures were approved by the Ethical Committee of the School of Medicine, University of Belgrade, in accordance with the Declaration of Helsinki.

Immunohistochemical staining

Immunohistochemical staining was performed on 4 mm-thick paraffin tissue sections. Tissue sections were first deparaffinized with overnight incubation in xylene and rehydrated through graded concentrations of ethanol. For antigen retrieval, slides were warmed in antigen retrieval buffer in the microwave for 3 min at 95°C, followed by cooling at room temperature for 20 min and washing 3 times with PBS. Endogenous peroxidase activity was blocked for 5 min using Dako Dual Endogenous Enzyme Block. After blocking, tissue sections were washed 3 times with PBS then incubated for 90 min at room temperature with primary Monoclonal Mouse Anti-CMV antibody (1:100, clones CCH2 + DDG9, Dako, code M0854) and Anti IL-6 antibody (1:100, sc-130326, Santa Cruz Biotechnology, USA). Following this, the tissues were washed 3 times with PBS and incubated with labeled polymer HRP Rabbit/Mouse (Dako EnVision+ Dual Link System-HRP DAB+) for 30 min. Staining was achieved by adding 100 ml of DAB+ chromogen diluted 1:100 in substrate buffer (Dako EnVision+ Dual Link System-HRP DAB+) for 10 min. Nuclei were counterstained with hematoxylin. The same procedure was performed at the same time on the slides with a lung fragment from a patient who had died due to CMV pneumonia which served as a positive control. Negative control was performed on the salivary gland tumor tissue by applying PBS instead of primary antibody. The level of expression was scored according to both the intensity of staining and the proportion of positive staining of carcinoma cells within the entire slide. Cases were considered positive if more than 10% of tumor cells immunoreacted. Positive reactivity was graded as weak, moderate, and strong

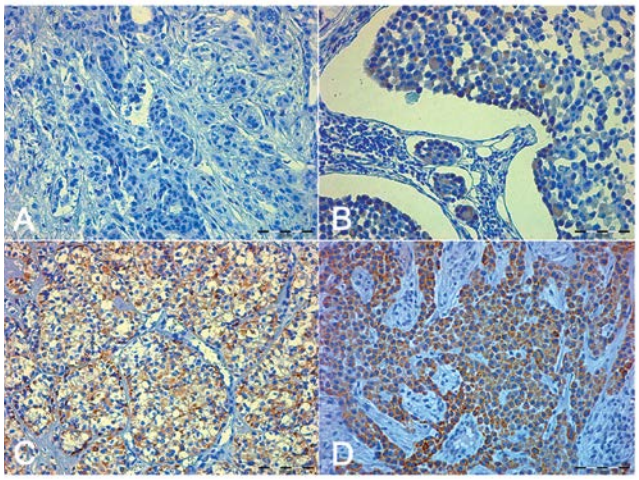

Figure 1. Immunohistochemistry of CMV proteins in SGC tissue. **(A)** Negative CMV expression, **(B)** Weak CMV positivity, **(C)** Intermediate CMV positivity, **(D)** Strong CMV positivity (original magnification x100).

according to the relative strength of the immunoreactivity (Figure 1).

Statistics

Positivity to CMV was determined for each sample based on the immunohistochemistry. Frequencies and percentages were calculated to summarize CMV expression for each histological type. Differences in positivity of expression of CMV and IL-6 between SGC and normal tissue were determined using the Pearson chi-square test. The statistical significance of the association of the presence of CMV and IL-6 expression in SGC was determined by Pearson chi-square test and Linear-by-Linear Association. A two-sided p value < 0.05 was considered as statistacally significant.

Results

Pathological examinations were performed on 92 patients with SGC (49 male/53.3% and 43 female/46.7%). The most common histological type was adenoid cystic carcinoma diagnosed in 35 cases (38%), followed by myoepithelial carcinoma diagnosed in 15 cases (16.3%). The prevalence of all diagnosed histological types of SGC is presented in Table 1. SGC was found in both big and small salivary glands, localized in epipharyngx, oral cavity, parotid and submandibular glands (prevalence shown in Table 1).

To determine the presence of CMV in SGC, as well as in normal salivary gland tissue, we performed immunohistochemical investigation using the CCH2 antibody, which reacts with an early nuclear protein identical with non-structural DNA-binding protein p52, highly conserved among CMV strains, and the DDG9 antibody, which reacts with an immediate early nuclear protein of about 76 kDa.

Our results showed that CMV was detected

Table 1. Clinical and pathological characterization of all diagnosed salivary gland carcinomas

Parameters		*Prevalence*	*%*
Gender	Male	49	53.3
	Female	43	46.7
Salivary gland size	Small	47	51.1
	Big	45	48.9
SGC localization	Epipharynx	30	32.6
	Oral cavity	17	18.5
	Parotid	38	41.3
	Submandibular	7	7.6
SGC histological type	Polymorphous low-grade adenocarcinoma	10	10.9
	Adenoid cyctic carcinoma	35	38.0
	Salivary duct carcinoma	3	3.3
	Mucinous adenocarcinoma	1	1.1
	Acinic cell carcinoma	7	7.6
	Mucoepidermoid carcinoma	8	8.7
	Myoepithelial carcinoma	15	16.3
	Adenocarcinoma, N.O.S	9	9.8
	Basal cell adenocarcinoma	2	2.2
	Oncocytic carcinoma	1	1.1
	Cribriform cystadenocarcinoma	1	1.1

SGC: salivary gland carcinoma, NOS: not otherwise specified

Table 2. Comparison of detected CMV expression in salivary gland carcinomas with clinical and pathological characteristics

Clinicopathological characteristics		*CMV expression*			
		Negative	*Weak*	*Moderate*	*Strong*
Gender	Male	12	11	10	16
	Female	14	9	10	10
Salivary gland size	Small	12	8	12	15
	Big	14	12	8	11
SGC localization	Epipharynx	8	2	8	12
	Oral cavity	4	6	3	4
	Parotid	10	10	8	10
	Submandibular	4	2	1	0
SGC histological type	Polymorphous low-grade adenocarcinoma	5	2	0	3
	Adenoid cystic carcinoma	8	4	10	13
	Salivary duct carcinoma	0	3	0	0
	Mucinous adenocarcinoma	1	0	0	0
	Acinic cell carcinoma	2	2	1	2
	Mucoepidermoid carcinoma	0	2	4	2
	Myoepithelial carcinoma	5	5	3	2
	Adenocarcinoma, N.O.S	4	1	0	4
	Basal cell adenocarcinoma	2	0	0	0
	Oncocytic carcinoma	0	0	1	0
	Cribriform cystadenocarcinoma	0	1	0	0

For abbreviations see footnote of Table 1

Table 3. Expression of IL-6 in salivary gland carcinomas and controls

	IL-6 expression		
	Negative N (%)	*Positive N (%)*	*Total N (%)*
SGC	27 (29.3)	65 (70.7)	92 (100)
Control	16 (80)	4 (20)	20 (100)

Pearson x^2= 17.820, p=0.000. SGC: salivary gland carcinoma

in 65 out of 92 SGC cases (70.6%). Detailed immunohistochemical results are presented in Table 2. There was no statistically significant difference in the presence of CMV with respect to gender, tumor localization or the size of affected salivary gland. There was a statistically significant difference in the presence of CMV with respect to histological types (p<0.05). In all 20 cases of the control group, strong CMV positivity was found only in salivary gland ductal epithelium. On the contrary, in the gland acinar tissue, CMV positivity was not detected in any sample.

IL-6 expression was present in 65 out of 92 SGC cases (70.7%), and only in 4 cases of normal salivary gland (20%). Detailed immunohistochemical results are presented in Table 3. The analysis revealed that IL-6 was present much more frequently in the cancer tissues than in normal tissue, and the difference was highly significant (p<0.01).

The presence of CMV in SGC compared with the expression of IL-6 is presented in Table 4. The results showed a high association of CMV antigen presence with the presence of IL-6 and intensity, which both showed a highly statistical significant (p<0.01).

Discussion

Many reports show a very high prevalence of CMV nucleic acids and proteins in different types of malignancies of various organs, such as breast, brain, colon, prostate, and lung [11-17]. Since salivary glands are exposed to many infectious agents through the oral cavity, and especially since CMV is frequently present in the salivary gland ductal epithelium [21], along with the fact that inflammation is always present in cancer [6], and that other herpesviruses, such as EBV and HHV8, have been proven to cause malignancies, we hypothesized that CMV could have an important role in SGC carcinogenesis and the increase of IL-6 mediated inflammatory process. In this study, it was shown that the CMV antigens are present in different histological types of SGC and are highly associated with increase of IL-6 expression.

Protein products of several CMV genes have the ability to disrupt mechanisms involved in mutagenesis, apoptosis, cell cycle, angiogenesis, cell

Table 4. Comparison of the intensity of IL-6 and CMV antigen expression in salivary gland carcinomas

		IL-6 expression				
		Negative N (%)	*Weak N (%)*	*Moderate N (%)*	*Strong N (%)*	*Total N (%)*
CMV expression	Negative	16 (61.5)	8 (30.8)	0 (0)	2 (7.7)	26 (100)
	Positive	11 (16.7)	22 (33.3)	21 (31.8)	12 (18)	66 (100)
Total	27 (29.3)	30 (32.6)		21 (22.8)	14 (15.2)	92 (100)

Linear-by-linear association value=16.398, p=0.000

invasion and host antitumor response [22]. It has been shown that the products of MIE CMV genes have many similarities with proteins of other DNA oncogenic viruses, and target members of the Rb and p53 families, and therefore promote cell cycle, induce DNA mutations and block apoptotic pathways.

Some studies show that CMV has the ability to express its genome only in cells that are in a specific stage of the cell cycle. In these cells CMV products inhibit cell death by promoting anti-apoptotic pathways and could lead to the initiation and/or propagation of cancer. CMV was found in high percentage in preneoplastic prostatic epithelium, and favors the transformation into prostatic cancer [22]. Also, in *in vitro* studies of human colonic adenocarcinoma cells (Caco-2 cells) CMV presence was found only in cells that were in a specific state of differentiation [23]. Another *in vivo* study of colorectal cancer and its precancerous lesions showed that CMV IE1-72 protein was always found in cancer tissue. In benign lesions, such as adenomas, IE1-72 immunoreactivity was detected in areas of dysplastic epithelium, and was not present in areas of normal-appearing colonic crypts in which the cells did not show any sign of cellular atypia [24]. In our study we also found that CMV was absent in normal gland tissue, both in healthy controls and in unchanged surrounding peritumoral tissue; however in tumor tissue CMV antigens expression was detected.

A detailed investigation of the role of CMV in salivary gland tumors was performed by Melnick et al. [25]. However, the authors focused their research only on one histological type of SGC, mucoepidermoid carcinoma (MEC). They found that CMV antigens were present in 38 out of 39 cases of MEC (97%) in the tumor cells, while the adjacent nonmalignant tissue was unaffected. In an animal model they also showed that the presence of CMV in MEC cells led to upregulation and activation of the oncogenic COX-2/AREG/EGFR/ERK signaling pathway [26]. We have also found positive CMV protein expression in all of our MEC cases (8/8), but we also investigated other types of SGC. We found a high CMV positivity percentage of 77.1% in adenoid cyctic carcinoma (27/35 cases), 71% in acinic cell carcinoma (5/7 cases), 66.6% in myoepithelial carcinoma (10/15 cases), 55.6% in adenocarcinoma NOS (5/9 cases), in all 3 cases of salivary duct carcinoma. Unfortunately, in this 10-year period, some histological types were very rare, and therefore accurate statistical evaluation could not be performed. In the majority of our positive cases the CMV expression was weak or moderate, which is in accordance with a study of CMV presence in glioma tissues, where the CMV infection was always present in low level [22].

Due to its activation of the STAT3 signaling pathway, IL-6 can lead to various consequences that favor cell proliferation and the progression to neoplasia, such as activating the anti-apoptotic Bcl-2 proteins, cell survival factors and c-myc [27]. Some reports show a higher expression of IL-6 in cases of breast, prostatic, colon and oral cavity cancers [28-31]. We have also found a significantly higher expression of IL-6 in SGC in comparison to the normal control group. *In vitro* and *in vivo* studies have shown that CMV immediate early (IE) gene products significantly enhanced the expression of IL-6 various cells [32,33]. We have also found an important connection between CMV presence and IL-6 expression. In SGC cases where CMV virus was present, the intensity of IL-6 expression was moderate to strong in comparison to SGC cases where CMV was not detected, and where IL-6 expression was either negative or weak. This difference showed highly statistical significance (Table 4).

Even though CMV is extremely frequently present in SG ducts, SGC remains a rather rare malignancy. On the other hand, positive expression of CMV antigens in a high percentage of malignant cells of SGC suggests that it might play an important role in carcinogenesis, especially in some histological types such as myoepithelial carcinoma, adenoid cyctic carcinoma and acinic cell carcinoma. We suspect that some environmental or intrinsic pathological factors can alter the sali-

vary gland cells and make them more susceptible to CMV infection, allowing CMV products to further disrupt cell mechanisms and lead to an increase of IL-6 production, inducing inhibition of apoptosis and resulting in tumor development.

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Conflict of interests

The authors declare no confict of interests.

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