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The Role of Cytomegalovirus and Epstein-Barr virus in the Development and Progression of Salivary Gland Cancer

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Uloga Citomegalovirusa i Epštajn-Bar virusa u nastanku i progresiji karcinoma pljuvačnih žlezda

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SUMMARY

The Role of Cytomegalovirus and Epstein-Barr virus in the Development and Progression of Salivary Gland Cancer

Introduction: Salivary gland carcinomas (SGC) are rare tumors characterized by an enormous morphological diversity between different subtypes going along with diverse clinical courses. The etiology of SGC is still unknown, although a correlation has been shown between the occurrence of SGC and some environmental factors, however, only a very small percentage of malignancies develop as a direct result of these factors. There is growing evidence that infectious agents are frequently associated with human cancer. Recent studies show that Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) CMV infections are associated with various epithelial malignancies. It has been shown that in various tissues CMV and EBV could increase the expression of IL-6, NFκB, MMP-2 and MMP-9. These proteins are associated with certain types of cancer. We hypothesized that CMV and EBV could cause inflammation and deregulation of genes involved in cell proliferation and could induce the development of salivary gland cancer.

Aims: The aims of this study were to determine the prevalence of CMV and EBV in SGC tissues, to compare the immunohistochemical and nested PCR detection of CMV and EBV in SGC, to investigate the viral protein expression in different histological types of salivary gland cancer, to investigate the immunohistochemical expression of prognostic markers IL6, MMP2, MMP9 and NFκB, to investigate the association of the presence of CMV and EBV and the immunohistochemical expression of these prognostic markers and to analyze the polymorphisms of IL6, MMP2 and MMP9 genes and investigate the association of the polymorphisms and the corresponding protein expression**.**

Material and Methods: This cross-section study included 93 patients diagnosed with salivary gland cancer (SGC), surgically treated at the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia from 2004 to 2013. For the immunohistochemical analysis, the control consisted of healthy salivary gland tissue from 20 autopsy cases with no malignancies and salivary gland pathology. For the analysis

concerning DNA polymorphisms, the control group consisted of buccal swabs from 100 healthy volunteers, matching the study group in sex and age. CMV and EBV detection was performed by immunohistochemistry and by nested PCR. Immunohistochemistry (IHC) was performed to demonstrate the expression of IL6, MMP2, MMP9 and NFκB. 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 according to the relative strength of the immunoreactivity.

Results: Using IHC staining, CMV antigens were detected in 66 out of 92 SGC cases (71.7%), while all control cases were negative. EBV was detected in 32 (34,8%) from our 92 cases and in 1 case of control. The nested PCR results did not differ significantly from IHC. In 16 of the 92 (17.4%) SGC cases there was no expression of viral antigens, neither CMV nor EBV, while 76 cases (82.6%) expressed antigens of at least one virus. However, there were 22 cases that expressed both CMV and EBV antigens. The prevalence and intensity of expression of IL-6 and NFκB were significantly higher in SGC than in control cases ($p<0.01$), while the expressions of MMP-2 and MMP-9 did not show a difference between SGC and control cases. SGC cases that were CMV positive showed a significantly higher expression of IL-6, NFκB and MMP-2 ($p<0.01$), while MMP-9 expression did not differ between SGC and control cases. There was no difference in the expression of any prognostic marker in SGC and control cases. However, in cases that expressed both CMV and EBV antigens, the expressions of IL-6, NFκB and MMP-2 were higher than in cases that expressed only CMV antigens.

The C allele for the IL-6 -174G>C polymorphism increases the risk for SGC both in homozygote CC and heterozygote GC. This increase in susceptibility was also confirmed using the dominant model (OR 3.77, 95% CI 1.91-7.44, $p<0.001$for the CT+TT genotypes). The T allele for the MMP-2 -1306 C>T polymorphism exhibits its effect in heterozygous carriers, increasing the risk for SGC (OR 1.98, 95% CI 1.07-3.65, P=0.03). According to the dominant model, CT+TT genotypes had a 2-fold increased risk of developing SGC ($p=0.02$).When the dominant model is applied for the MMP2 -1575 G>A, individuals with

GA+AA genotypes exhibited a 1.77-fold increase in cancer risk, but with borderline significance (P=0.049). Heterozygous carriers of the variant T allele for the MMP-9 -1562 C>T polymorphism had roughly a two-fold increase in susceptibility for SGC compared to wild type homozygotes (CC). (p=0.02). However, the polymorphisms of IL-6 and MMP-2 were not associated with a higher expression of their protein products, while the CC genotype of the MMP-9 -1562 C>T polymorphism was associated with a higher expression of MMP, but only with border line significance (p=0.049).

Conclusions: Both CMV and EBV are found more often in SGC than in normal salivary gland tissue. Since the results of IHC and nested PCR coincide in a high percentage, both methods can be used in the detection of viruses from formalin-fixed, paraffin embedded tissues. IL-6 and NFκB could be considered as good positive prognostic factors for SGC, while MMP-2 and MMP-9 expression does not differ between SGC and normal tissue. While EBV does not seem to influence the expression of any of the tested markers, CMV positive SGC cases showed a higher expression of IL-6, NFκB and MMP-2.

IL-6 -174 G>C, MMP-2 -1306 C>T and MMP-9 -1562 C>T polymorphisms genotypes seem to influence the development of SGC, whereas MMP-2 -1575 G>A seems to be of a minor importance. None of the analyzed polymorphisms led to a higher protein production.

Key words: Cytomegalovirus, Epstein- Barr virus, salivary gland cancer, IL-6, NFκB, MMP-2, MMP-9

Scientific field: Molecular medicine\ Microbiology

SAŽETAK

Uloga Citomegalovirusa i Epštajn-Bar virusa
u nastanku i progresiji karcinoma pljuvačnih žlezda

Uvod: Karcinomi pljuvačnih žlezda su retki tumori koji u različitim histološkim tipovima pokazuju spektar različitih morfoloških promena koje odgovaraju različitom kliničkom ispoljavanju i toku. Etiologija karcinoma pljuvačnih žlezda je još uvek nepoznata. Brojna istraživanja su pokazala značajan uticaj faktora sredine na njihov nastanak i progresiju, ali je samo za mali procenat jasno pokazano da je tumor nastao upravo direktnim uticajem nekog od faktora sredine. Sve je više istraživanja koja ukazuju na čestu i tesnu povezanost infektivnih agenasa sa pojavim maligniteta. Novija istraživanja pokazala su direktnu povezanost infekcija Citomegalovirusom (CMV) i Epštajn-Barr virusom (EBV) sa nastankom različitih oblika karcinoma. U različitim tkivima pokazano je da CMV i EBV mogu uzrokovati povećanje ekspresije IL-6, NFκB, MMP-2 i MMP-9, proteina koji su direktno povezani sa određenim tipovima karcinoma. Naša hipoteza je bila da CMV i EBV mogu dovest do zapaljenja i do deregulacije gena ćelijske proliferacije, te uticati na nastanak i razvoj karcinoma pljucačnih žlezda.

Ciljevi: Ciljevi ove studije bili su da se utvrdi prisustvo CMV i EBV u tkivu karcinoma pljuvačne žlezde, da se uporedi immunohistohemijska i nested PCR detekcija CMV i EBV u karcinomu pljuvačne žlezde, da se ispita ekspresija virusnih proteina u različitim histološkim tipovima karcinoma pljuvačnih žlezda, da se ispita imunohistohemijska ekspresija prognostičkih markera IL6, MMP2, MMP9 i NFκB, da se ispita povezanost prisustva CMV i EBV sa imunohistohemijskom ekspresijom pomenutih prognostičkih markera i da se uradi analiza polimorfizma IL6, MMP2 i MMP9 gena i ispita asocijacija polimorfizama i odgovarajuće ekspresije proteina.

Materijal i metode: Ova studija preseka obuhvatila je 93 pacijenta kod kojih je dijagnostikovan karcinom pljuvačne žlezde, koji su operisani na Klinici za otorinolaringologiju i maksilofacialnu hirurgiju, Kliničkog centra Serbije u periodu od 2004. do 2013. Detekcija CMV i EBV analizirana je imunohistohemijskom i nested PCR metodom.

Imunohistohemijska metoda korišćena je da se utvrdi ekspresija IL-6, MMP2, MMP9 i NFκB. Jačina ekspresije određivana je prema intenzitetu bojenja sa jedne strane, i utvrđivanju broja pozitivno obojenih karcinomskih ćelija u odnosu na ukupan broj ćelija. Slučajevi su ocenjeni kao pozitivni ako je nađeno više od 10% pozitivno obojenih tumorskih ćelija. Shodno jačini imunoreaktivnosti pozitivnost je ocenjena kao slaba, umerena i izražena.

Rezultati: Imunohistohemijski CMV antigeni su detektovani u 66 od 92 slučaja karcinoma pljuvačne žlezde (71.7%), dok su svi kontrolni slučajevi bili negativni. CMV je češće bio prisutan u adenoidno cističnom karcinomu nego u drugim histološkim tipovima ($p<0.05$). EBV je detektovan u 32 (34,8%) od 92 slučaja i u 1 kontrolnim slučaju. Rezultati nested PCR ispitivanja se nisu značajno razlikovali od imunohistohemijskih. U 16 od 92 (17.4%) slučajeva tumora pljuvačnih žlezda nije nađena ekspresija ni CMV ni EBV virusnih antigena, dok je u 76 slučajeva (82.6%) detektovana ekspresija antigena bar jednog od ispitivanih virusa. U 22 slučaja nađena je ekspresija i CMV i EBV antigena. Imunohistohemijska pozitivnost i intenzitet ekspresije IL-6 i NFκB bili si značajno viši u karcinomima pljuvaćnih žlezda nego u kontrolnim slčajevma ($p<0.01$), dok se ekspresija MMP-2 i MMP-9 nije razlikovala u tumorima i kontrolnoj grupi. U slučajevima tumora pljuvačnih žlezda u kojima je nađena CMV pozitivnost zapažena je značajno veća ekspresija IL-6, NFκB i MMP-2 ($p<0.01$), dok razlika u ekspresiji MMP-9 u kontrolnoj grupi i slučajevima karcinoma nije primećena. Nije utvrđena razlika ekspresije ni jednog prognostičkog markera između EBV pozitivnih i EBV negativnih slučajeva karcinoma pljuvačnih žlezda. Međutim, u slučajevima u kojima je nađena ekspresija i CMV i EBV antigena, ekspresija IL-6, NFκB i MMP-2 je bila veća nego u slučajevima u kojima je nađena samo ekspresija CMV antigena.

C alel IL-6 -174G>C polimorfizma povećava rizik oboljevanja od carcinoma pljuvačne žlezde i u formi homozigota CC i heterozigota GC. Povećan rizik je potvrđen i u dominantnom modelu (OR 3.77, 95% CI 1.91-7.44, $p<0.001$ za CT+TT genotip). T alel MMP-2 -1306 C>T polimorfizma pokazuje efekat u heterozigotnim nosiocima, povećevajući rizik za dobijanje carcinoma pljuvačne žlezde (OR 1.98, 95% CI 1.07-3.65, $p=0.03$). Prema dominantnom modelu, CT+TT genotipovi imaju 2 puta veći rizik od razvijanja carcinoma

pljuvačne žlezde (p=0.02). Prema dominantnom modelu MMP2 -1575 G>A polimorfizma, osobe koje poseduju GA+AA genotipove pokazuju 1.77 puta veći rizik od razvitka kancera, ali je statistička značajnost granična (P=0.049). Heterozigotni nosioci T alela MMP-9 -1562 C>T polymorfizmaimaju oko 2 puta veći rizik od oboljevanja od carcinoma pljuvačne žlezde u poređenju sa nosiocima homozigota CC (p=0.02). Međutim, ni jedan od ispitivanih genotipova IL-6 i MMP-2 gena nije povezan sa većom ekspresijom svog proteinskog produkta, dok je CC genotip MMP-9 -1562 C>T polimorfizma povezan sa jačom ekspresijom MMP-9, ali je statistička značajnost granična gymorphism was associated with a higher expression of MMP, but only with border line significance (p=0.049).

Zaključci: Prisustvo CMV i EBV nađeno je češće u karcinoma pljuvačnih žlezda nego u normalnom tkivu pljuvačnih žlezda. Kako se rezultati IHH i PCR poklapaju u velikom procentu, za detekciju virusa u tkivu iz parafinskog bloka mogu se koristiti obe metode podjednako uspešno. IL-6 i NFκB mogu se smatrati dobrim pozitivnim prognostičkim markerima u slučajevima karcinoma pljuvačnih žlezda, dok se ekspresija MMP-2 i MMP-9 ne razlikuje u slučajevima karcinoma i u tkivu normalne pljuvačne žlezde. Dok EBV izgleda da nema neki značajniji uticaj na ekspresiju bilo kog ispitivanog prognostičkog markera, u slučajevima CMV pozitivnih karcinoma plljuvačnih žlezda nađena je povećana ekspresija IL-6, NFκB i MMP-2.
IL-6 -174 G>C, MMP-2 -1306 C>T i MMP-9 -1562 C>T polimorfizmi izgleda da utiču na razvoj karcinoma pljuvačnih žlezda, dok MMP-2 -1575 G>A da ima manji značaj. Nijedan od ispitivanih polimorfizama nije uticao na povišenu proteinsku produkciju.

Ključne reči: Citomegalovirus, Epštajn-Bar virus, karcinom pljuvačnih žlezda, IL-6, NFκB, MMP-2, MMP-9

Naučna oblast\Uža naučna oblast: Molekularna medicina\Mikrobiologija

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1. INTRODUCTION

1.1 Salivary Gland Tumors

1.1.1 Anatomy and Histology of Salivary Gland

Salivary glands are exocrine organs which produce and secrete saliva. There are three pairs of major glands, the parotid, submandibular and sublingual, and the numerous minor glands widely distributed throughout the mouth, oropharynx, in the upper respiratory and sinonasal tracts, and the paranasal sinuses. Secretory acinus (that can be serous, mucous or mixed), with related ducts and myoepithelial cells, is the functional unit of salivary glands. Serous acini form wedge-shaped secretory cells with basal nuclei whose role is to secrete amylase into the intercalated ducts. The cytoplasm of serous cells contains periodic acid Schiff positive and diastase resistant, densely basophilic, refractile zymogen granules. Mucous acinar cells also have basally placed nuclei, their cytoplasm is clear and contains vacuoles of sialomucin. The secretions of these cells pass through the intercalated ducts lined by a single layer of cuboidal cells with relatively large, central nuclei. The intercalated ducts are linked to the much larger striated ducts responsible for modifying the salivary secretions. The striated ducts are lined by tall, columnar, eosinophilic cells that are rich in mitochondria. They have parallel infoldingsof the basal cytoplasm and they join the interlobular excretory ducts, which are lined by pseudostratifiedcolumnar epithelium that often contains few mucous cells. Contractile myoepithelial, or basket cells, are located between the basement membrane and the basal plasma membrane of the acinar cells. Their morphology varies, and in hematoxylin and eosin (HE) sections they seem to be unremarkable. They contain smooth muscle actin, myosin and intermediate filaments including keratin- 4. Their long dendritic processes embrace the secretory acini. Myoepithelial cells also surround the intercalated ducts, but their presence in striated ducts is not firmly established (Figure 1).

The parotid gland is almost purely serous. Its parenchymal lobules are divided by fibrous septa. The parotid gland also contains intralobular and extralobular adipose tissue, randomly distributed lymphoid aggregates and one to more than 20 in number lymph nodes.

Submandibular gland is mixed serous and mucous, although the serous element predominates (~90%). In mixed acini the serous cells form caps, or demilunes, on the

periphery of the mucous cells. The intercalated ducts are shorter and the striated ducts more conspicuous than those of the parotid gland.

Sublingual gland is also mixed but is predominantly mucous in type. The mucous acini form elongated tubules with peripheral serous demilunes.

Minor salivary glands are not encapsulated and they are most numerous at the junction of the hard and soft palate, buccal mucosa and lips. Those of the lateral aspects of the tongue, lips and buccal mucosa are seromucous, whereas those in the ventral tongue, palate, glossopharyngeal area and retromolar pad are predominantly mucous. Salivary glands related to the circumvallate papillae (von Ebner's glands) are serous in type.

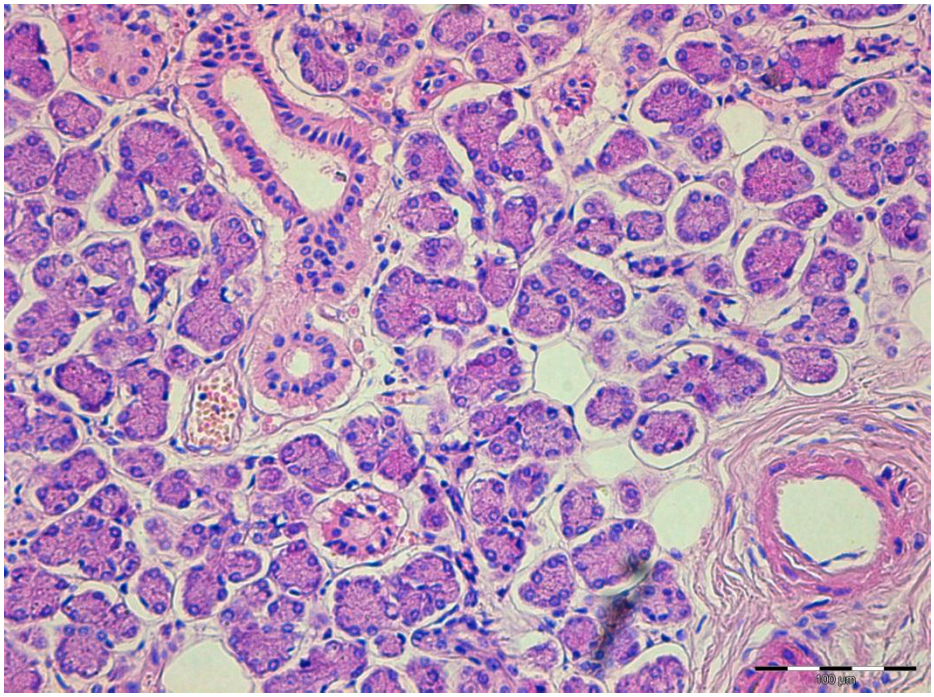

Figure 1. Normal salivary gland. HE x100

1.1.2 Classification of Salivary Gland Tumors

WHO classification of salivary gland tumors is presented on Table 1 (Rousseau and Badoual,2011).

Table 1. Histological Classification of Salivary Gland Tumors

Malignant epithelial tumors	Benign epithelial tumors
Acinic cell carcinoma	Pleomorphic adenoma
Mucoepidermoid carcinoma	Myoepithelioma
Adenoid cystic carcinoma	Basal cell adenoma
Polymorphous low-grade adenocarcinoma	Warthin tumor
Epithelial-myoepithelial carcinoma	Oncocytoma
Clear cell carcinoma, not otherwise specified	Canalicular adenoma
Basal cell adenocarcinoma	Sebaceous adenoma
Sebaceous carcinoma	Lymphadenoma
Sebaceous lymphadenocarcinoma	Sebaceous
Cystadenocarcinoma	Non-sebaceous
Low-grade cribriform cystadenocarcinoma	Ductal papillomas
Mucinous adenocarcinoma	Inverted ductal papilloma
Oncocytic carcinoma	Intraductal papilloma
Salivary duct carcinoma	Sialadenomapapilliferum
Adenocarcinoma, not otherwise specified	Cystadenoma
Myoepithelial carcinoma	**Soft tissue tumors**
Carcinoma ex pleomorphic adenoma	Haemangioma
Carcinosarcoma	**Haematolymphoid tumors**
Metastasizing pleomorphic adenoma	Hodgkin lymphoma
Squamous cell carcinoma	Diffuse large B-cell lymphoma
Small cell carcinoma	Extranodal marginal zone B-cell lymphoma
Large cell carcinoma	**Secondary tumors**
Lymphoepithelial carcinoma	

1.1.3 Epidemiology of Salivary Gland Cancers

The epidemiology of salivary gland cancers (SGCs) is limited and not well documented. SGCs are relatively rare. The global annual incidence when all salivary gland tumors were considered varied from 0.4-13.5 cases per 100,000 population (Elamin, et al., 1998). The annual incidence of malignant salivary neoplasms rates in the world vary from 0.4-2.6 cases per 100,000 population (Koivunen, et al.,2002) (Östman, et al., 1997) (Eneroth, 1971) (Pinkston, Cole, 1999) (Sun, et al., 1999)

SGCs are mostly adenocarcinomas. Parotid gland is the most common site where between 64% and 80% of all primary epithelial salivary gland tumors occur, usually in its superficial, lateral lobe; 7-11% occur in the submandibular glands; fewer than 1% occur in the sublingual glands; and 9-23% occur in minor glands (Auclair, at al., 1991) (Eneroth, 1971) (Eveson andCawson, 1985) (Seifert, et al.,1986) (Spiro, 1986). Malignant tumors represent 21-46% and their proportion varies greatly by site. So, 15-32% of salivary gland malignant tumors belong to parotid tumors, 41-45% to submandibular tumors, 70-90% to sublingual tumors, and 50% to minor gland tumors. Eighty to 90% of tumors that occur in the tongue, floor of mouth, and retromolar areas are malignant. Females are more frequently affected. The average age of patients with SGCs is46 years, and the peak incidence of most of the specific types is in the sixth and seventh decades. However, the highest incidence of mucoepidermoid carcinomas, and acinic cell carcinomas is in the third and fourth decades. In patients under 17 years of age, the frequency of mesenchymal tumors of the major glands is similar to that of epithelial tumors (Kessler and Handler, 1994) (Lack and Upton, 1988) (Seifert, Okabe andCaselitz, 1986) (Shikhani and Johns, 1988). Among all patients, in most large studies, mucoepidermoid carcinoma is the most common malignant tumor (Auclair, et al., 1991) (Eneroth, 1971) (Eveson and Cawson, 1985) (Seifert, et al, 1986) (Spiro, 1986).

1.1.4 Etiology and pathogenesis of SGCs

1.1.4.1 Radiation

Exposure to ionizing radiation is found to show impact in the development of SGCs. Follow-up studies of the patients exposed to ionizing radiation showed that there was a high frequency of both mucoepidermoid carcinomas and Warthintumors in these patients (Saku, et al., 1997). The risk was directly related to the level of exposure to ionizing radiation. Therapeutic radiation, particularly of the head and neck region, has been linked with a significantly increased risk of developing salivary gland cancers (Mihailescu, et al., 2002) (Modan, et al, 1998) (Ron and Saftlas, 1996) (Schneider, et al., 1977). There appears to be a risk from Iodine-131 used in the treatment of thyroid disease, as the isotope is also concentrated in the salivary glands (Hoffman, et al., 1982). There is evidence that exposure to routine dental radiographs is associated with an increased risk of salivary gland carcinoma (Preston-Martin, et al., 1988) (Preston-Martin, and White, 1990). Exposure to ultraviolet radiation has also been implicated (Nagler, and Laufer, 1997) (Spitz, Sider, and Newell, 1990) (Spitz, et al., 1988). There appears to be no excess risk in those exposed to radon (Miller,et al., 1993), or the microwaves of cellular telephones (Auvinen, et al., 2002) (Johansen, et al., 2001).

1.1.4.2 Occupation

Occupation was shown to have an impact on SGC development.It has been shown that workers in a variety of industries have an increased incidence of SGCs, particularly those employed in rubber manufacturing (Horn-Ross, Ljung, and Morrow, 1997) (Horn-Ross, Morrow, and Ljung, 1997) (Mancuso, and Brennan, 1970), those exposed to metal in the plumbing industry and nickel compounds (Horn-Ross, Ljung, and Morrow, 1997), and those involved in woodworking in the automobile industry (Swanson, and Belle, 1982). Employment in hairdressing and beauty shops also was reported to be of an influence on salivary gland carcinogenesis (Swanson, and Burns, 1995) (Swanson, and Burns, 1997). An increased risk of salivary gland cancers was reported in people living in certain Quebec counties where asbestos was mined, and the risk was inversely proportional to the distance from the mines (Graham, Blanchet and Rohrer, 1977).

1.1.4.3 Role of Lifestyle and Nutrition

Role of lifestyle and nutrition was also investigated in etiology of SGCs. Although there is a strong association between smoking and Warthin tumor, no association was found between tobacco use and alcohol consumption and salivary gland cancers in a case/control study (Muscat, and Wynder, 1998). Exposure to silica dust and kerosene as a cooking fluid increased the risk of developing salivary malignancy in a Chinese population (Zheng,et al., 1996). Higher level of risk of parotid carcinomas was associated with exposure to nickel, chromium, asbestos and cement dust in a European study (Dietz, et al., 1993). Also, an increased level of risk has been postulated in those with a high cholesterol intake (Horn-Ross, Morrow, and Ljung, 1997).

1.1.1.4.4 Hormones

Hormones were also investigated in SGC genesis, particularly the role of estrogen and progesteron, but the results were conflicting.

1.1.4.5 Viruses

Etiology of SGCs has been investigated for a long time, and still remains incompletely explained. The role of many viruses have been investigated in the pathogenesis of SGCs. Epstein Barr virus (EBV), cytomegalovirus, or SV40 sequences have been demonstrated, especially in human pleomorphic adenomas (Martinelli, et al., 2002), but there is no convincing association between human SGCs and other viruses, including polyoma virus and papilloma virus.

1.1.5. Clinical aspects of SGCs

1.1.5.1 Symptoms

Signs and symptoms of salivary gland cancer may include:

- A lump or swelling on or near your jaw or in your neck or mouth
- Numbness in part of your face
- Muscle weakness on one side of your face
- Persistent pain in the area of a salivary gland
- Difficulty swallowing
- Trouble opening your mouth widely

Having a lump or area of swelling near the salivary gland is the most common sign of a salivary gland tumor, but it doesn't mean that the patient has cancer. Most salivary gland tumors are noncancerous (benign). Many non-tumor related conditions may lead to a swollen salivary gland, including an infection or a stone in a salivary gland duct.

1.1.5.2 Diagnosis

Tests and procedures used to diagnose salivary gland cancer include:

- A physical exam.
- Imaging tests, such as magnetic resonance imaging (MRI) and computerized tomography (CT), may help to determine the size and location of salivary gland ca. While for ductal inflammatory disease plain radiography and sialography are useful, for evaluation of suspected neoplastic disease computed tomography (CT), ultrasonography, CT sialography, and magnetic resonance imaging (MRI) are better.
- Collecting a sample of tissue (biopsy) for laboratory testing. During an aspiration biopsy, the doctor inserts a needle into the suspicious area and draws out fluid or cells for pathohistological examination. Fine needle aspiration biopsy (FNA) can provide clinicians with rapid, non-surgical diagnoses. FNA can be used both as a diagnostic test and as a screening tool to triage patients into different treatment groups. It is useful in recognizing inflammatory process from neoplastic, or a lymphoma from an epithelial

malignancy. However, a specific diagnosis can only be made in approximately 60-75% of cases (Ellis, and Auclair, 1996).

- Frozen section examination is a useful diagnostic method, but the accuracy of frozen section diagnoses of the salivary gland is the most controversial. While the accuracy rate (98.7%, excluding deferred diagnoses) is excellent for the benign lesions, in the malignant tumor group the accuracy rate (85.9%) is suboptimal (Gnepp, Brandwein, and Henley, 2001).
- Pathohistological analysis of tumor sample obtained by biopsy is the golden standard in determining the type, the extent of SGC of salivary gland tumors.

Biopsy provides important data such as the types of salivary gland tumor allowing their classification, the grade of cellular atipia, and using slides from paraffin blocks, makes possible further immunohistochemical analyses, often crucial for a precise diagnosis. Cancer stages are identified by Roman numerals, with stage I indicating a small, localized tumor and stage IV indicating an advanced cancer that has spread to the lymph nodes in the neck or to distant parts of the body.

1.1.6 Prognosis and predictive factors

Prognosis was shown to correlate most strongly with clinical stage. Emphasizing the importance of early diagnosis in cases of salivary gland carcinomas are extremelyimportant for optimal survival. The microscopic grade, which is often in positive correlation with clinical stage, and tumor type have been shown to be independent predictors of behavior and often play an important role in optimizing treatment (Kane, et al., 1991) (O'Brien, et al., 1986) (Spiro RH, 1995) (Spiro,Huvos, and Strong, 1982) (Spiro, et al. 1991) (Szanto, et al., 1984). Other important tumor-related predictors of survival are extra-parotid tumor extension, cervical node involvement and facial nerve paralysis. Apart from the histopathological degree of malignancy, a major factor of both local recurrences and spreading is the invasiveness of the tumor (margin status, perineural invasion, angiolymphatic invasion, tumor necrosis).

Lymphatic spread can be frequent in some particular histotypes such as ductal carcinomas, high-grade mucoepidermoid carcinomas, carcinomas ex pleomorphic adenomas, squamous cell carcinomas. On the contrary, it is not frequent in polymorphus low-grade adenocarcinoma, in low-grade mucoepidermoid carcinoma and in adenoid cystic carcinoma.

Distanthematogenous metastases localize most frequently in lungs (80%), in bone (15%), in liver and other sites (5%). Depending on the degree of malignancy, they are the main cause of death of patients with malignant salivary gland tumors. Adenoid cystic carcinoma, adenocarcinoma not otherwise specified (NOS), carcinoma ex-mixed tumor, small cell carcinoma and ductal carcinoma have the highest distant metastases rate (up to 50%).

Survival of patients with salivary gland carcinomas strongly correlates with clinical stage and tumor grade. Histopathology is shown to be a predictor of the tumor behavior and it contributes to optimize treatment.

1.2 Human Herpesviruses

Human herpesviruses (lat. *Herpesviridae*) are large family of enveloped DNA viruses. The main characteristic of herpesviruses is the ability to cause lytic, persistent, but also latent and recurrent infections. Based on the structure of the genome, tropism for certain tissues, cytopathologic effect, latency site as well as clinical manifestation of the infection, the Herpesviridae family is divided into three subfamilies, namely alpha, beta and gamma herpesviruses (Roizmann, et al. 1992) (Table 2).

Table 2: Taxonomy of Human herpesviruses

Subfamily	Genus	Virus	Disease
Alphaherpesvirinae	*Simplexvirus*	Herpes Simplex 1 (HSV1), Herpes Simplex 2 (HSV2)	Herpes simplex
	Varicellovirus	Varicella Zoster (VSZ; HHV3)	Chickenpox, Shingles
Betaherpesvirinae	*Cytomegalovirus*	Cytomegalovirus (CMV; HHV4)	CMV infections, Mononucleosis
	Roseolovirus	Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7)	Roseola (exanthemsubitum)
Gammaherpesvirinae	*Lymphocryptovirus*	Epstein-Barr Virus (EBV; HHV5)	infectious mononucleosis, Burkitt's lymphoma and other malignancies
	Rhadinovirus	Kaposi's sarcoma-associated HV (KSHV; HHV8)	Kaposi's sarcoma (KS)

The virion of herpesviruses is large, approximately 150 nm in diameter and has a very characteristic morphology. The genetic material is a linear, double-stranded DNA, and is surrounded by an icosahedral capsid with 162 capsomeres. The surface of the virion is covered by a glycoprotein-containing envelope. Between the capsid and envelope herpesviruses have a specific protein structure called the tegument that helps initiate replication (Figure 2). Similarly to other enveloped viruses, herpesviruses are sensitive to drying, detergents, solvents and acids (Whitley, 1996).

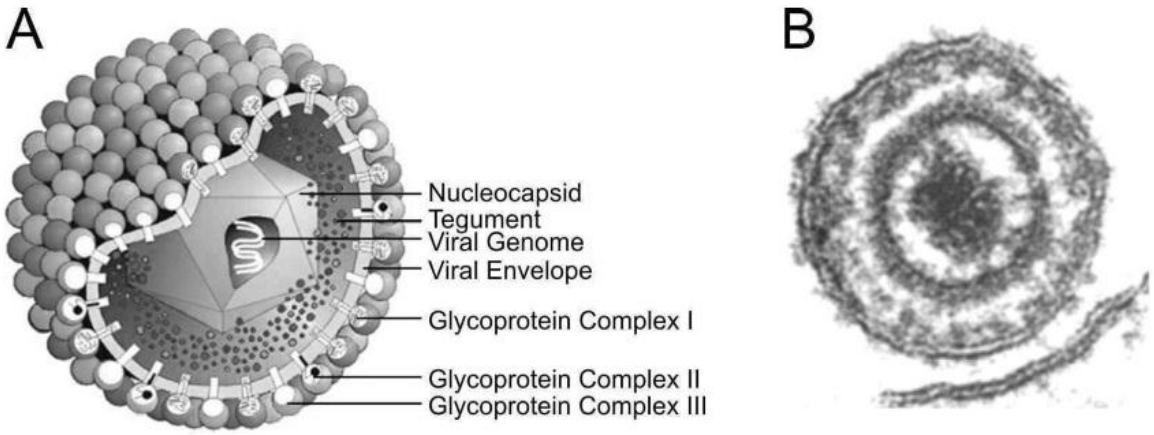

Figure 2. Structure of a CMV Virion. (A) Schematic representation of a CMV particle (Reschke, 1997). (B) Electron microscopic image of a CMV virion (Flint 2004).

The replication of herpesviruses begins with the interaction of viral glycoprotein antireceptors with cellular tissue-specific receptors, followed by the fusion of the envelope with the plasma membrane and the release of the nucleocapsid into the cytoplasm. The nucleocapsid bonds to the nuclear membrane and delivers the genome into the nucleus, where transcription and replication takes place. Once the genome enters the nucleus, the terminal regions ligate, and the genome changes from linear to circular form. Since circularization occurs in the absence of viral gene expression, one of the host-cell ligases is likely to be responsible (Garber, Beverley, Coen, 1993). Transcription is a result of the cellular DNA-dependent RNA polymerase, but it is regulated by both viral encoded and cellular nuclear factors. This interaction between cellular and viral factors determines if a lytic, persistent or

latent infection will take place. In a productive infection (lytic or persistent) transcription occurs in three phases:

1. Production of Immediate-early proteins (IE) that are responsible for regulating gene transcription and taking over the cell metabolism
2. Production of Early proteins (E) that include most of the transcription factors and enzymes and
3. Production of Late proteins (L) that consist of structural proteins that are produced after genome replication has begun.

In cells where latent infection takes place, a special set of viral genes is transcribed, producing "latency proteins" without genome replication.

Viral genome replication takes place in the nucleus due to the activity of the viral-encoded DNA polymerase, in a "rolling-circle" manner. This process results in the formation of concatemers, long linear strands of DNA, consisting of a large number of repeating viral linear DNA. Late proteins are synthetized and form empty procapsids in the nucleus. The terminal proteins of the procapsids have the ability to specifically cut a monomer DNA from the concatemer that enters the procapsid. They obtain the envelope on the nuclear or Golgi membrane, and can exit the cell either by lysis or exocytosis (Challberg, 1996).

1.2.1 Human Cytomegalovirus (CMV)

The human cytomegalovirus (CMV) is genetically an extraordinary complex virus and the largest of all herpesviruses (Geelen, et al., 1978). CMV, also known as human herpesvirus 5 (HHV-5), is a member of the subfamily Betaherpesvirinae. Primary isolation and cultivation of CMV was isolated from the salivary gland tissue in 1956 (Rowe, et al., 1956). CMV is a ubiquitous pathogen (Weller, 1971), and socio-economic status greatly influences the prevalence of CMV infection within a population. Approximately 50% of adult individuals in industrialized countries and up to 100% in less developed countries are found to be seropositive for CMV (Pass 1985; Staras et al. 2006). It has been shown that there is a 1% cumulative rise in chance of infection per year of age. The ubiquitous nature of this virus is demonstrated by the fact that a great percentage of population was found seropositive even in the most isolated tribal communities (Black et al., 1974).

Like all other herpesviruses, after primary infection and lytic replication, CMV establishes lifelong latency, with phases of reactivation and virus secretion (Crough and Khanna, 2009). Also, frequently the same host can be reinfected with a different strain of CMV (Bale, et al., 1996; Novak, et al., 2008).

1.2.1.1 Structure and Morphology of CMV

With the size of approximately 220 kbp (kilo base bairs), the CMV genome is the virus with the longest genome. It has the ability to code for approximately 200 virus specific open reading frames (Murphy, et al., 2003). The virion structure is similar to other herpesviruses. The linear double-stranded DNA is surrounded by an icosahedral capsid, composed of five viral proteins (Irmiere and Gibson 1985) that assemble into subunits (capsomeres). The capsid is made up of two types of capsomers, 150 hexons and 12 pentons. Between the nucleocapsid and the envelope, there is the tegument, a structure characteristic for all herpesviruses. The predominant virion protein is the tegument phosphoprotein (pp) 65. The tegument is a low density structure consisting of 20 to 25 proteins that have important roles in the activation of transcription (Homer, et al., 1999), but also might interfere with the immune response (Browne and Shenk 2003). The envelope consists of a lipid bilayer derived

from the host cell membrane and incorporates viral glycoproteins associated into three glycoprotein complexes (Gretch et al. 1988). Glycoprotein complex (gC), made up of homotrimeric glycoprotein B, gC II, composed of gM and gN linked by disulfide bonds, and gC III, consisting of gH, gL and gO or gH, gL, and UL128-131A

1.2.1.2 Epidemiology and Pathogenesis of CMV infections

Most often, the primary CMV infection in immunocompetent hosts passes without clinical manifestations (Kumar et al. 1973). Even though the virus is ubiquitous, it is not highly contagious and requires direct contact with bodily fluids, most commonly with saliva, but also with others (Hayes et al. 1972; Reynolds et al. 1973; Lang and Kummer 1975; Pass 1985; Hamprecht et al. 2005). Since CMV can be found in vaginal fluid and semen, CMV infection is considered a sexually transmitted infestion (Sohn, et al., 1991). It can be transmitted by blood transfusion, solid organ or hematopoietic stem cell transplantation and also vertically, from mother to child. (Meyers, et al., 1990) (Rubin, 1998) (Schleiss 2003). The highest risk for infection of a newborn is during a primary infection of the mother during pregnancy. The risk for the newborn is greatest in primary infection of the mother during pregnancy (Boppana, et al., 1992). The infection can lead to cytomegalic inclusion disease (CID) whose symptoms include retinitis, hearing loss or mental retardation. Also, infants can develop pneumonitis. Besides congenital morbidity, CMV infections can lead to serious morbidity in immunocompromised patients, such as AIDS patients or seronegative transplant recipients (Landolfo, et al., 2003). Affected recipients often develop life-threatening complications such as interstitial pneumonia, gastroenteritis or hepatitis, and CMV infection can often lead to allograft loss (Simmons, et al., 1977). (Rubin 1998).

1.2.1.3 Persistent and latent CMV infections

Permissive cells allow all phases of viral replication to take place, which results in the production of new viruses. Permissive cells for CMV include endothelial cells, fibroblasts, smooth muscle cells and some epithelial cells. On the other hand, after primary infection, the virus remains present in the organism due to persistent and latent infection that is established in various cells in the organism. It has been shown that CMV establishes

latency in certain cells of the myeloid lineage, while a persistent infection develops in certain endothelial cells (Sinclair and Sissons, 2006). Endothelial cells, therefore, represent a reservoir of CMV in the host. It has been shown that CMV has the ability to replicate in endothelial cells causing a minimal cytopathogenic effect, and cell death occurs after a long period of time. The tropism for endothelial cells is determined by the envelope proteins pUL, 128 and pUL130, while pUL 131A is thought to have a role in forming a firm bond with the cell receptor and is very important in replication.

There is evidence that CMV acts differently even in various endothelial cells. The CMV infection of brain microvascular endothelial cells (BMVECs) differs greatly from the infection of aortic macrovascular endothelial cells (AECs) by the same virus.

BMVECs, together with astrocytes, form the blood-brain barrier, and therefor contain transport systems for various metabolites, a characteristic that is not present in AECs. Although CMV can fully replicate in both types of endothelial cells, in BMVECs sites of intracellular accumulation of the virus are seen, which disrupts the cellular metabolism and cell death occurs more rapidly. On the other hand, AECs do not show this accumulation, which shows that these cells have developed an efficient mechanism of secreting newly synthetized viruses. Infected AECs survive much longer, and are thought to be a source of viral persistency in the organism.

Viruses that are released from endothelial cells most often infect peripheral blood monocytes, in which they establish latency. It is believed that products of viral IE1 and IE2 genes react with and activate the promoter of ICAM-1 gene, and lead to a greater expression of ICAM-1 adhesion molecule. This molecule is responsible for a stronger bond that develops between monocytes and the infected endothelial cells, and makes CMV infection of monocytes much easier (Jarvis and Nelson, 2002).

For a long period of time it was not certain whether CMV can establish a latent infection. However, with an increasing number of organ transplants, it has been shown that a large percentage of seronegative organ acceptors develop CMV infection, after receiving organs from seropositive donors. Also, CMV reactivation has been shown in

immunocompromised patients. Even though all the cells where CMV can establish latency are not defined, in vitro and in vivo studies show that CMV establishes latency in peripheral blood CD14+ monocytes. The viral genome is present in 1 out of 104 monocytes, however the number of viral gene copies in these cells is very low, 6-13 copies per cell. When in latency, the viral genome is located in the cell nucleus in episomal form. Very few genes are expressed, and all belong to the group of immediate early (IE) genes. The products of these genes have an anti-apoptotic, as well as an immunomodulatory effect, especially on the expression of class I major histocompatibility complex (MHC-I)molecules, adhesion and co-stimulatory molecules (ICAM-1, LFA-1, B7). There is an increased production of inhibitory factors that prevents the lytical effect of complement (CD55), but also an increased production of cytokines (TGF beta, TNF alpha) in infected cells.

Some viral gene products expressed during latency are homologs of cell molecules. For example, a protein coded by the viral ORF UL111a gene is a homolog of the cellular IL-10, and is named LAcmvIL-10. Even though the viral and cellular protein show a structural similarity of only 27%, LAcmvIL-10 binds to the receptor of its homolog and has the same biological effects. One of those effects is the induction of monocyte differentiation into macrophages. Macrophages are permissive for CMV, so CMV reactivation occurs, the virus enters the lytic phase of replication and new viruses are produced. Besides LAcmvIL-10, other factors such as IL-2, TNFα, IFNγ and NFκB can induce the differentiation into macrophages, and therefore lead to the reactivation of CMV (Ouyang, et al., 2014).

Since monocytes are present in peripheral blood only a short period of time, it was assumed that CMV can also infect and establish latency in the monocyte bone marrow progenitors. This was proven by finding the latent CMV genome in the pluripotent CD34+ cells. These cells have the ability to differentiate into many different cells in the myeloid and lymphocytic lineage, so there is a question of how is latent CMV found only in monocytes. One explanation is that the virus favors the differentiation of CD34+ cells into monocytes. Also, it is possible that progenitor cells also differentiate into other cells, but are not able to survive with the virus present, or they might have developed some antiviral mechanism to eliminate the viral genome (Mendelson, et al., 1996)

1.2.1.4 CMV infection and malignancies

CMV is thought to be an etiological factor in carcinogenesis. Specifically, in vivo and in vitro studies have shown that CMV alters the expression of factors regulating cell survival, replication, motility and adhesion (Söderberg-Nauclér, 2006). CMV may also promote carcinogenesis by impairing the hosts' cellular immunity, by inducing T-cell anergy (Koch, et al., 2007) and the deregulation the host inflammatory response.

Recently, growing evidence shows that active CMV infection is associated with various malignancies, such as tumors of the breast, brain, colon, prostate, and lung (Bishop, et al., 2015) (Richardson, et al., 2015) (Söderberg-Nauclér and Johnsen, 2015) (Cobbs, Matlaf and Harkins, 2014) (Arastefar, et al., 2015) (Samanta, et al., 2003) (Giuliani, Jaxmar and Casadio, 2007). 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 (Soroceanu and Cobbs, 2011) (Slinger, et al., 2011). In contrast, noncancer cells surrounding the tumor appear to be CMV negative. This suggests that a persistently active CMVinfection may be present in certain tumors. Several initial studies were not able to prove that CMV has the ability to transform normal human cells, and therefor CMV is not considered to be oncogenic. However, there is evidence that this virus can modify tumor cell biology, in a fashion not involving direct transformation, and hence has a significant role in oncomodulation (Cinatl, et al., 2004)

1.2.2 Epstein-Barr virus

Epstein-Barr virus (EBV) also known as Human Herpesvirus 4 (HHV4) was discovered in 1964 by electron microscopy of cells cultured from Burkitt's lymphoma (Epstein, Achong, and Barr, 1964). EBV is one of the most widely spread viruses, with the infection prevalence of over 90%. Since EBV is constantly shed into saliva (Hadinoto, et al., 2009), transmission of EBV most often occurs in early childhood, through contact with family members. When acquired in childhood, the infection passes without symptoms or with a mild pathology, however, when primary infection occurs later in life, most often in adolescence, the infection often manifests as infectious mononucleosis, a self-limiting, lymfoproliferative disease.

The target cells for EBV are B lymphocytes. EBV infections have been associated with lymphoproliferative disease in immunocompromised patients, and also some malignancies, the most common being lymphoma (Burkitt, Hodgkin, NK/T cell), but also nasopharyngeal and gastric carcinoma (Küppers, 2003).

1.2.2.1 Structure and Morphology of EBV

EBV has a similar structure as other herpesviruses. It has a linear double-stranded DNA with terminally repeating sequences, surrounded by an icosahedral capsid with 162 capsomeres that is separated from the viral envelope by a tegument consisting of protein fibrils. The envelope contains 11 glycoproteins: membrane antigen (MA) complex that is composed of gp350/220 (BLLF-1) and gH (gp85, BXLF-2), responsible for viral adhesion to the cells and penetration, the gN (BLRF-1) and gM (BBRF-3) complex, necessary for the maturing of the virus, gB (gp110, BALF-4), needed for the virus to exit the cell, and 4 other proteins, gp78 (BILF-2), gp150 (BDLF-3), gp60 (BILF-1) and BMRF-2, whose role is not fully explained. It is thought that BMRF-2 is needed for the infection of epithelial cells, since it reacts with integrins (Lawrence, et al., 2007).The viral capsid antigen (VCA) is comprised of four proteins, p160, p18, p23 and p40. The tegument proteins are both viral (BNRF-1, BPLF-1) and cellular proteins such as actin, cofilin, tubulin and Hsp70 (Rickinson and Kieff, 2007).

1.2.2.2 Pathogenesis EBV infections

As stated above, EBV is a B-lymphotropic virus, however it has the ability to also infect epithelial cells. During primary infection, viral replication takes place in the oropharyngeal epithelium (Sixbey, 1984). The virus then enters the subepidermal connective tissue, where the viral envelope glycoprotein (gp350) binds to the receptor on B lymphocytes, CD21. However, in order for EBV to enter the cell, another signal must be formed, it is necessary for the viral gp42 to bind to II class MHC molecule. Once it enters the cell, the virus can cause a productive (active) or latent infection

The events following primary infection and the initial productive phase that lead to latency in the memory B cell population are still not fully understood. It is thought that the virus uses a pattern of gene expression that mimics antigen-driven activation and differentiation of B cells. In the germinal center the infected naïve B cell is driven to become a B cell blast, undergoes somatic hypermutation and class-switch and finally gives a latently infected memory B cell that carries the virus but that does not express viral genes (Thorley-Lawson and Gross, 2004). The signal that leads to the reactivation of the virus in vivo has still not been identified, although the belief is that it is an external, and a not viral factor. Actually, the reactivation of the latent virus occurs when the infected memory B lymphocyte differentiates into plasma cells (Laichalk and Thorley-Lawson, 2005), and a productive, lytical EBV infection is established. The first protein indicators of EBV reactivation are BZLF-1 and BRLF-1 that actually regulate transition from latency to a lytic infection. They act as transactivators of other viral genes, including the gene for DNA-polymerase that is necessary for the replication of the viral DNA. (Kutok and Wang, 2006).

1.2.2.3 Latent EBV infections

When latency is established the EBV genome is present in the cell nucleus in episomal form. During latency, there is a constitutive expression of certain viral genes, and a production of latency associated proteins. These proteins include 6 Epstein-Barr Nuclear Antigen (EBNA) proteins (EBNA-1, EBNA-2, EBNA-3A, 3B, 3C and EBNA-LP) that act as co-activators of transcription, and 3 Latency Membrane Proteins (LMP-1, LMP-2A and LMP-2B) that are analogs of membrane receptors. It has been shown that during latency, some RNA products are transcribed that do not code proteins, but have a regulatory role in the cell. They include 2 EBV-encoded small RNAs (EBER-1 and EBER-2) and microRNAs, products of BART and BHRF1 gene. However, all of these products are not always expressed in cells infected with latent EBV (Rickinson and Kieff, 2007). Because of the different patterns of expression of certain markers three types of latency, known as latency programs have been established:

1. Type III latency- the growth program
2. Type II latency- the default program
3. Type I latency- the latency program

The gene products expressed in certain latency programs are shown in Table 3.

Table 3: EBV gene expression during various latency programs

Latency type	EBNA-1	EBNA-2	EBNA-3 (A,B,C)	EBNA-LP	LMP-1	LMP-2A	LMP-2B	EBER
III	+	+	+	+	+	+	+	+
II	+	-	-	+/-	+	+	+	+
I	+	-	-	-	-	-	-	+

Once EBV infects a naïve B lymphocyte it has a tendency to bring the cell to a state in which the viral latency can be most easily maintained. When B lymphocytes are activated by a pathogen, first clonal proliferation occurs, followed by the differentiation into either

effector plasma cells, with a short life span, or long lasting memory cells. When establishing latency, EBV mimics these processes in the 3 latency programs. When EBV first infects a naïve B cell, type III latency is established. B lymphocytes are activated and they proliferate, and the cell expresses all latency associated proteins. After proliferation, type II latency is established. Some proteins are not produced, while the cell expresses EBNA-1, all three LMPs and EBER. During this program the B lymphocyte differentiates into memory cells. After differentiation, the cell establishes type I latency, during which only EBNA-1 and EBER are produced, which is sufficient to maintain latency. These products enable the replication of viral genome during cell replication.

The roles of latency associated proteins are still investigated. It is known that EBNA-1 is present in all latency programs. It is a nuclear phosphoprotein that facilitates viral replication by binding the viral episome to the host cell chromosome. It binds to the OriP region, where replication is initiated. It also binds to viral promoters and regulates the expression of other EBNA proteins as well as LMP-1 (Young and Rickinson, 2004). Besides this, it has been shown to have an important role in the inhibition of apoptosis, by not allowing the HAUSP/p53 bond to form, and therefore leading to the degradation of the proapoptotic protein p53 (Kennedy, Komano and Sugden, 2003).

EBNA-2 is present only in type III latency, and is crucial for proliferation. It binds to RBP Jκ, and activates the Notch signaling pathway. An overexpression of certain cell transcription factors occurs (c-myc, hes-1, CD21, CD23), that leads to cell proliferation. It has been shown that EBNA-3 has a similar role to EBNA-2 and is important for cell proliferation (Hofelmayr, et al., 2001).

LMP-1, present in types III and II latency, is crucial for B lymphocyte immortalization. In physiological conditions, in order for B cell activation, it is necessary for a bond to be formed between CD40 receptor on B lymphocytes and CD40L on T lymphocytes, after which the Jak/Stat signaling pathway is activated. LMP-1 is a structural analog of CD40, however it does not require to bond with a ligand to activate the Jak/Stat signaling pathway (Figure 3). It also inhibits proapoptotic molecules such as bcl-2, mcl-1 and Bfl-1. LMP-2A, also present in types III and II latency, also has a role in B lymphocyte

immortalization. This protein is a structural analog of the BCR, and leads to the activation of B lymphocytes, without the need to bind a specific antigene (Young and Rickinson, 2004).

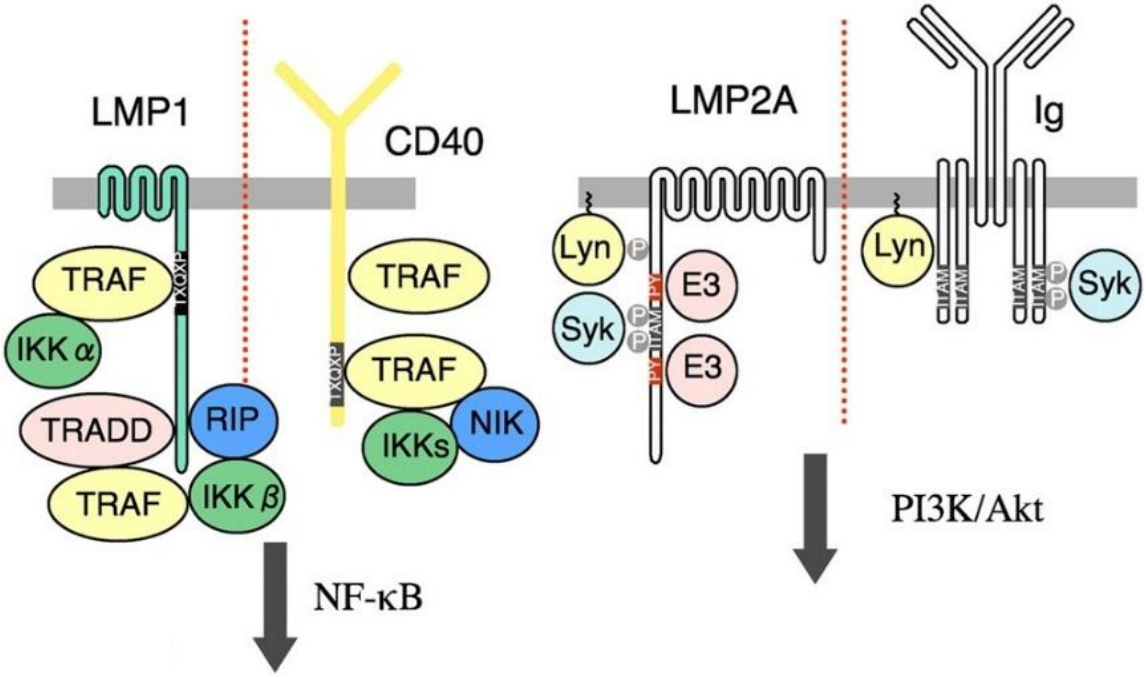

Figure 3. LMP1 mimics CD40, while LMP2A mimics Ig receptors in B cells. Modified from (Gordon and Longnecker, 2012)

1.2.2.4. EBV infections and malignancies

EBV was first discovered in Burkitt lymphoma cells, and is the first virus that was considered capable to lead to malignant transformation. Present day, a much greater number of lymphomas are associated with EBV infection. In these malignancies, the viral genome was detected, however, it has been shown that in various malignancies, EBV expresses different latency programs. T cell immunodeficient patients, especially patients on immunosuppressive therapy after organ transplantation, very often develop B cell lymphoma. These tumors develop very rapidly, often within a year of the operation, and all have been shown to be EBV positive. These tumors express EBNA-2 and LMP-1, and therefore type III latency program is established. This explains how B lymphocytes can proliferate in the absence of T lymphocytes. Similar reports have been seen in patients with AIDS. They usually develop lymphoproliferative diseases, especially of the CNS. In the affected cells, EBV establishes type III latency program.

Hodgkin lymphoma is also associated with EBV infection, since EBV genome was found in 40% of cases. Since only a small portion of B lymphocytes are infected with EBV in the organism (1-100 out of 106), it is statistically impossible that the presence of EBV in these tumors is accidental. In tumors that were EBV positive, the virus was found in all Reed-Sternberg cells. In these cells, EBV establishes type II latency program (EBNA-1, LMP-1 and 2 positive, EBNA-2 negative). In these malignancies LMP-1 is considered to play the most important role in cell transformation by activating NF-κB.

In Burkitt lymphoma cells, latent EBV is present very often, especially in endemic regions in Africa, where the virus is present in 85% of the lymphomas, while in other parts of the world in only about 15%. In these tumors EBV establishes type I latency program, however the role of EBV in malignancy development is still unknown. EBV latency was also found in T cell and NK cell lymphomas. They develop immediately after primary infection, and in the transformed lymphocytes, EBV establishes type I or type II latency program (Niedobitek, Young and Herbst, 1996) (Carbone, Gloghini and Dotti, 2008).

Besides lymphocyte transformation, EBV has a role in the development of epithelial carcinomas. In Southeast Asia and China most cases of undifferentiated nasopharyngeal carcinoma are associated with EBV infection. Recent studies also show the connection between other carcinomas, such as gastric and breast cancer, and EBV infection.

1.3. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of zinc and calcium dependent proteolytic enzymes that have the ability to degrade the extracellular matrix and basement membrane. Different MMPs can digest various components of the extracellular matrix, including elastin, fibronectin, vitronectin, laminin, proteoglycans and collagen. MMPs have an important role in many physiological processes, such as embryogenesis, uterine involution and angiogenesis, but they also have a vital role in pathological processes such as invasion, migration, metastasis and tumorigenesis (Woessner, 1991) (Stetler-Stevenson, 2001). MMPs are divided into five groups based on their structure, substrate specificity and on the cellular localization (Christiansen and Rajasekaran, 2006). These groups are:

1. collagenases (MMP-1, 8, 13 and 18) that degrade triple-helical fibrillar collagen into distinctive 3/4 and 1/4 fragments.
2. gelatinases (MMP-2 and MMP-9) that degrade type IV collagen and gelatin. These enzymes have a specific structure, since they have an additional domain inserted into the catalytic domain.
3. stromelysins (MMP-3, 10 and 11) that degrade a broad spectrum of extracellular matrix proteins (proteoglycans, laminin and fibronectin), but are unable to cleave the triple-helical fibrillar collagens.
4. matrilysins (MMP-7) that degrades the broadest spectrum of proteins. This MMP binds to cholesterol sulfate in cell membranes, and is therefore usually found membrane associated, and
5. membrane-type MMPs (MT-MMPs) (MMP-14, 15, 16, 17, 24, and 25) that are transmembrane proteins, and have the ability to activate other MMPs.

1.3.1. Activation of Matrix Metalloproteinases

MMPs are produced as inactive pro-enzymes and require proteolytic degradation, in the presence of calcium ions, in order to produce the active form of the enzyme (Freije et al, 1994). Two MMPs that are most often associated with malignancies are MMP-2 (gelatinase A) and MMP-9 (gelatinase B). These proteases have the ability to degrade type IV collagen,

which is the main component of the basement membrane of blood vessels, and are therefore thought to have a major role in the development of metastasis. There is a strict control over the proteolytic activity of these MMPs, and the deregulation of this process leads to an uncontrolled degradation of the extracellular matrix, which can lead to the spread of tumors. This control occurs on many levels, from the regulation of gene expression, the stability of mRNA, the activation of pro-enzymes, to the inhibition of proteolytic activity by specific endogenous tissue inhibitors of metalloproteinases (TIMPs). Even though MMP-2 and MMP-9 have the same substrate, the regulation of gene expression of these two MMPs is different. Certain transcription factors have important roles in the regulation of their expression, and bind to activator protein-1 and 10 (AP-1 and AP-10). MMP-2 has several regulatory sequences that are specific only for this enzyme, and its expression is mostly regulated by activators (Woessner, 1991). MMP-9, on the other hand, in the promoter region has a specific binding site for NFκB and SP-1 protein, through which Tumor Necrosis Factor α (TNFα) induces MMP-9 expression (Ries and Petrides, 1995).

The structure of MMPs consists of a signal sequence, a prodomain, a catalytic domain, and usually a hemopexin-like domain. Matrix metalloproteinases are primarily synthesized in the latent form (zymogen). They are secreted as proenzymes (pro-MMPs) and require extracellular activation. The activation of pro-MMPs varies, and is a result of the biological activity of various tissue or plasma proteinases, including other MMPs. MMPs are kept inactive (latent) because a conserved cysteine residue in the prodomain displaces the catalytic water molecule from the active site zinc ion (Springman, et al. 1990).

A well-known process is the activation of MMP-2, that occurs at the cell surface due to the activity of MT-MMPs. It is believed that MT1-MMP has the most important role in this activation (Sternlicht and Werb, 2001) (Strongin, et al., 1995). TIMP-2, adhered to the catalytic site of one MT1-MMP molecule, acts as a receptor for MMP-2, specifically by binding with the noncatalytic COOH-terminal domain. A second molecule of MT1-MMP then partially cleaves the MMP-2 prodomain, which is then fully cleaved by autoproteolysis. It has been shown that TIMP-2 is the only TIMP that has this ability to activate MMP-2 (Worley Thompkins, et al., 2003). Besides this mechanism, MMP-2 can also be activated by MMP-1, MMP-7, thrombin and activated protein C. MMP-9 can be activated by plasmin,

trypsin- 2, MMP-2, MMP-13 (activated by MMP-2) and MMP-3 (activated by plasmin) (Björklund and Koivunen, 2005) (Fridman, et al., 2003).

However, in vitro and in vivo studies show that certain MMPs can also be activated by physical or chemical agents, such as low pH, binding of sodium dodecyl sulfate or 4-aminophenylmercuric acid. These agents provoke structural unwinding, autocatalytic cleavage of the NH2-terminal prodomain and hence opens the so-called "cysteine switch" (Springman, et al., 1990). A physiological example of chemical activation may be the ability of nitric oxide to activate MMP-9 in the brain (Gu, et al., 2002). Reactive oxygen species (ROS) potentially released from macrophages can also activate MMP-2 and -9 (Rajagopalan, et al., 1996).

1.3.2. Matrix Metalloproteinases in Malignancies

The role of many MMPs has been investigated in cancer pathology, however, most attention has been given to the gelatinases because they are overexpressed in a variety of malignant tumors and they are associated with tumor aggressiveness and a poor prognosis. As mentioned earlier, gelatinases have the ability to degrade various components of the extracellular matrix and non-matrix proteins (Roy, Yang and Moses, 2009) (Rodríguez, Morrison and Overall, 2010) (Deryugina and Quigley, 2010). Due to their proteolytic activity, gelatinases are thought to be very important for cell migration and invasion. These processes are very complex, and involve numerous components, such as the extracellular matrix, chemokines, proteinases, adhesion receptors and basement membrane. A crucially important process for both tumor growth and metastatic spreading is angiogenesis, the formation of new blood vessels from preexisting ones. It was shown that gelatinases are able to degrade type IV collagen and therefore has a role in the invasion of the basement membrane (Egeblad and Werb, 2002).

However, recent studies have showed that gelatinases also indirectly have a role in cell signaling and affect cell growth, migration, inflammation and angiogenesis. Gelatinases degrade the extracellular matrix, and some products of the degradation have the ability to influence cell migration, angiogenesis and cancer progression. For example, the digestion of type IV collagen by MMP-9 reveals cryptic sites, that are crucial for angiogenesis (Hangai,et

al., 2002). Similarly, the digestion of laminin-5 by MMP-2 reveals a cryptic epitope that increases the migration of endothelial cells (Giannelli, et al., 1997). By degrading the extracellular matrix, MMP-9 can release certain factors, including VEGF, TGF-β and FGF-2, which promote angiogenesis and tumor growth by stimulating proliferation and endothelial cell migration (Bergers, et al., 2000) (Yu and Stamenkovic, 2000) (Ardi, et al., 2009).

There is evidence of increased levels of MMP-2 and/or MMP-9 in many malignancies, such as melanoma, bladder, brain, breast, colorectal, lung, ovarian, pancreas and prostate cancers (Turpeenniemi-Hujanen, 2005). Besides in solid tumors, increased levels of MMPs were also found in hematological malignancies such as acute lymphoblastic leukemia, adult T-cell leukemia, chronic B lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myeloid leukemia, myelodysplastic syndromes and Hodgkin's and non-Hodgkin's lymphoma (Yu and Han, 2006) (Klein, et al., 2004).

1.4 Inflammation and cancer

A role of inflammation in the etiology and progression of cancer is well established (Balkwill, Charles and Mantovani, 2005) (Coussens and Werb, 2002). In vitro and in vivo studies show that chronic inflammation can influence carcinogenesis in several ways: directly inducing mutagenesis by producing radicals, or indirectly, inactivating DNA repair proteins and other mechanisms (Grivennikov, Greten and Karin, 2010) (Colotta, et al., 2010). Inflammatory cells are always present in the tumor microenvironment, and can produce molecules that inhibit tumor cell death, and promote tumor cell proliferation and angiogenesis (Mantovani, et al., 2008). There is numerous epidemiological evidence that inflammation has an etiological role in carcinogenesis. Many studies show a positive association between chronic inflammatory diseases, such as pancreatitis or inflammatory bowel diseases, and the risk of cancer (Bernstein,et al., 2001) (Lowenfels, et al., 1993). The severity of inflammation was found to be a factor for colorectal cancer incidence (Rutter, et al., 2004) In total, 20% of cancers can be associated with chronic infections (Aggarwal, et al., 2009). The role of infective agents that do not have direct oncogenic potential, such as Helicobacter pylori or hepatitis B and C viruses, in carcinogenesis can be explained by inflammation (Ernst and Gold, 2000)

Furthermore, there is evidence that long-term use of non-steroidal anti-inflammatory drugs reduces the incidence and mortality of certain malignancies, including colorectal, breast, prostate, lung, stomach, esophageal, and pancreatic cancers (Elwood, et al., 2009). Several studies showed that people with higher levels of circulating inflammatory markers have a higher risk of cancer incidence and mortality (Heikkilä, et al., 2009) (Il'yasova, et al., 2005) (Erlinger, et al., 2004). In the majority of these studies systemic inflammation was measured using levels of circulating non-specific inflammatory markers, such as C-reactive protein (CRP) or total white blood cells count. Other studies have examined the association between certain cytokines and other acute phase reactants such as erythrocyte sedimentation rate, serum globulin, and fibrinogen, and cancer incidence and mortality.

The immune systems primary role is the defense against microorganisms and foreign structures. The immune process is controlled by the synthesis and release of immune

response mediators, mainly cytokines, also known as interleukins, and chemokines (Schindler and Strehlow, 2000). Cytokines bind to and activate cell receptors known as Janus kinases (JAK) that belong to the family of tyrosine kinases (Liu, Gaffen and Goldsmith, 1998). Chemokines bind to and activate heterotrimeric GTP-binding proteins (Soriano, et al., 2003). The inflammatory response is reinforced by prostaglandins and leukotrienes. The functions of these pathways are often overlapping and redundant, but insure a stable defense against the ever attacking microorganisms.

However, it is now known that the functions of cytokines and chemokines are much broader, and include many metabolic processes including carcinogenesis (L in and Karin, 2007). For example, it has been shown that cytokines are able to act as survival and growth factors for some premalignant cells (Karin and Greten, 2005), promote angiogenesis and metastasis (Yu, Pardoll and Jove, 2009) (Karin, 2006). Over 50% of malignancies show anomalous activation of NF-κB and/or STAT3, which leads to a resistance to apoptosis in both premalignant and fully transformed cells, and an increase in tumor growth due to the increased rate of proliferation (Grivennikov and Karin, 2010) (Bollrath and Greten, 2009). Mutations in genes encoding NF-κB and STAT3 are very rare, so some autocrine or paracrine factor must be responsible for the activation of these transcription factors. In fact, tumor necrosis factor-α (TNF- α) and interleukin 6 (IL-6) are cytokines most associated with cancer initiation and propagation, since they were first proven to be able to activate the oncogenic transcription factors NF-κB, AP-1 and STAT3 in epithelial cells (Balkwill, 2009) (Bromberg, et al., 1999). TNF and IL-6 can influence all stages of carcinogenesis, including initiation, promotion, progression and metastasis.

IL-6 is a cytokine that has a 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 (Hideshima, et al., 2001). IL-6 is an inflammatory cytokine that is very potent, and has a multitude of physiological functions, including the developmental differentiation of lymphocytes, cell proliferation, and cell survival and inhibition of apoptotic signals (Heinrich, et al., 1998). Besides this, IL-6 shows an effect on the general metabolism and can affect many cells of

other tissues and organ systems. As shown in Figure 4, 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) (Yang, et al., 2003).

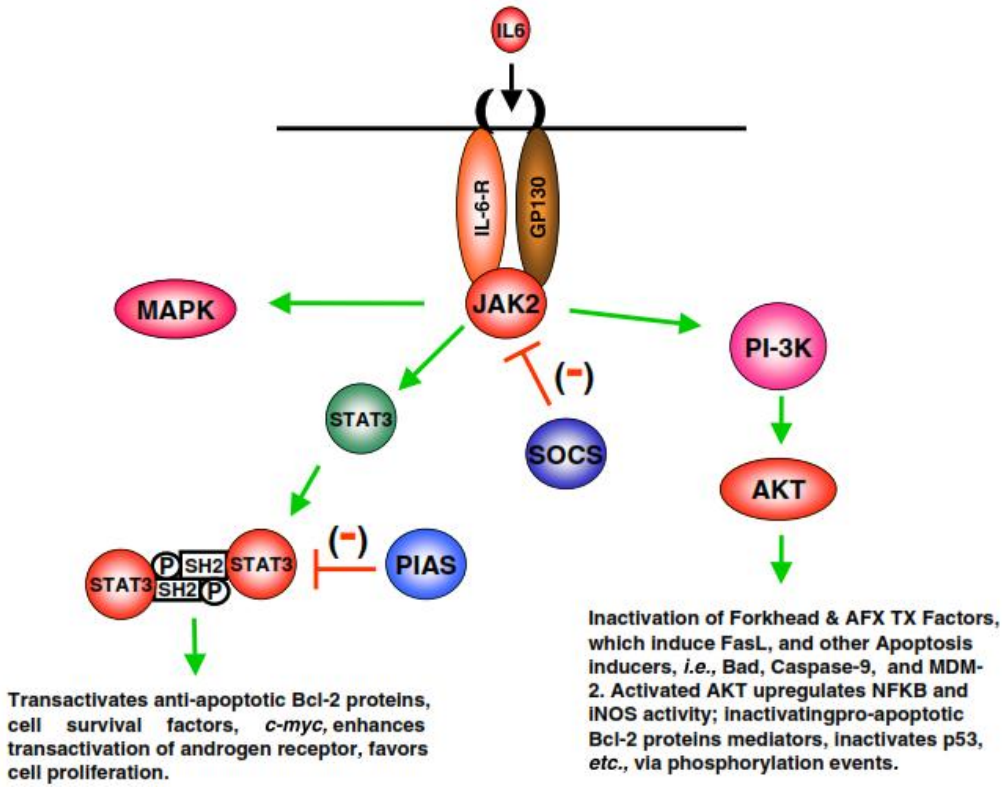

Figure 4. IL-6 activates anti-apoptotic and proliferative pathways (Hodge, et al., 2005)

As mentioned earlier, IL-6 binds to and activates the JAK signal receptor, which leads to the activation of the signal transducers and activators of transcription (STAT) factors STAT1 and STAT3. This activated signaling pathway leads 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 (Eustace, et al., 1993) (Leu, et al., 2003). The receptor for IL-6 is a heterodimer that contains two Ig-like proteins, the IL-6 specific chain gp80 (CD126), and gp130 (CD130). It is present on many cells in different tissues, including astrocytes, lung, ovary, endometrial stromal cells, heart, amnion-derived cells, macrophages, monocytes, microglia, kupffer cells, osteoblasts, multiple myeloma, mast cells, leydig cell

precursors (testes), fibroblasts (dental pulp, gingival, nasal turbinate, polyps, synovial), mesangial cells (kidney), human endothelial cells, hepatocytes, and prostatic cells. Also, there are many molecules that induce the secretion of IL-6, such as lipopolysacharides (LPS), IL-1b, prostaglandin E-2 (PGE-2), vascular endothelial growth factor (VEGF), and tumor necrosis factor-a (TNF-a) (Suganuma, et al., 2002). IL-6 can be present in many normal tissues, however its excessive and long term production during chronic inflammation is strongly associated with many types of cancer.

Tumor necrosis factor- α (TNF- α) is a major inflammatory cytokine whose first identified characteristic was the ability to induce necrosis of experimental cancers. Because of this characteristic, research was focused on utilizing this molecule in cancer treatments. However, it was noticed that, not only does this cytokine not ameliorate the condition, it paradoxically acts as a tumor promoter. TNF-α is a transmembrane protein with an intracellular amino terminus, however, after proteolytic cleavage it can be released as a soluble cytokine. There are two TNF-αreceptors: TNFR1, present on most cells in the body, and TNFR2, present mostly on hematopoietic cells (Harrison, et al., 2007). TNFR1 is activated by soluble ligand, and TNFR2 primarily binds transmembrane TNF-α. Depending on the affected cell, once TNFR1 is activated, two different end results can occur, apoptosis or inflammation and cell survival (Figure 5) (Balkwill, 2009).

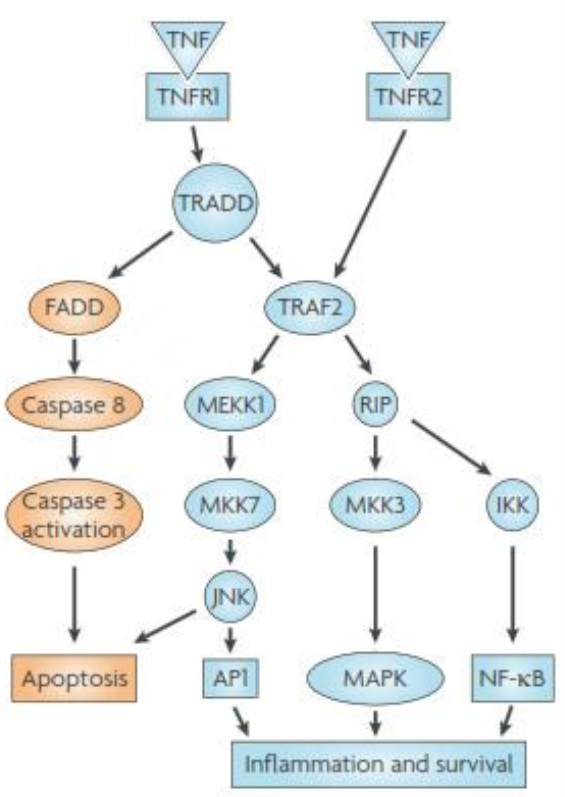

Figure 5. Intracellular tumor necrosis factor signaling (Balkwill, 2009)

The default program leads to the activation of genes involved in inflammation and cell survival. TNFR1, activated by TNF-α, leads to the activation of the AP1 transcription factors or IκB kinases (IKKs) that. These factors have a role in activating an important transcription factor, nuclear factor-kappaB (NF-κB) (Pikarsky, et al., 2004). This has an antiapoptotic effect on the cell, since NF-κB induces antiapoptotic regulators such as FLIPL, BCL-2 and superoxide dismutase. The inability to activate NF-κB in an adequate manner leads to apoptosis. This could be achieved through the activation of caspase 8 and Jun amino-terminal kinase (JNK), through the accumulation of reactive oxygen species (ROS)or mitochondrial pathways.

2. AIMS OF THE STUDY

Since CMV and EBV are often present in ductal epithelial cells of the salivary gland, we hypothesized that these viruses could cause inflammation and deregulation of genes involved in cell proliferation and could induce the development of salivary gland cancer. Having this in mind, the aims of this research were:

1. To determine the prevalence of CMV and EBV in salivary gland cancer tissues,
2. To compare the immunohistochemical and nested PCR detection of CMV and EBV in SGC ,
3. To investigate the viral protein expression in different histologic types of salivary gland cancer.
4. To investigate the immunohistochemical expression of IL6, MMP2, MMP9 and NFκB
5. To investigate the association of the presence of CMV and EBV and the immunohistochemical expression of IL6, MMP2, MMP9 and NFκB,
6. To analyze the polymorphisms of IL6, MMP2 and MMP9 genes and investigate the association of the polymorphisms and the corresponding protein expression.

3. MATERIAL AND METHODS

3.1 Study subjects

This cross-section study included 93 patients diagnosed with salivary gland cancer (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. Histopathologic diagnosis and immunohistochemical analysis were performed in the Institute of Pathology, School of Medicine, while the DNA polymorphism analysis and CMV and EBV PCR detection were performed in the Institute for Human Genetics, School of Medicine, and Microbiology Laboratory, School of Dental Medicine.

Histopathologic diagnosis of the histological type of SGC was performed in accordance with the 2005 World Health Organization (WHO) classification. The antibodies used for the diagnosis of SGC were anti-CK7, anti-p63, anti-CK5/6, anti-calponin and anti-SMA.

For the immunohistochemical analysis, the control consisted of healthy salivary gland tissue from 20 autopsy cases with no malignancies and salivary gland pathology. For the analysis concerning DNA polymorphisms, the control group consisted of buccal swabs from 100 healthy volunteers, matching the study group in sex and age.

All procedures were approved by the Ethical Committee of the School of Medicine, University of Belgrade, in accordance with the Declaration of Helsinki.

3.2 Methods

3.2.1 Histologic processing of the SGC samples

After the surgical obtainment of the cancer biopsies, the samples were fixed for 12-24 h in 4% buffered formalin, washed in phosphate-buffered saline (PBS) and then dehydrated in growing concentrations of ethanol (starting form 70% ethanol, to absolute ethanol, during 24 hours). The samples were then lyophilized in xylene, and placed in paraffin blocks. The paraffin blocks were then cut into 3-5 μm sections using a standard microtome, that were then stained with standard hematoxylin and eosin (HE) stain.

3.2.1.1 Hematoxylin and eosin (HE) stain

The procedure of HE staining is the following:

- Slides containing paraffin sections were placed in a slide holder
- Deparaffinization and rehydration was achieved by placing sections:
  - 3 x 5 minutes in xylene (excess xylene was blotted before going into ethanol)
  - 2 x 3 minutes in 100% ethanol
  - 1 x 3 minutes in 96% ethanol
  - 1 x 3 minutes in 70% ethanol
  - Rinse thoroughly with deionized water
- Hematoxylin staining:
  - 1 x 5 minutes in Mayer's hematoxylin
  - Rinse thoroughly with deionized water
  - Dip 8-12 times into 0.2% ammonia water solution
  - Rinse thoroughly with deionized water
- Eosin staining and dehydration:
  - 1 x 10-15 minutes in 1% eosin
  - Rinse thoroughly with tap water
  - 1x 3 minutes in 70% ethanol
  - 1x 3 minutes in 96% ethanol
  - 2x 3 minutes in 100% ethanol
  - 3x 5 minutes in xylene
- A drop of Canada balsam was placed on the slide and covered with the coverslip.

3.2.1.2. Immunohistochemical staining

All immunohistochemical staining was performed in the Institute of Pathology, School of Medicine, University of Belgrade. The immunohistochemical preparation of the sample is a multistep process that includes the following procedures:

- Deparaffinization and rehydration
- Antigen unmasking (proteolytic digestion)

- Endogenous peroxidase quench
- Immunohistochemical staining

Deparaffinization and rehydration:

Formalin-fixed, paraffin-embedded (FFPE) samples were cut into 5 µm thick paraffin tissue sections. Deparaffinized with incubation in xylene and rehydrated through graded concentrations of ethanol as shown in the following protocol:

2 x 5 minutes in xylene
2 x 3 minutes in 100% ethanol
2 x 3 minutes in 96% ethanol
1x 30 seconds in deionized water

Antigen unmasking

For antigen retrieval, slides were warmed in antigen retrieval citrate buffer (10mmol/L, pH 6,0) in the microwave for 2x 5 min on maximum temperature, followed by cooling at room temperature for 30 min and washing 3 times with PBS.

Endogenous peroxidase quench

Endogenous peroxidase activity was blocked by submerging the samples in 3% hydrogen peroxide (diluted in deionized water) for 5 minutes. After blocking, tissue sections were washed once in deionized water and 3 times with PBS.

Labeled streptavidin-biotin (LSAB) immunohistochemical staining

For the immunohistochemical staining a commercially available kit was used (Dako LSAB2 System-HRP). The staining procedure consists of the following several phases:

Phase I-applying the primary antibody in the adequate dilution:

- The samples were covered with 20-30 µl of primary antibody to cover specimen

- 30 minutes incubation on room temperature
- Carefully rinsed with PBS

Phase II- applying the biotinylated antibody

- The slide around the sample was dried
- The samples were covered with 20-30 µl of secondary biotinylated antibody (named Link antibody) to cover specimen
- 30 minutes incubation on room temperature
- Carefully rinsed with PBS

Phase III-applying the streptavidin conjugated to horseradish peroxidase (HRP)

- The slide around the sample was dried
- The samples were covered with 20-30 µl of Streptavidin-HRP to cover specimen
- 30 minutes incubation on room temperature
- Carefully rinsed with PBS

Phase IV- applying the 3,3'-diaminobenzidine (DAB)-Substrate-Chromogen

- The slide around the sample was dried
- The samples were covered with 20-30 µl of DAB-Substrate-Chromogen
- 30 minutes incubation on room temperature
- Carefully rinsed with PBS

Phase V- hematoxylin counterstain

- The slides were immersed in Mayer's hematoxylin and incubated 10 minutes at room temperature
- Carefully rinsed with deionized water
- Dip 8-12 times into 0.2% ammonia water solution
- Rinse thoroughly with deionized water

As the end products, the sites where the antibodies bonded gave a brown color, and the cell nuclei were counterstained blue with hematoxylin.

The following antibodies (in the adequate dilution) were used in immunohistochemical staining:

- CMV (M0854, Dako, UK), 1:80
- EBV (LMP M0897, Dako, UK), 1:80
- MMP-2 (NCL-MMP2-507, Leica Biosystems, UK) 1:80
- MMP-9 (NCL-MMP9-439, Leica Biosystems, UK) 1:80
- IL-6 (sc-130326, Santa Cruz Biotechnology, USA), 1:100
- NFkB (sc-8008, Santa Cruz Biotechnology, USA), 1:100

A positive control was provided for all applied antibodies. 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 according to the relative strength of the immunoreactivity.

3.2.2 DNA analysis

3.2.2.1 DNA extraction from FFPE tissue sections

DNA was extracted from FFPE tissue as shown in the following protocol:

- 10 µm thick FFPE tissue sections were collected in a plastic microtube (1.5 ml)
- 1 ml of xylene was added to the microtube containing tissue sections for 30 min with two changes,
- 100% and 75% ethanol were added for 30 min with two changes
- The ethanol was discarded and the samples were washed twice with PBS
- 500 µl of lysis buffer was added (proteinase K 20 mg/ml, 50 µl, 1 M Tris-HCl solution 10 µl, 0.5 M EDTA 2 µl, 10% SDS 100 µl, and distilled water 838 ml), and the mixture was incubated at 52°C overnight until all tissue fragments were dissolved completely
- 500 µl phenol:chloroform: isopropanolalcohol was added at 25:24:1 to the de-waxed tissue, and the mixture vortexed
- The samples were centrifuged at RT, 12,000 x g for 10 min
- The supernatant fluid was transferred to a new autoclaved microtube
- One volume of chloroform was added to the supernatant, and vortexed
- The samples were centrifuged at RT, 12,000 x g for 5 min
- The upper aqueous supernatant was transferred to a new microtube
- 0.1 volume of 3 M sodium acetate was added to the new tube and vortexed
- 1 volume of isopropanol was added, and incubated at -20C overnight.
- The precipitated DNA was centrifuged at 12,000 x g at 4C.
- The supernatant fluid was discarded and washed once with 75% ethanol
- The extracted DNA was collected after further centrifugation.
- The final yield of DNA was diluted in 50 µl distilled water

3.2.2.2 Genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to evaluate the single nucleotide polymorphisms (SNP) of IL-6, MMP-2 and MMP-9. PCR was carried out in a total volume of 25 µl, containing 300 ng genomic DNA, 0.4 µM of each primer and 1U Taq DNA polymerase (Thermo Fisher Scientific Inc). The solution was incubated for 3 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 45 s at primer specific annealing temperature (Table 4) and 45 s at 72 °C, with a final extension of 72 °C for 7 min.

The primer sequences and annealing temperatures are given in Table 4.

Table 4: Primer sequences and annealing temperatures for IL-6 -174G>C, MMP-2 -1306 C>T, MMP-2 -1575 G>A and MMP-9 -1562 C>T polymorphisms

SNP		Primer sequence	Annealing temp.
IL-6 -174 G>C	forward	5'-ATGCCAAGTGCTGAGTCACTA-3'	54 ºC
	reverse	5'-TCGAGGGCAGAATGAGCCTC-3'	
MMP-2 -1306 C>T	forward	5'-CTTCCTAGGCTGGTCCTTACTGA-3'	63ºC
	reverse	5'-CTGAGACCTGAAGAGCTAAAGAGCT-3'	
MMP-2 -1575 G>A	forward	5'-GTCTGAAGCCCACTGAGACC-3'	63ºC
	reverse	5'-CTAGGAAGGGGGCAGATAGG-3'	
MMP-9 -1562 C>T	forward	5'-GCCTGGCACATAGTAGGCCC-3'	60ºC
	reverse	5'-CTTCCTAGCCAGCCGGCATC-3'	

IL-6 -174G>C genotyping

The 226bp PCR product was then digested with 3 U NlaIII (Thermo Fisher Scientific Inc) overnight at 37 ◦C and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. The GG genotype showed a 226bp product; the GA genotype had 226bp, 117bp, and 109 bp fragments; and AA had 117bp and 109bp fragments.

MMP-2 -1575G >A genotyping

The 175 bp PCR product was then digested with 3 U NlaIII (Thermo Fisher Scientific Inc) overnight at 37 ◦C and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. The GG genotype showed a 175 bp product; the GA genotype had 175 bp, 112 bp, and 63 bp fragments; and AA had 112 bp and 63 bp fragments.

MMP-2 -1306C > T genotyping

The 193 bp product was digested with 3 U BfaI (Thermo Fisher Scientific Inc) overnight at 37 ◦C and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. The CC genotype showed a 193 bp digestion product, the CT genotype had 193 bp, 167 bp, and 26 bp fragments and the TT genotype had 167 bp and 26 bp fragments.

MMP-9 -1562 C>T genotyping

The 435 bp PCR product was digested with 3 U of SphI (Thermo Fisher Scientific Inc) overnight and the fragments were separated on an 8% polyacrylamide gel stained with ethidium bromide. After digestion, CC homozygotes showed 1 band of 435 bp, TT homozygotes had 2 bands (247 and 188 bp) and CT heterozygotes had 3 bands (435, 247 and 188 bp).

Genotypes were confirmed by randomly re-genotyping 10% of the samples. There were no discrepancies between the genotypes determined in duplicate.

3.2.2.3 Detection of CMV DNA using nested PCR

A nested PCR method was used to detect CMV in the samples. Primers were designed to amplify sequences from the CMV gBgene. The sequences of primers and nested PCR conditions are given in Table 5.

Table 5.Primer sequences and nested PCR conditions for CMV gB gene

Primer sequence *		**Product size (bp)**	**PCR conditions**					
			Temperature profile **					Cycles
			ID	D	A	E	FE	
OF	5'-CAGTTGACCGTACTGCAC-3'	489	95 °C	95 °C	52 °C	72 °C	72 °C	35
OR	5'-GGTATCAAGCAAAAATCT-3'		5 min	30 sec	30 sec	1 min	5 min	
IF	5'-GAAACGCGCGGCAATCGG-3'	301	95 °C	95 °C	60 °C	72 °C	72 °C	35
IR	5'-TGGAACTCGAACGTTTGG-3'		5 min	30 sec	30 sec	1 min	5 min	
* OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse ** A, annealing; D, denaturation; E, extension; FE, final extension; ID, initial denaturation								

The first round of PCR amplification was performed in a total volume of 25-mL mixture that contained 5 mL sample, 1x KAPA Taq buffer A with 1.5 mmol/L MgCl2, 1.5 mmol/L MgCl2,0.4 mmol/L deoxynucleoside triphosphates (dNTP Set; Qiagen, Venlo, The Netherlands), 400 nmol/L of each primer, and 1 U Taq DNA polymerase (KAPA Taq DNA Polymerase; KapaBiosystems). The second round of PCR amplification was performed in a total volume of 25-mL mixture that contained 3 mL of the first-round PCR product, 1 mmol/L MgCl2 (KapaBiosystems), and all other components from the first round with identical concentrations.

3.2.2.4. Detection of EBV DNA using nested PCR

A nested PCR method was used to detect CMV in the samples. Primers were designed to amplify sequences from the EBV LMP-1 gene. The sequences of primers and nested PCR conditions are given in Table 6.

Table 6.Primer sequences and nested PCR conditions for EBV LMP-1 gene

Primer sequence *		Product size (bp)	PCR conditions					
			Temperature profile **					Cycles
			ID	D	A	E	FE	
OF	5'-GCTAAGGCATTCCCAGTAAA-3'	663	95 °C	95 °C	47 °C	72 °C	72 °C	40
OR	5'-GATGAACACCACCACGATG-3'		5 min	1 min	1 min	1 min	10 min	
IF	5'-CGGAACCAGAAGAACCCA-3'	506	95 °C	95 °C	47 °C	72 °C	72 °C	40
IR	5'-TCCCGCACCCTCAACAAG-3'		5 min	1 min	1 min	1 min	10 min	
*** OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse** **** A, annealing; D, denaturation; E, extension; FE, final extension; ID, initial denaturation**								

The first round of PCR amplification was performed in a total volume of 25-mL mixture that contained

- 12,5 µl QiagenTaqPCR Master Mix (QIAGEN GmBH, Hilden, Germany),
- 1 µl of both outer primers in the concentration of 25µM/ml ("forward" and "reverse") (Invitrogen by Life technologies, Carlsbad, California, USA),
- 5 µl of extracted sample DNA
- 5,5 µl DNA-free water

The second round of PCR amplification was performed in a total volume of 25-mL mixture that contained

- 12,5 µl QiagenTaqPCR Master Mix (QIAGEN GmBH, Hilden, Germany),
- 1 µl of both inner primers in the concentration of 25µM/ml ("forward" and "reverse") (Invitrogen by Life technologies, Carlsbad, California, USA),
- 5 µl of extracted sample DNA
- 5,5 µl DNA-free water
- 5,5 µl DNA-free water

3.2.3 Statistical Analysis

In this study we used descriptive and analytical statistical methods.

Descriptive statistical methods used in study are:

- Counts and percentages (n, %)
- Central tendency measures (mean)
- Variability measurements (standard deviations)
- Analytical statistical methods included nonparametric tests
- Pearson chi-square test
- Cochran-Armitage chi square test for trend
- All data were analyzed using SPSS 20.0 (IBM corp.) statistical package.
- All p values less than 0,05 were considered significant.

4. RESULTS

We obtained a total of 93 samples from patients with SGC. Surgical procedures were performed in the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia during time period from 2004 to 2013. For each patient we recorded the patients' gender, age, affected salivary gland size and localization (Table 7).

Table 7.Distribution of SGC patients by gender, affected salivary gland size, and localization.

		Prevalence	Percentage	χ^2	P
Gender	Male	49	52.7%	0,391	0,532
	Female	44	47.3%		
SG size	Small	47	50,6%	0,043	0,835
	Big	46	49.4%		
SGC localization	epifarings	30	32.3%	24,609	**0,001**
	oral cavity	17	18.3%		
	parotid gland	39	41.9%		
	submandibular gland	7	7.5%		

Distribution of SGC patients by gender and age

From 93 patients with SGC 44 (47,3%) were female, and 49 (52,7%) were male patients, however this difference did not show statistical significance. The youngest patient was 15 years old, and the oldest was 86 years old (Mean +/- SD = 60,02 +/- 1,45). Higher prevalence was among older patients (the highest was between 61 and 80 years). However, in the first two age groups (0-20 and 21-40) SGC was diagnosed sporadically (Table 8).

Table 8. Distribution of SGC patients by age

Patient's age	**0-20**	**21-40**	**41-60**	**61-80**	**over 80**	**Total**
Prevalence	1	7	29	53	3	**93**
Percentage	1.1%	7.5%	31.2%	57%	3.2%	**100%**

Distribution of SGC tumor localization and size of affected salivary gland

SGC was found more often in small in comparison to big salivary glands (47 vs. 46) but without a significant difference. The most common localization of the cancer was parotid gland, followed by epifaringeal glands, while SGC was very rare in submandibular gland. (Table 7).

Distribution of SGC by histologic type

Among all types of salivary gland carcinoma adenoid cystic carcinoma was the most common (35 cases), followed by myoepithelial carcinoma (15 cases), while some types like acinic cell carcinoma, sebaceus carcinoma, carcinoma ex pleomorphic adenoma, squamous cell carcinoma, small cell carcinoma, large cell carcinoma, or lymphoepithelial carcinoma, were not found in our patients. Some types like acinic cell carcinoma, basal cell adenocarcinoma, cribriform cystadenocarcinoma and salivary duct carcinoma were sporadically diagnosed (Chart 1).

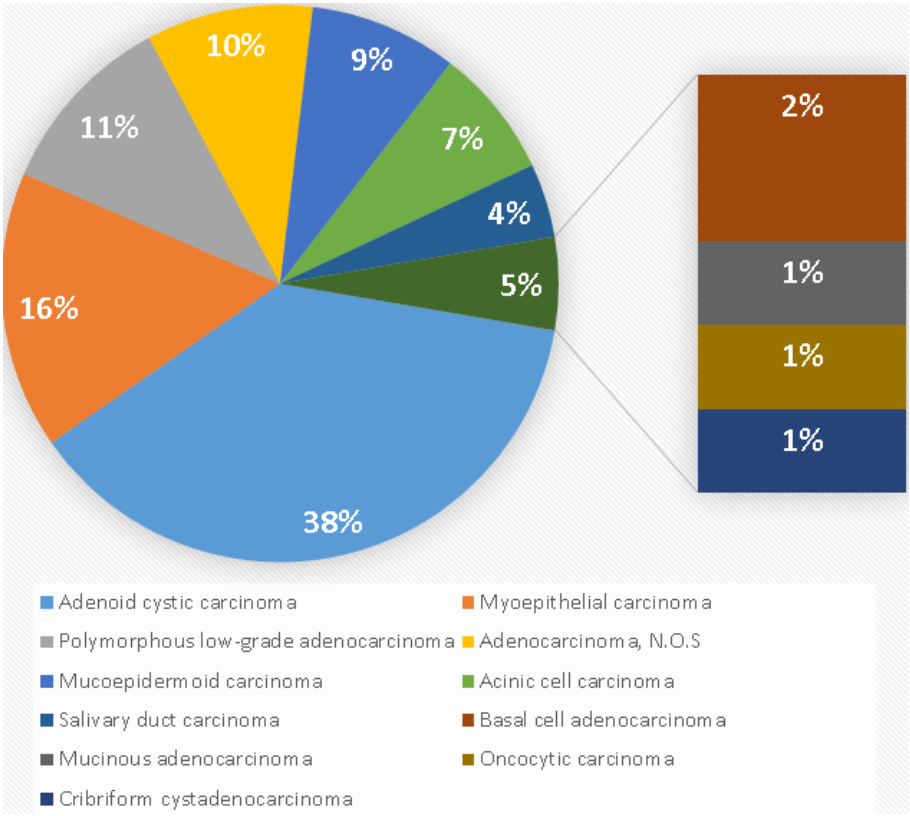

Chart 1. Distribution of SGC histologic types

4.1 Detection of CMV and EBV in SGC tissue

4.1.1 CMV antigen expression in SGC

Using immunohistochemical staining, CMV antigens were detected in 66 out of 92 SGC cases (71.7%). The differences in positivity strength can be seen in Figure 6. Detailed immunohistochemical results are presented in Table 9. There was no statistically significant difference in the presence of CMV with respect to gender, tumor localization or the size of affected salivary gland. Statistical analysis of the presence of CMV with respect to histological types cannot be performed due to small number of samples in certain groups.
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 tissue positivity was not detected in any sample.

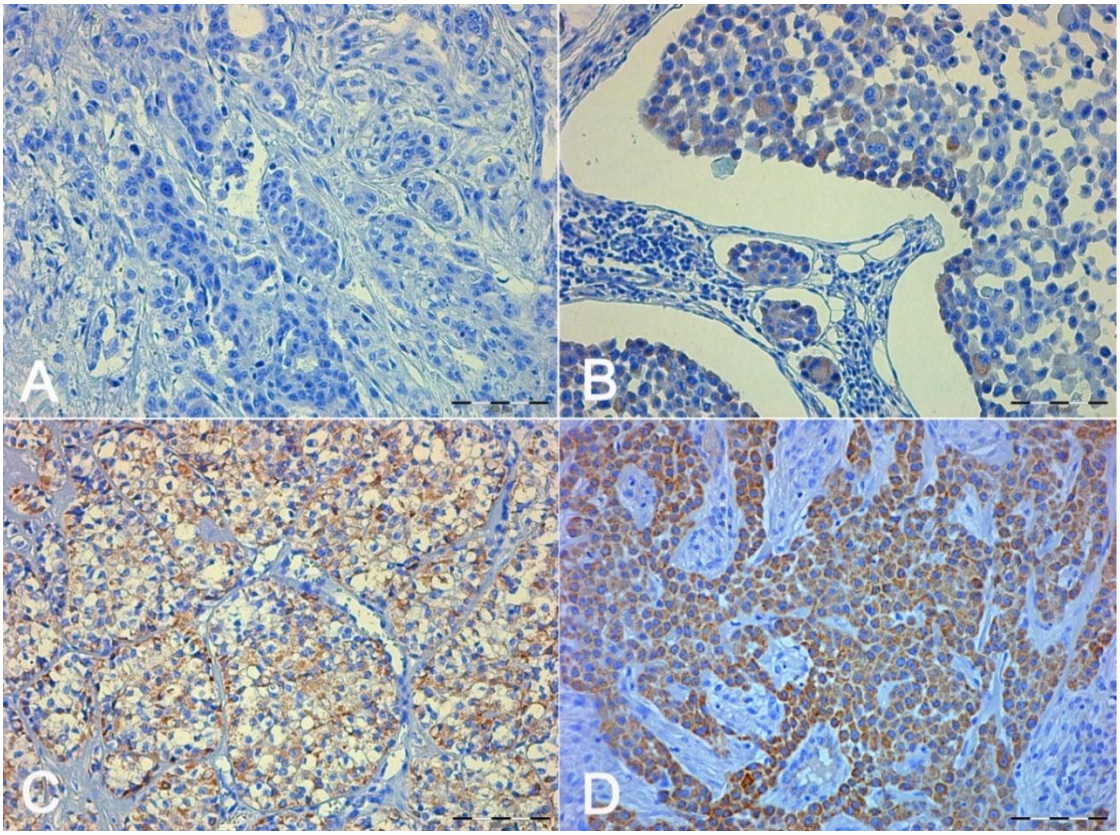

Figure 6. Immunohistochemistry of CMV proteins in SGC tissue.(A) Negative expression, (B) Weak positivity, (C) Intermediate positivity, (D) Strong. (IHCx100)

Table 9.Comparison of detected CMV antigen expression in SGC with clinical and pathological 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	Epipharinx	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	4	5	4	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

4.1.2 CMV genome presence in SGC tissue

Using nested PCR, CMV genome was detected in 62 out of 92 SGC cases (67.4%). The distribution by gender, salivary gland size, localization and histologic type are presented in Table 10. There was no statistically significant difference in the presence of CMV with respect to gender, tumor localization or the size of affected salivary gland. Statistical analysis of the presence of CMV with respect to histological types cannot be performed due to small number of samples in certain groups.

Table 10.Comparison of detected CMV DNA in SGC with clinical and pathological characteristics.

		CMV DNA	
		Negative	Positive
Gender	Male	13	35
	Female	17	27
Salivary gland size	Small	12	35
	Big	18	27
SGC localization	Epipharinx	7	23
	Oral cavity	5	12
	Parotid	14	24
	Submandibular	4	3
SGC histological type	Polymorphous low-grade adenocarcinoma	6	4
	Adenoid cystic carcinoma	7	28
	Salivary duct carcinoma	1	2
	Mucinous adenocarcinoma	1	0
	Acinic cell carcinoma	2	5
	Mucoepidermoid carcinoma	1	7
	Myoepithelial carcinoma	5	10
	Adenocarcinoma, N.O.S	4	5
	Basal cell adenocarcinoma	2	0
	Oncocytic carcinoma	0	1
	Cribriform cystadenocarcinoma	1	0

When comparing CMV detection by immunohistochemistry and nested PCR it is seen that the results are in agreement in 86/92 cases (94%). In 1 case, PCR detected CMV, while the IHC expression was negative, and in 5 cases the IHC gave a positive result, but CMV could not be detected by PCR (Table 11). The Cohen's Kappa, the measure of agreement, was 0.846, with a high statistical significance ($p<0.01$).

Table 11. Comparison of CMV prevalence in SGC obtained by IHC and nested PCR

		CMV PCR		Total
		Negative	Positive	
CMV IHC	Negative	25	1	26
	Positive	5	61	66
Total		30	62	92

4.1.3 Epstein Bar Virus (EBV) expression in salivary gland carcinomas

EBV was detected in 32 (34,8%) from our 92 cases of SGC. The differences in positivity strength can be seen in Figure 7. Detailed immunohistochemical results are presented in Table 12. There was no statistically significant difference in the presence of EBV with respect to gender, tumor localization or the size of affected salivary gland. Statistical analysis of the presence of EBV with respect to histological types can be performed only between adenoid cystic carcinoma and other histologic types. EBV is present in adenoid cystic carcinoma tissue more often than in other types of SGC ($p<0.01$).

In normal salivary gland tissues, EBV antigen expression was found only in one case, while 19 were negative. However, antigens of the latent EBV were sporadically found in lymphocytes of the normal salivary gland.

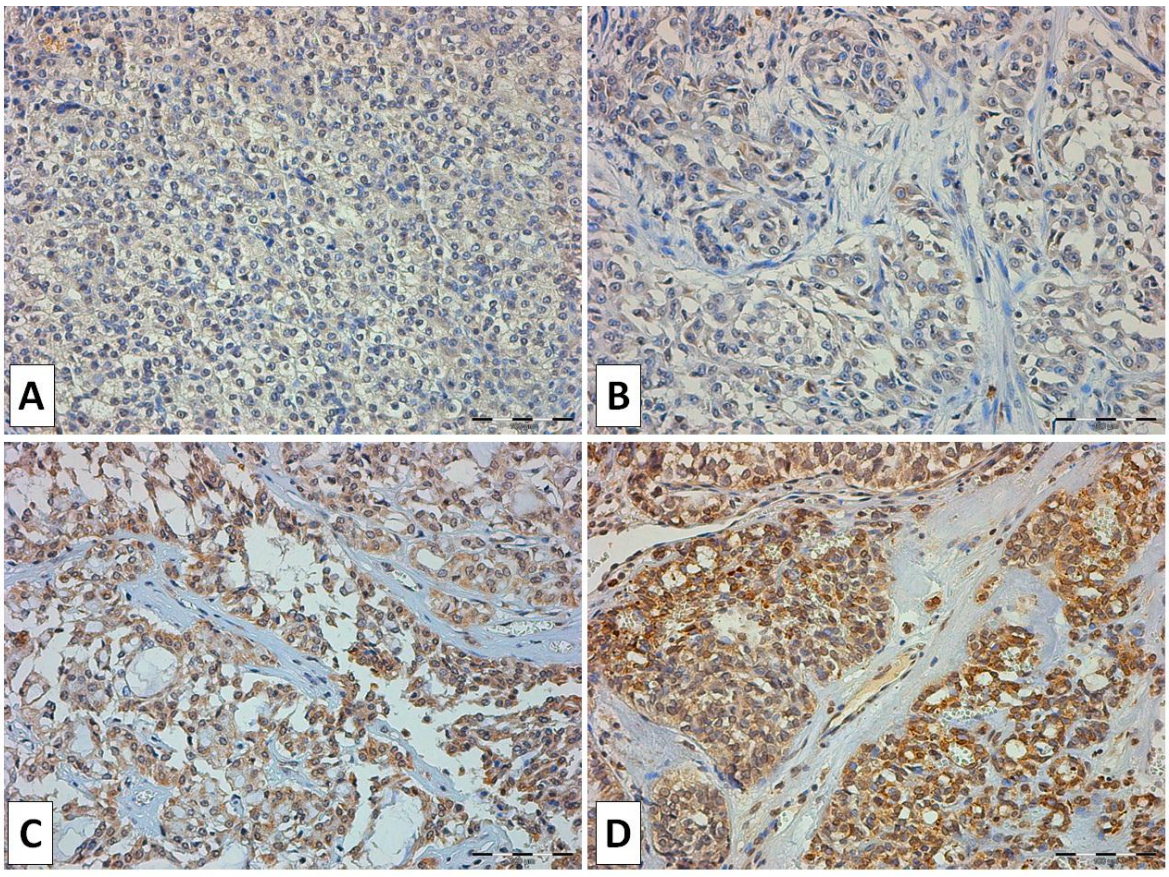

Figure 7.Immunohistochemistry of EBV proteins in SGC tissue.(A) Negative expression, (B) Weak positivity, (C) Intermediate positivity, (D) Strong. (IHCx100)

Table 12.Comparison of detected EBV expression in SGC with clinical and pathological characteristics.

		Negative	Weak	Moderate	Strong
Gender	Male	33	6	6	3
	Female	27	9	6	2
Salivary gland size	Small	31	7	7	2
	Big	29	8	5	3
SGC localization	Epipharinx	19	5	5	1
	Oral cavity	10	4	2	1
	Parotid	26	5	5	2
	Submandibular	5	1	0	1
SGC histological type	Polymorphous low-grade adenocarcinoma	8	1	0	1
	Adenoid cystic carcinoma	16	6	9	4
	Salivary duct carcinoma	2	1	0	0
	Mucinous adenocarcinoma	0	1	0	0
	Acinic cell carcinoma	7	0	0	0
	Mucoepidermoid carcinoma	6	2	0	0
	Myoepithelial carcinoma	13	0	2	0
	Adenocarcinoma, N.O.S	4	4	1	0
	Basal cell adenocarcinoma	2	0	0	0
	Oncocytic carcinoma	1	0	0	0
	Cribriform cystadenocarcinoma	1	0	0	0

4.1.4 EBV genome presence in SGC tissue

Using nested PCR, EBV genome was detected in 35 out of 92 SGC cases (38%). The distribution by gender, salivary gland size, localization and histologic type are presented in Table 10. There was no statistically significant difference in the presence of EBV with respect to gender, tumor localization or the size of affected salivary gland. Also here, statistical analysis of the presence of EBV with respect to histological types can be performed only between adenoid cystic carcinoma and other histologic types. EBV is present in adenoid cystic carcinoma tissue more often than in other types of SGC ($p<0.01$).

Table 13.Comparison of detected EBV DNA in SGC with clinical and pathological characteristics.

		EBV DNA	
		Negative	Positive
Gender	Male	33	15
	Female	24	20
Salivary gland size	Small	29	18
	Big	28	17
SGC localization	Epipharinx	17	13
	Oral cavity	11	6
	Parotid	25	13
	Submandibular	4	3
SGC histological type	Polymorphous low-grade adenocarcinoma	7	3
	Adenoid cystic carcinoma	15	20
	Salivary duct carcinoma	2	1
	Mucinous adenocarcinoma	1	0
	Acinic cell carcinoma	7	0
	Mucoepidermoid carcinoma	5	3
	Myoepithelial carcinoma	12	3
	Adenocarcinoma, N.O.S	4	5
	Basal cell adenocarcinoma	2	0
	Oncocytic carcinoma	1	0
	Cribriform cystadenocarcinoma	1	0

When comparing EBV detection by immunohistochemistry and nested PCR it is seen that the results are in agreement in 87/92 cases (94,6%). In 4 cases, PCR detected EBV, while the IHC expression was negative, and in 1 casee the IHC gave a positive result, but EBV could not be detected by PCR (Table 14). The Cohen's Kappa value that measures inter-rater agreement, or in our case the agreement between two methods used, was high (0.883) with a high statistical significance ($p<0.01$). IHC results were considered as golden standard. The sensitivity of nested PCR is 96.8%, the specificity 93.3%, the positive predictive value 88.5%, and negative predictive value 98.2%.

Table 14. Comparison of EBV prevalence in SGC obtained by IHC and nested PCR

		EBV PCR		Total
		Negative	Positive	
EBV IHC	Negative	56	4	60
	Positive	1	31	32
Total		57	35	92

In 16 of the 92 (17.4%) SGC cases there was no expression of viral antigens, neither CMV nor EBV, while 76 cases (82.6%) expressed antigens of at least one virus. However, there were 22 cases that expressed both CMV and EBV antigens (Table 15).

Table 15. Comparison of CMV and EBV antigen expression

		EBV		Total
		Negative	Positive	
CMV	Negative	16	10	26
	Positive	44	22	66
Total		60	32	92

4.2 Expression of prognostic markers in SGC

4.2.1 Expression of IL-6 in SGC

IL-6 expression was positive in 65 out of 92 SGC cases (70.7%), and only 4 control cases (20%). The difference in expression between SGC and control cases showed a high statistical significance (p<0,01) (Table 16).

Table 16. Expression of IL-6 in SGC and control

	IL-6 Expression		Total
	Negative	Positive	
SGC	27 (29.3%)	65 (70.7%)	92 (100%)
Control	16 (80%)	4 (20%)	20 (100%)
Pearson Chi-Square Value= 17.820, p=0.000			

When analyzing the difference between the SGC and the control group in the intensity of IL-6 expression, it can be seen that in the control group all the positive cases showed a weak expression, while in the group with SGC, there was a greater percentage of cases with moderate and strong expression. The difference showed a high statistical significance (p<0,01) (Table 17). The focal expressions of IL-6 in SGC tissue can be seen in Figures 8 and 9, and diffuse in Figures 10 and 11.

Table 17. IL-6 expression intensity in SGC and control

	IL-6 Expression				Total
	Negative	Weak	Moderate	Strong	
SGC	27 (29.3%)	30 (32.6%)	21 (22.8%)	14 (15.2%)	92 (100%)
Control	16 (80%)	4 (20%)	0 (0%)	0 (0%)	20 (100%)
Pearson Chi-Square Value= 19.448, p=0.000					

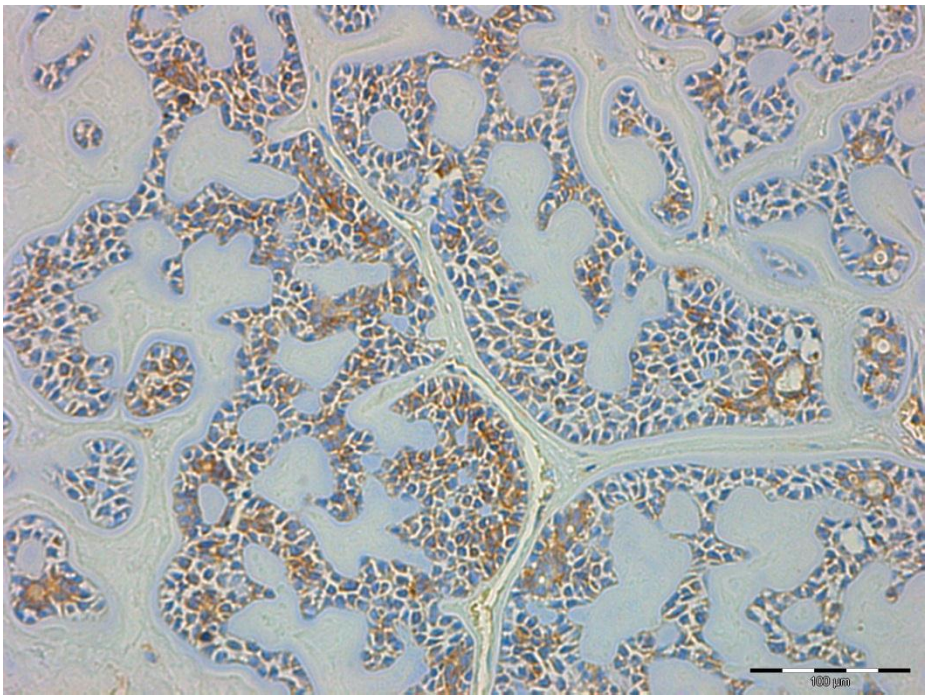

Figure 8.Focal moderate IL-6 expression in well differentiated adenoid cystic carcinoma of big submandibular salivary gland. IHC X 200

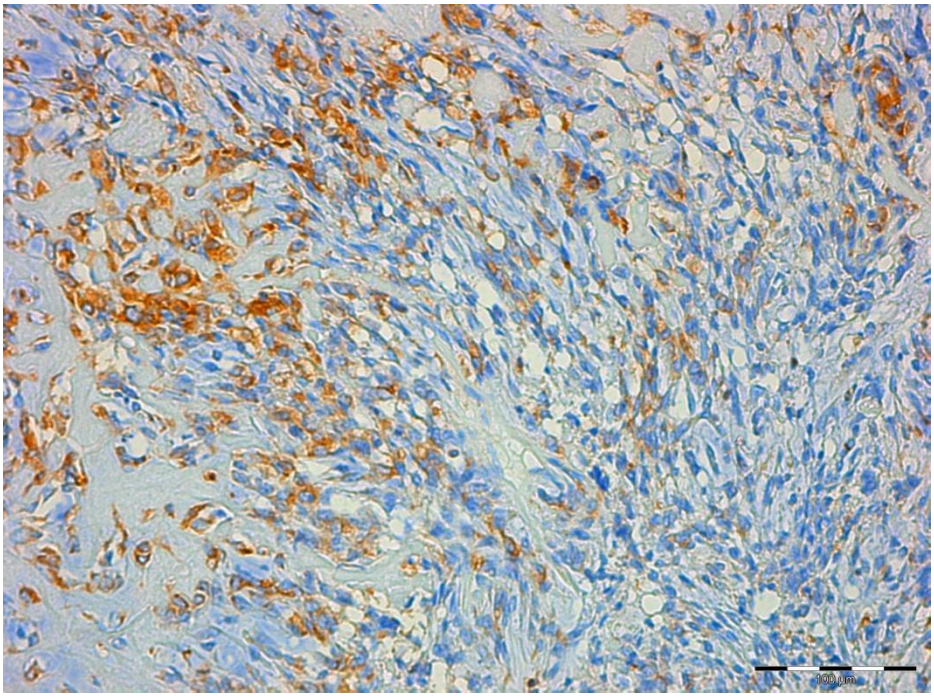

Figure 9.Focal strong IL-6 expression in low grade myoepithelial carcinoma of parotid gland. IHC X 200.

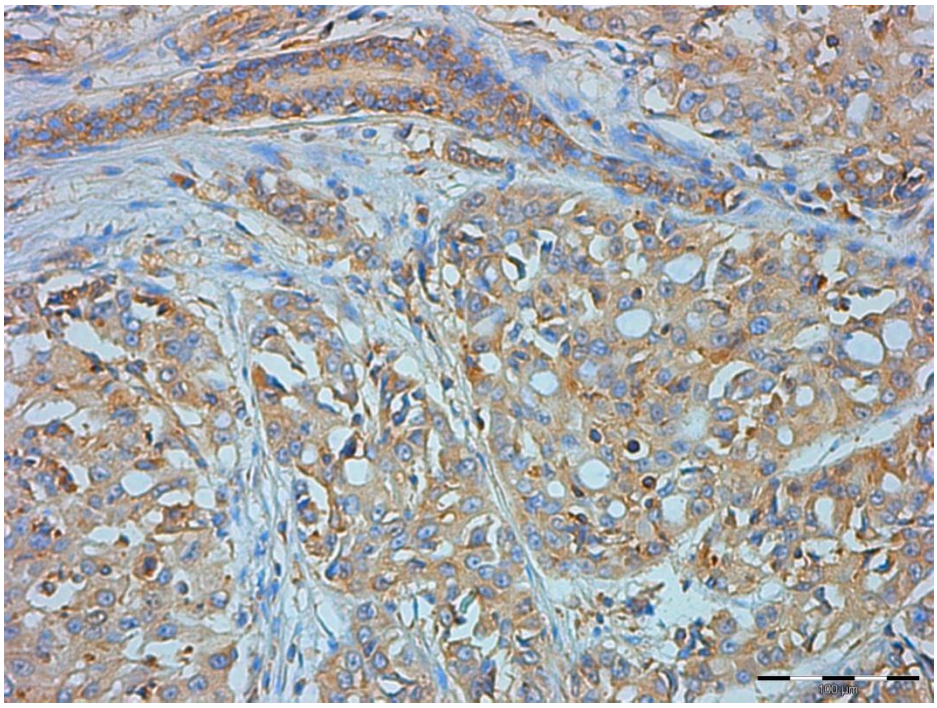

Figure 10. Weak diffuse IL-6 expression in acinic cell carcinoma of parotid gland. IHC X 200

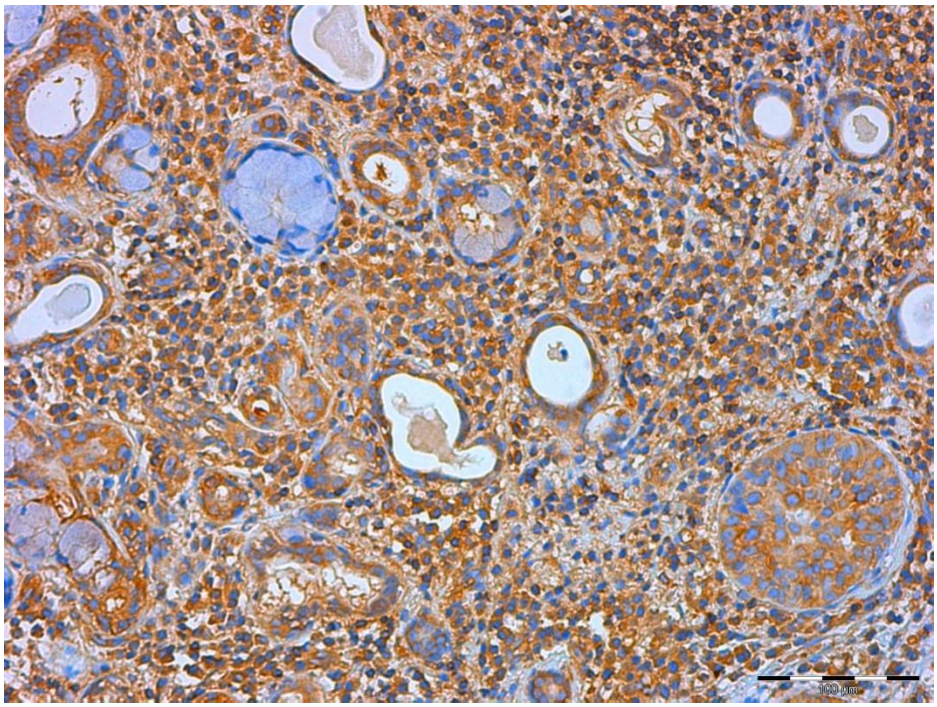

Figure 11.Strong diffuse IL-6 expression in adenoid cystic carcinoma of in oral cavity small salivary gland. IHC X 200

4.2.2 Expression of NFκB in SGC

NFκB expression was positive in 85 out of 92 SGC cases (92.4%), and only 2 control cases (10%). The difference in NFκB expression between SGC and control cases showed a high statistical significance (p<0,01) (Table 18). Figure 12 shows the expression of NFκB in low-grade polymorph adenocarcinoma, and Figure 13 in acinic cell carcinoma.

Table 18. Expression of NFκB in SGC and control

	NFκB Expression		Total
	Negative	Positive	
SGC	7 (7.6%)	85 (92.4%)	92 (100%)
Control	18 (90%)	2 (10%)	20 (100%)
Pearson Chi-Square Value= 64.319, p=0.000			

There was also a highly significant difference in the intensity of NFκB expression (p<0.01). While both positive control cases showed weak NFκB expression, weak expression was found only in 32.6% of SGC cases, while 59.7% showed moderate or strong expression (Table 19).

Table 19. NFκB expression intensity in SGC and control

	NFκB Expression				Total
	Negative	Weak	Moderate	Strong	
SGC	7 (7.6%)	30 (32.6%)	35 (38%)	20 (21.7%)	92 (100%)
Control	18 (90%)	2 (10%)	0 (0%)	0 (0%)	20 (100%)
Pearson Chi-Square Value= 64.857, p=0.000					

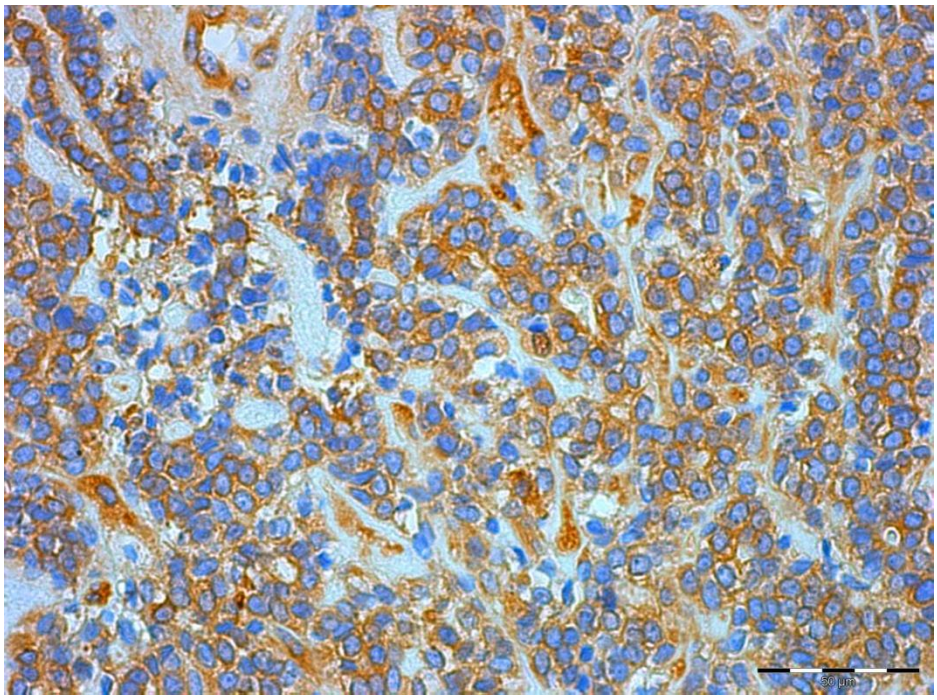

Figure 12.NFκB expression (moderate to strong cytoplasmatic positivity) in low-grade polymorph adenocarcinoma of small nasal salivary gland. IHC X 400

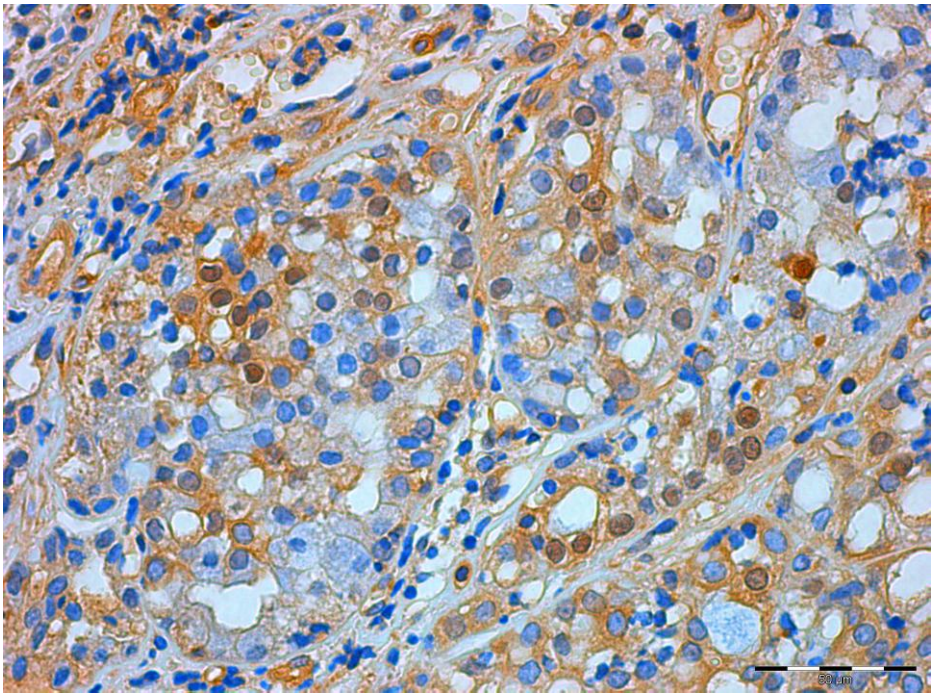

Figure 13.Strong NFkB positivity in acinic cell carcinoma of parotid gland. IHC X 200

4.2.3 Expression of MMP-2 in SGC

MMP-2 expression was positive in 27 out of 92 SGC cases (29.3%), and in 7 control cases (35%). The difference in MMP-2 expression between SGC and control cases did not show a statistical significance ($p>0.05$) (Table 20). Strong focal MMP-2 expression is shown in Figure 12.

Table 20. Expression of MMP-2 in SGC and control

	MMP-2 Expression		Total
	Negative	Positive	
SGC	65 (70.7%)	27 (29.3%)	92 (100%)
Control	13 (65%)	7 (35%)	20 (100%)
Pearson Chi-Square Value= 0.248, p=0.618			

The intensity of MMP-2 expression was weak in all 7 positive control cases. In SGC group 7 out of 27 positive cases showed moderate or strong expression, however due to small number of positive samples this value did not show statistical significance ($p>0.05$) (Table 21).

Table 21. MMP-2 expression intensity in SGC and control

	MMP-2 Expression				Total
	Negative	Weak	Moderate	Strong	
SGC	65(70.7%)	20 (21.7%)	5(5.4%)	2 (2.2%)	92 (100%)
Control	13 (65%)	7 (35%)	0 (0%)	0 (0%)	20 (100%)
Pearson Chi-Square Value= 2.795, p=0.396					

In several SGC cases even when there was no expression of MMP-2 in cancer tissue, a strong MMP-2 expression was noticed in the inflammatory cells in surrounding nonmalignant tissue (Figure 14). Also in several SGC cases the expression of MMP-2 was stronger in the peripheral areas than in the rest of the tumor (Figure 15).

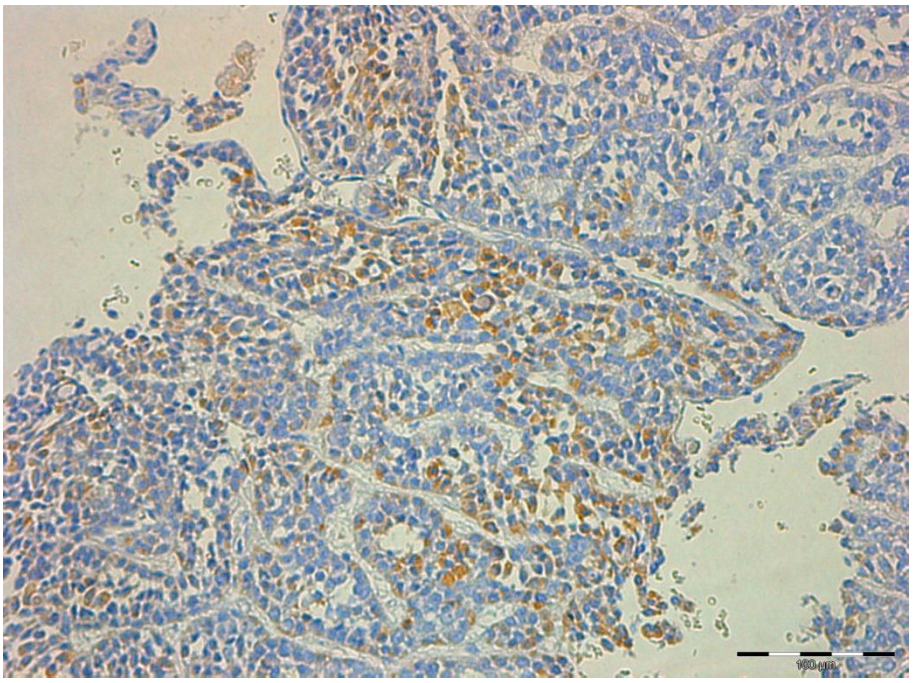

Figure 14. Strong focal MMP2 expression in myoepithelial carcinoma of parotid gland. IHC X 200

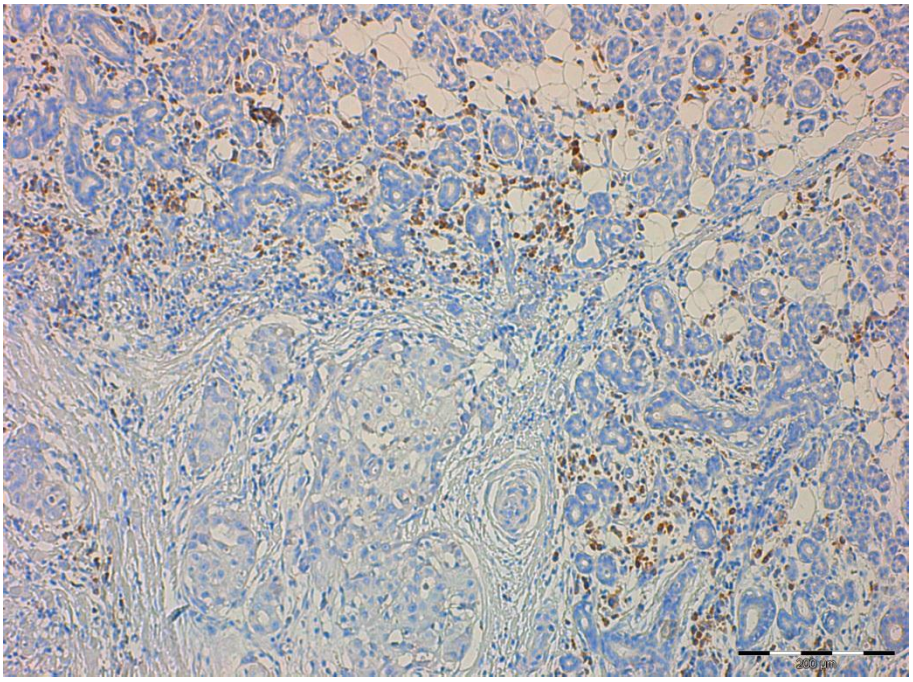

Figure 15. MMP-2 expression in acinic cell carcinoma of parotid gland. MMP-2 is strongly positive in inflammatory cells in surrounding nonmalignant tissue, while it is negative in tumor cells. IHC X 200

4.2.4 Expression of MMP-9 in SGC

MMP-9 expression was positive in 21 out of 92 SGC cases (22.8%), and in 5 control cases (25%). The difference in MMP-9 expression between SGC and control cases did not show a statistical significance ($p>0.05$) (Table 22). Strong diffuse expression in myoepithelial carcinoma is shown in Figure 16.

Table 22. Expression of MMP-9 in SGC and control

	MMP-9 Expression		Total
	Negative	Positive	
SGC	71 (77.2%)	21 (22.8%)	92 (100%)
Control	15 (75%)	5 (25%)	20 (100%)
Pearson Chi-Square=0.044, p=1.000			

The intensity of MMP-2 expression was weak in all 5 positive control cases. In SGC group 4 out of 21 positive cases showed moderate or strong expression, however due to small number of positive samples this value did not show statistical significance ($p>0.05$) (Table 23).

Table 23. MMP-9 expression intensity in SGC and control

	MMP-9 Expression				Total
	Negative	Weak	Moderate	Strong	
SGC	71 (77.2%)	17 (18.5%)	2 (2.2%)	2 (2.2%)	92 (100%)
Control	15 (75%)	5 (25%)	0 (0%)	0 (0%)	20 (100%)
Pearson Chi-Square Value= 1.235, p=0.892					

In cases where MMP-9 expression was positive, MMP-9 was located either only in the peripheral zones of the cancer (Figure 17), or it showed a diffuse distribution, but the intensity of the expression was stronger in the peripheral zones of the tumor (Figure 18).

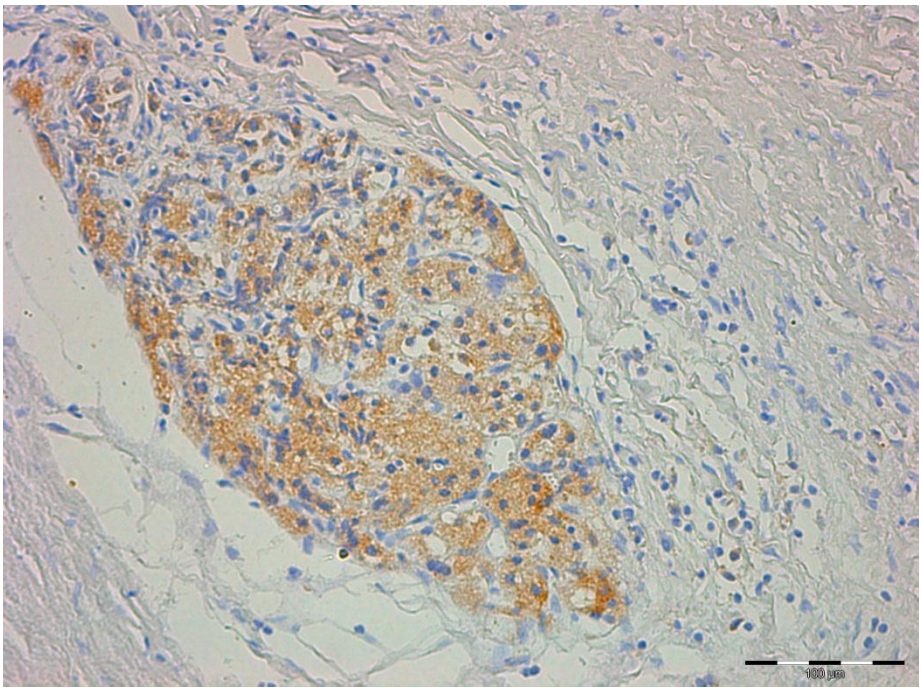

Figure 16.MMP-9 strong expression in myoepithelial carcinoma of the parotid gland. IHC X 200.

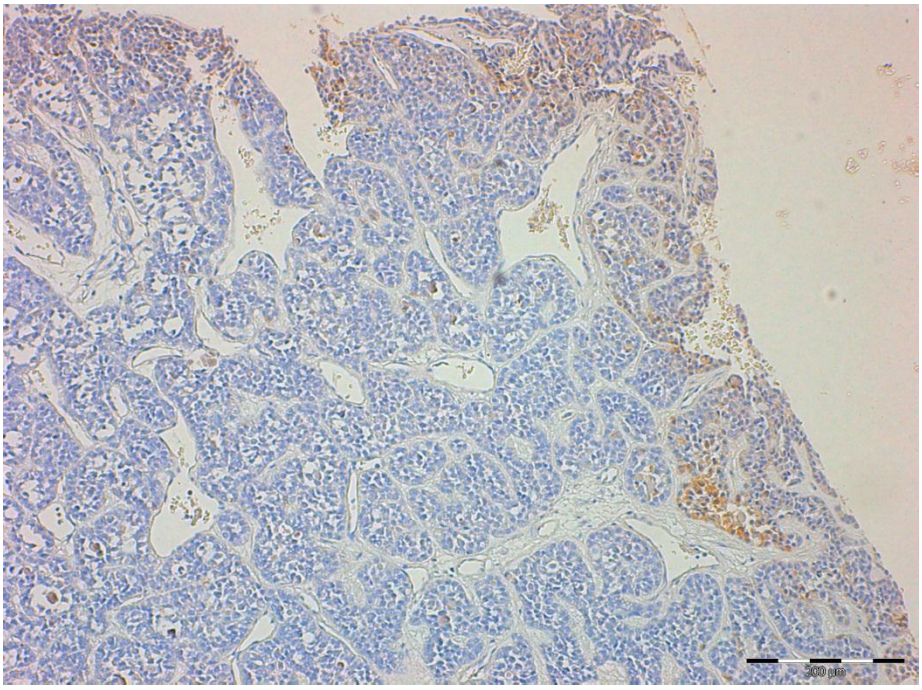

Figure 17.MMP9 moderate expression only in peripheral zones of in myoepithelial carcinoma of parotid gland.IHC X 200.

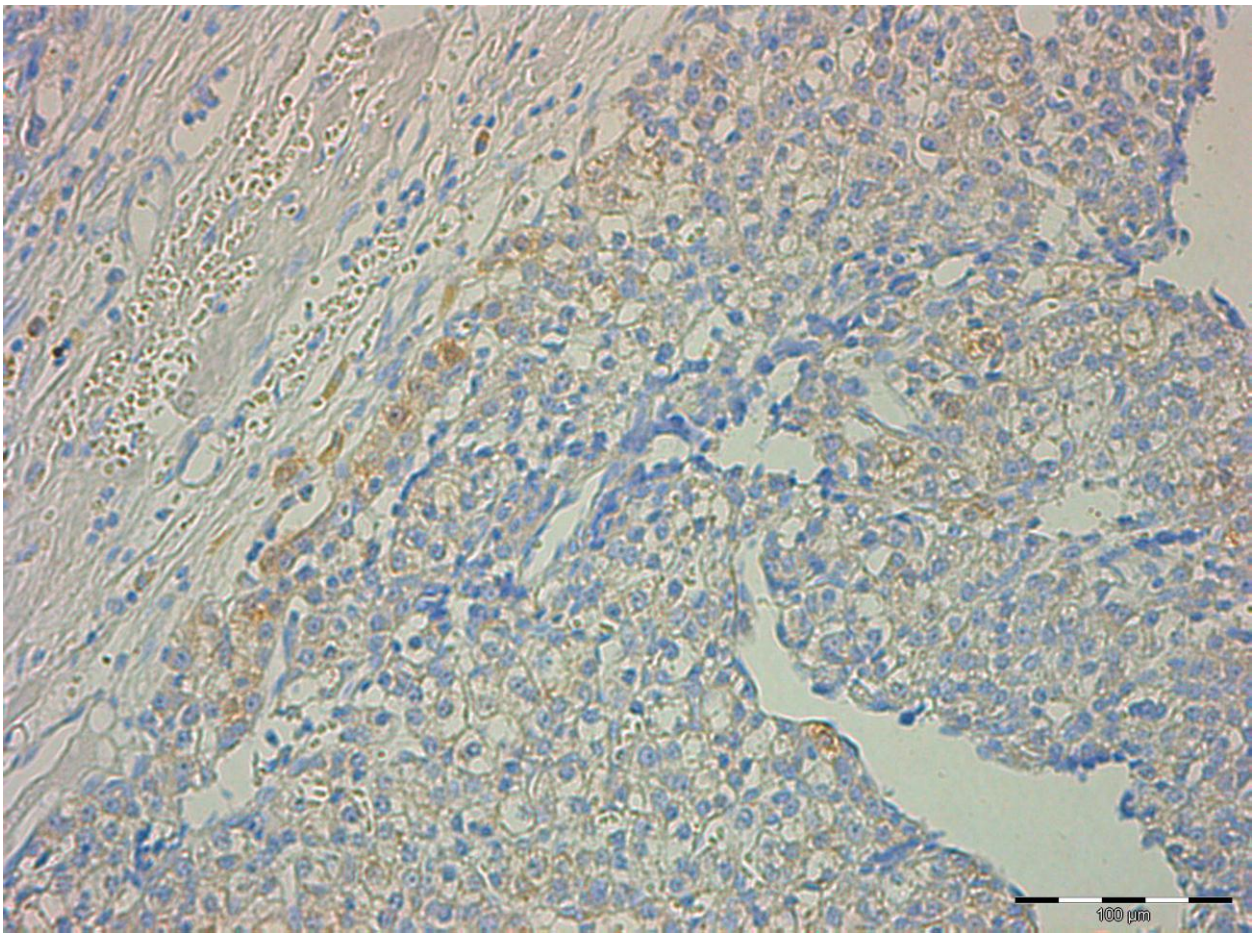

Figure 18. Moderate peripheral MMP-9 positivity and weak diffuse MMP-9 positivity in acinic cell carcinoma of parotid gland. IHC X 200

4.3 Expression of prognostic markers in CMV and EBV infected SGC

4.3.1 Expression of IL-6 in CMV and EBV infected SGC

In 92 SGC cases the expression of IL-6 in CMV infected and non-infected tissues was analyzed. Out of the 27 cases that did not show IL-6 expression, 16 also did not show CMV expression. Out of the 65 cases that showed IL-6 positivity, only 10 did now express CMV antigens, while 55 were positive for CMV. The association between IL-6 expression and the presence of CMV showed a high statistical significance ($p<0.01$) (Table 24).

Table 24. Comparison of IL-6 and CMV antigen expression in SGC

		IL-6 Expression		Total
		Negative	Positive	
CMV Expression	Negative	16 (61.5%)	10 (31.5%)	26 (100%)
	Positive	11 (16,7%)	55 (83.3%)	66 (100%)
Total		27 (29.3%)	65 (70.7%)	92 (100%)
Pearson Chi-Square Value= 18,112, p=0.000				

When analyzing the trend of IL-6 expression intensity in SGC cases, it has been shown that in cases that in did not express CMV antigens, most cases (8/10) showed a weak expression of IL-6. On the other hand, in cases where CMV antigens were present, the majority of cases (33/55) showed a moderate or strong IL-6 expression. (Table 25). The difference in the intensity of IL-6 expression in CMV positive and negative cases showed a high statistical significance ($p<0.01$).

Table 25. Comparison of the intensity of IL-6 and CMV antigen expression in SGC

		IL-6 Expression				Total
		Negative	Weak	Moderate	Strong	
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						

In 92 SGC cases the expression of IL-6 in EBV infected and non-infected tissues was analyzed. Out of the 27 cases that did not show IL-6 expression, 20 also did not show EBV expression. Out of the 65 cases that showed IL-6 positivity, 40 did now express EBV antigens, while 25 were positive for EBV. The association between IL-6 expression and the presence of EBV did not show statistical significance ($p>0.05$) (Table 26).

Table 26. Comparison of IL-6 and EBV antigen expression in SGC

		IL-6 Expression		Total
		Negative	Positive	
EBV Expression	Negative	20 (33.3%)	40 (66.7%)	60 (100%)
	Positive	7 (21.9%)	25 (78.1%)	32 (100%)
Total		27 (29.3%)	65 (70.7%)	92 (100%)
Pearson Chi-Square Value= 1,321, p=0.250				

When analyzing the trend of IL-6 expression intensity in SGC cases, it has been shown that in most cases that in did not express EBV antigens, the expression of IL-6 was weak (39/60 cases). However, the difference in the intensity of IL-6 expression in EBV positive and negative cases did not show a statistical significance ($p>0.05$) (Table 27).

Table 27. Comparison of the intensity of IL-6 and EBV antigen expression in SGC

		IL-6 Expression				Total
		Negative	Weak	Moderate	Strong	
EBV Expression	Negative	20 (33.3%)	19 (31.7%)	15 (25.0%)	6 (10.0%)	60 (100%)
	Positive	7 (21.9%)	11 (34.4%)	6 (18.8%)	8 (25%)	32 (100%)
Total		27 (29.3%)	30 (32.6%)	21 (22.8%)	14 15.2%)	92 (100%)
Linear-by-Linear Association Value= 2.384, p=0.123						

The presence of both CMV and EBV viruses in the same SGC case and the expression of IL-6 was also analyzed (Table 28; Chart 2). Out of 16 cases that did not show expression of neither CMV nor EBV, 10 were also IL-6 negative. Only 1 case out of 22 did not show IL-6 expression when both CMV and EBV were present. These results showed a high statistical significance (p>0.01)

Table 28. Comparison of IL-6 and both CMV and EBV antigen expression in SGC

	IL-6 Expression		Total
	Negative	Positive	
CMV- EBV-	10 (62,5%)	6 (37,5%)	16 (100,0%)
CMV+ EBV-	16 (29,6%)	38 (70,4%)	54 (100,0%)
CMV- EBV+	10 (22,7%)	34 (77,3%)	44 (100,0%)
CMV+ EBV+	1 (4,5%)	21 (95,5%)	22 (100,0%)
Total	27 (29,3%)	65 (70,7%)	92 (100,0%)
Pearson Chi-Square Value= 15.010, p=0.001			

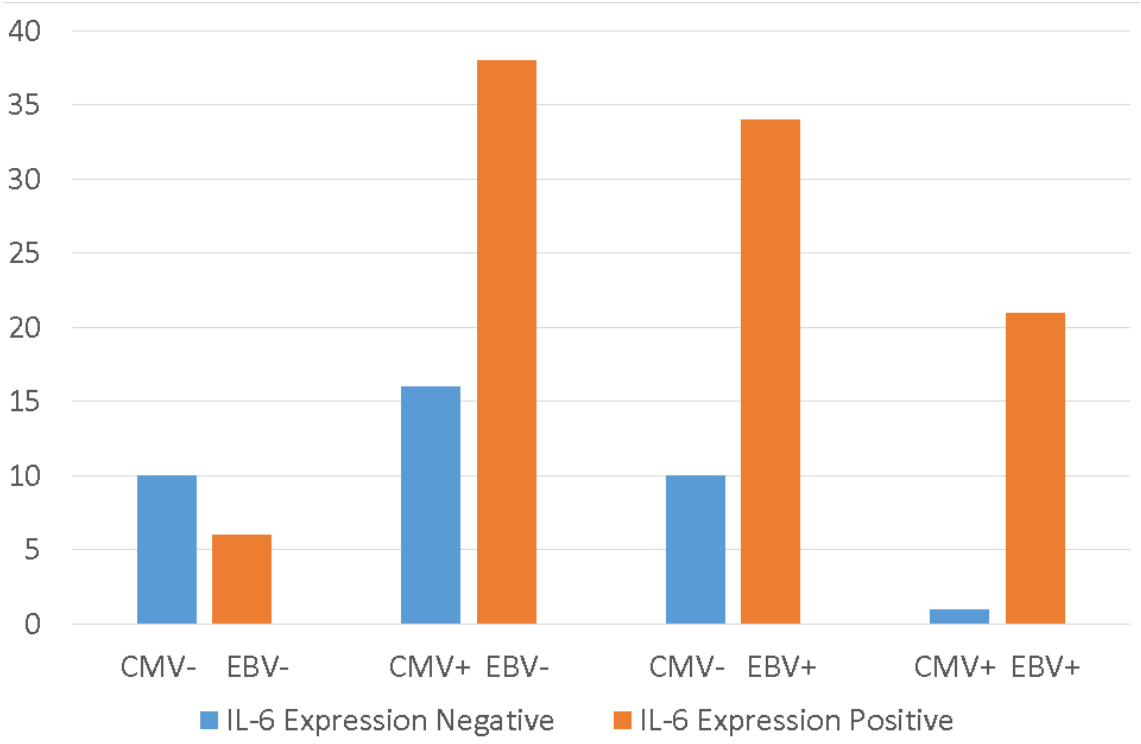

Chart 2. Comparison of IL-6 and both CMV and EBV antigen expression in SGC

4.3.2 Expression of NFκB in CMV and EBV infected SGC

In 92 SGC cases the expression of NFκB in CMV infected and non-infected tissues was analyzed. Out of the 7 cases that did not show NFκB expression, 5 also did not show CMV expression. Out of the 85 cases that showed NFκB positivity, 21 did not express CMV antigens, while 64 were positive for CMV. In 66 cases that were CMV positive, only 2 did not show NFκB expression. The association between NFκB expression and the presence of CMV showed a high statistical significance ($p<0.01$) (Table 29).

Table 29. Comparison of NFκB and CMV antigen expression in SGC

		NFκB Expression		Total
		Negative	Positive	
CMV Expression	Negative	5 (19,2%)	21 (80,8%)	26 (100%)
	Positive	2 (3,0%)	64 (97,0%)	66 (100%)
Total		7 (7,6%)	85 (92,4%)	92 (100%)
Pearson Chi-Square Value= 18,112, p=0.000				

When analyzing the trend of NFκB expression intensity in SGC cases, it has been shown that in cases that in did not express CMV antigens, 13 cases out of 26 cases (13/26) showed a weak expression of NFκB. On the other hand, in cases where CMV antigens were present, most cases (30/66) showed a moderate NFκB expression. (Table 30). The difference in the intensity of NFκB expression in CMV positive and negative cases showed a high statistical significance ($p<0.01$).

Table 30. Comparison of the intensity of NFκB and CMV antigen expression in SGC

		NFκB Expression				Total
		Negative	Weak	Moderate	Strong	
CMV Expression	Negative	5 (19,2%)	13 (50,0%)	5 (19,2%)	3 (11,5%)	26 (100%)
	Positive	2 (3,0%)	17 (25,8%)	30 (45,5%)	17 (25,8%)	66 (100%)
Total		7 (7,6%)	30 (32,6%)	35 (38,0%)	20 (21,7%)	92 (100%)
Linear-by-Linear Association Value= 6.888, p=0.009						

In 92 SGC cases the expression of NFκB in EBV infected and non-infected tissues was analyzed. Out of the 7 cases that did not show NFκB expression, 6 also did not show EBV expression. Out of the 85 cases that showed NFκB positivity, 31 did now express EBV antigens, while 54 were positive for EBV. The association between NFκB expression and the presence of EBV did not show statistical significance (p>0.05) (Table 31).

Table 31. Comparison of NFκB and EBV antigen expression in SGC

		NFκB Expression		Total
		Negative	Positive	
EBV Expression	Negative	6 (10,0%)	54 (90,0%)	60 (100%)
	Positive	1 (3,1%)	31 (96,9%)	32 (100%)
Total		7 (7,6%)	85 (92,4%)	92 (100%)
Pearson Chi-Square Value= 1,403, p=0.236				

When analyzing the trend of NFκB expression intensity in SGC cases, it has been shown that in cases that in did not express EBV antigens, most cases, 24 out of 60, showed a moderate expression of NFκB. In cases where EBV antigens were present, although 1 case showed negative NFκB expression, the distribution among weak, moderate and strong NFκB expression was similar (11:11:9 cases respectively). (Table 32). The difference in the intensity of NFκB expression in EBV positive and negative cases did not show statistical significance (p>0.05).

Table 32. Comparison of the intensity of NFκB and EBV antigen expression in SGC

		NFκB Expression				Total
		Negative	Weak	Moderate	Strong	
EBV Expression	Negative	6 (10,0%)	19 (31,7%)	24 (40,0%)	11 (18,3%)	60 (100%)
	Positive	1 (3,1%)	11 (34,4%)	11 (34,4%)	9 (28,1%)	32 (100%)
Total		7 (7,6%)	30 (32,6%)	35 (38,0%)	20 (21,7%)	92 (100%)
Linear-by-Linear Association Value= 1.149, p=0.516						

The presence of both CMV and EBV viruses in the same SGC case and the expression of NFκB was also analyzed (Table 33; Chart 3). Out of 16 cases that did not show expression of neither CMV nor EBV, 4 were also NFκB negative. In 22 cases where both CMV and EBV were present, all of them showed NFκB positivity. These results showed a statistical significance ($p<0.05$).

Table 33. Comparison of NFκB and both CMV and EBV antigen expression in SGC

	NFκB Expression		Total
	Negative	Positive	
CMV-, EBV-	4 (25,0%)	12 (75,0%)	16 (100,0%)
CMV+ EBV-	2 (4,5%)	42 (95,5%)	44 (100,0%)
CMV- EBV+	1 (10,0%)	9 (90,0%)	10 (100,0%)
CMV+, EBV+	0 (0,0%)	22 (100,0%)	22 (100,0%)
Total	7 (7,6%)	85 (92,4%)	92 (100,0%)
Pearson Chi-Square Value= 9.020, p=0.018			

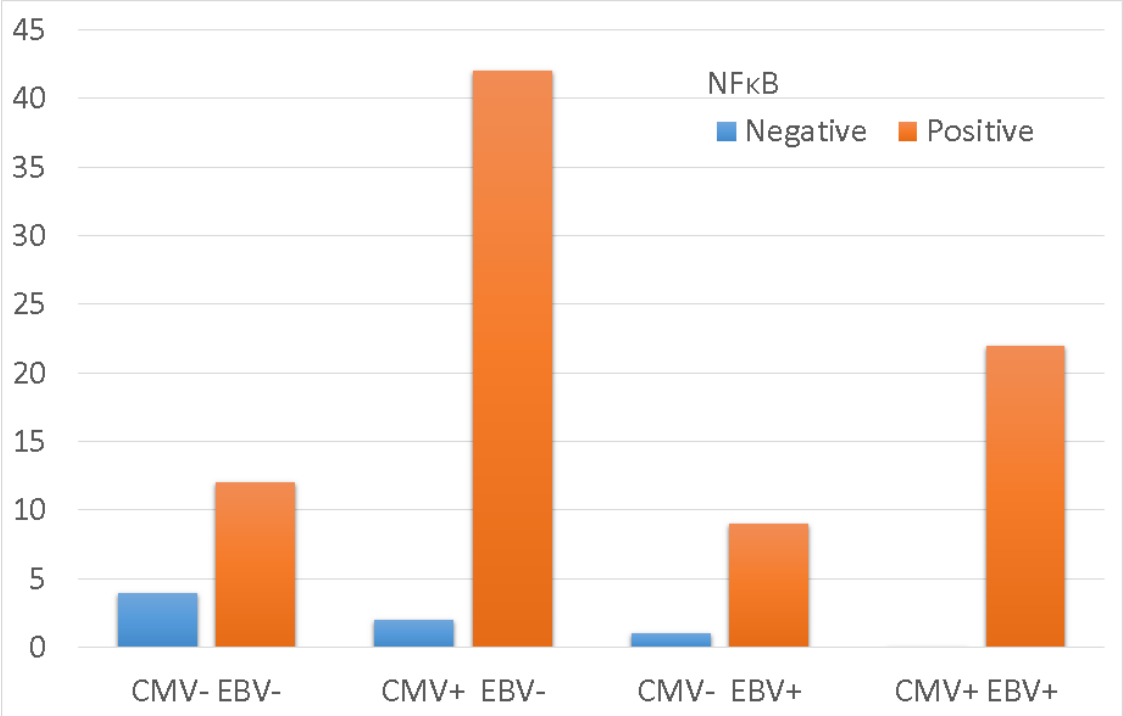

Chart 3. Comparison of NFκB and both CMV and EBV antigen expression in SGC

4.3.3 Expression of MMP-2 in CMV and EBV infected SGC

In 92 SGC cases the expression of MMP-2 in CMV infected and non-infected tissues was analyzed. Out of the 26 cases that did not show CMV expression, 25 also did not show MMP-2 expression. On the other hand, out of the 66 cases that showed MMP-2 positivity, 40 were CMV negative. The association between MMP-2expression and the presence of CMV showed a high statistical significance ($p<0.01$) (Table 34).

Table 34. Comparison of MMP-2 and CMV antigen expression in SGC

		MMP-2 Expression		Total
		Negative	Positive	
CMV Expression	Negative	25 (96,2%)	1 (3,8%)	26 (100%)
	Positive	40 (60,6%)	26 (39,4%)	66 (100%)
Total		65 (70.7%)	27 (29.3%)	92 (100%)
Pearson Chi-Square Value= 11,367, p=0.001				

When analyzing the trend of MMP-2 expression intensity in SGC cases, it has been shown that in cases that in did not express CMV antigens, the only case of MMP-2 positivity showed weak expression. In cases where both CMV and MMP-2 antigens were present, MMP-2 expression is most commonly weak (19/26), however, there were 7 cases where the MMP-2 expression was moderate or strong (Table 35). The difference in the intensity of MMP-2 expression in CMV positive and negative cases showed a high statistical significance ($p<0.01$).

Table 35. Comparison of the intensity of MMP-2and CMV antigen expression in SGC

		MMP-2 Expression				Total
		Negative	Weak	Moderate	Strong	
CMV Expression	Negative	25 (96,2%)	1 (3,8%)	0 (0,0%)	0 (0,0%)	26 (100%)
	Positive	40 (60,6%)	19 (28,8%)	5 (7,6%)	2 (3,0%)	66 (100%)
Total		65 (70,7%)	20 (21,7%)	5 (5,4%)	2 (2,2%)	92 (100%)
Linear-by-Linear Association Value= 9.350, p=0.001						

In 92 SGC cases the expression of MMP-2 in EBV infected and non-infected tissues was analyzed. Out of the 65 cases that did not show MMP-2 expression, 44 also did not show EBV expression. Out of the 27 cases that showed MMP-2 positivity, 16 did not express EBV antigens. The association between MMP-2 expression and the presence of EBV did not show statistical significance (p>0.05) (Table 36).

Table 36. Comparison of MMP-2 and EBV antigen expression in SGC

		MMP-2 Expression		Total
		Negative	Positive	
EBV Expression	Negative	44(73,3%)	16 (26.7%)	60 (100%)
	Positive	21 (65,6%)	11 (34.4%)	32 (100%)
Total		65 (70,7%)	27 (29.3%)	92 (100%)
Pearson Chi-Square Value= 0.598, p=0.439				

When analyzing the trend of MMP-2 expression intensity in SGC cases, it has been shown that in the 16 MMP-2 positive cases that in did not express EBV antigens, most cases (12/16) showed weak expression of MMP-2. Also, in MMP-2 positive cases where EBV antigens were present, most cases shoed weak expression of MMP-2 (8/ 11) (Table 37). The difference in the intensity of MMP-2expression in EBV positive and negative cases did not show statistical significance (p>0.05).

Table 37. Comparison of the intensity of MMP-2 and EBV antigen expression in SGC

		MMP-2 Expression				Total
		Negative	Weak	Moderate	Strong	
EBV Expression	Negative	44 (73,3%)	12 (20,0%)	3 (5,0%)	1 (1,7%)	60 (100%)
	Positive	21 (65,6%)	8 (25,0%)	2 (6,2%)	1 (3,1%)	32 (100%)
Total		65 (70,7%)	20 (21,7%)	5 (5,4%)	2 (2,2%)	92 (100%)
Linear-by-Linear Association Value= 0.610, p=0.539						

The presence of both CMV and EBV viruses in the same SGC case and the expression of MMP-2 were also analyzed (Table 38). Out of 16 cases that did not show expression of neither CMV nor EBV, only 1 showed MMP-2 positivity. In 22 cases where both CMV and EBV were present, half of them showed MMP-2 positivity (11/22). These results showed a statistical significance ($p<0.05$).

Table 38. Comparison of MMP-2 and both CMV and EBV antigen expression in SGC

	MMP-2 Expression		Total
	Negative	Positive	
CMV- EBV-	15 (93,8%)	1 (6,2%)	16 (100,0%)
CMV+ EBV-	29 (65,9%)	15 (34,1%)	44 (100,0%)
CMV- EBV+	10 (100,0%)	0 (0,0%)	10 (100,0%)
CMV+ EBV+	11 (50,0%)	11 (50,0%)	22 (100,0%)
Total	65 (70,7%)	27 (29,3%)	92 (100,0%)
Pearson Chi-Square Value= 8.706, p=0.013			

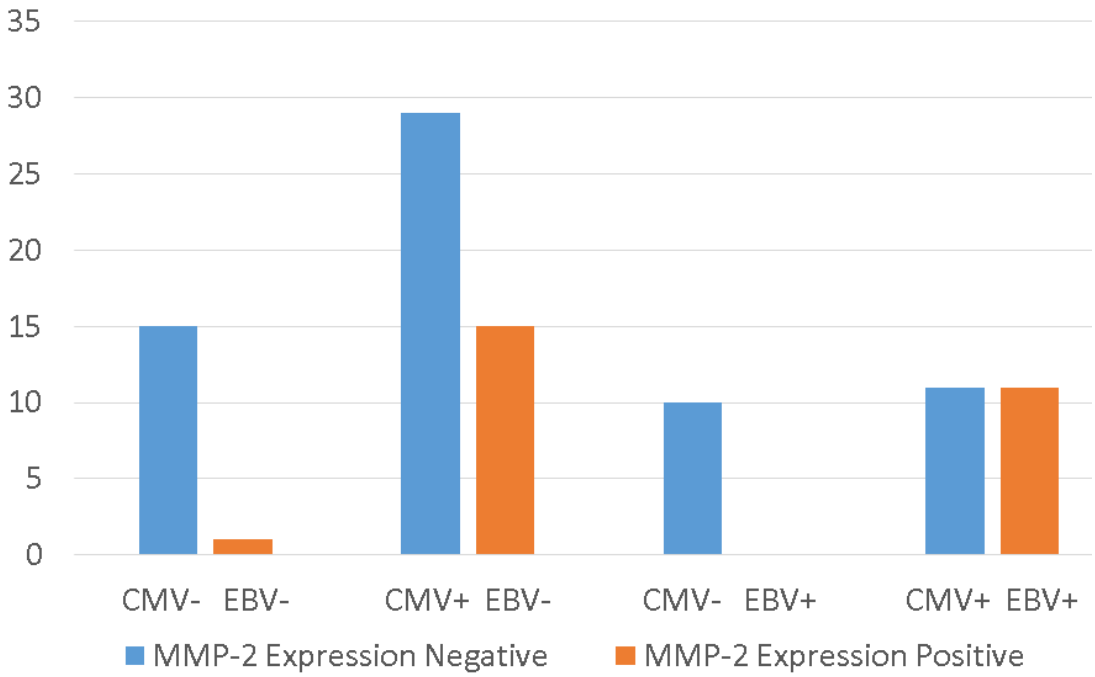

Chart 4.Comparison of MMP-2 and both CMV and EBV antigen expression in SGC

4.3.4 Expression of MMP-9 in CMV and EBV infected SGC

In 92 SGC cases the expression of MMP-9 in CMV infected and non-infected tissues was analyzed. Out of the 26 cases that did not show CMV expression, 22 also did not show MMP-9 expression. On the other hand, out of the 66 cases that showed MMP-9 positivity, 49 were CMV negative. The association between MMP-9expression and the presence of CMV did not show (p>0.05) (Table 39).

Table 39. Comparison of MMP-9 and CMV antigen expression in SGC

		MMP-9 Expression		Total
		Negative	Positive	
CMV Expression	Negative	22 (84,6%)	4 (15,4%)	26 (100%)
	Positive	49 (74,2%)	17 (25,8%)	66 (100%)
Total		71 (77,2%)	21 (22,8%)	92 (100%)
Pearson Chi-Square Value= 1,139, p=0.286				

When analyzing the trend of MMP-9 expression intensity in SGC cases, it has been shown that in cases that in did not express CMV antigens, the cases that were MMP-9 positive showed mostly weak expression (3/4). In cases where both CMV and MMP-9 antigens were present, MMP-9 expression was also most commonly weak (14/17) (Table 40). The difference in the intensity of MMP-9 expression in CMV positive and negative cases did not show statistical significance (p>0.05).

Table 40. Comparison of the intensity of MMP-9 and CMV antigen expression in SGC

		MMP-9 Expression				Total
		Negative	Weak	Moderate	Strong	
CMV Expression	Negative	22 (84,6%)	3 (11,5%)	0 (0,0%)	1 (3,8%)	26 (100%)
	Positive	49 (74,2%)	14 (21,2%)	2 (3,0%)	1 (1,5%)	66 (100%)
Total		71 (77,2%)	17 (18,5%)	2 (2,2%)	2 (2,2%)	92 (100%)
Linear-by-Linear Association Value= 0.370, p=0.602						

In 92 SGC cases the expression of MMP-9 in EBV infected and non-infected tissues was analyzed. Out of the 71 cases that did not show MMP-9 expression, 48 also did not show EBV expression. Out of the 21 cases that showed MMP-9 positivity, 12 did not express EBV antigens. The association between MMP-2 expression and the presence of EBV did not show statistical significance (p>0.05) (Table 41)

Table 41. Comparison of MMP-9 and EBV antigen expression in SGC

		MMP-9 Expression		Total
		Negative	Positive	
EBV Expression	Negative	48 (80%)	12 (20%)	60 (100%)
	Positive	23 (71.9%)	9 (28.1%)	32 (100%)
Total		71 (77.2%)	21 (22.8%)	92 (100%)
Pearson Chi-Square Value= 0.782, p=0.377				

When analyzing the trend of MMP-9 expression intensity in SGC cases, it has been shown that in the MMP-9 positive cases that did not express EBV antigens, most cases (11/12) showed weak expression of MMP-9. Also, in MMP-9 positive cases where EBV antigens were present, most cases showed weak expression of MMP-2 (6/9) (Table 42). The difference in the intensity of MMP-9 expression in EBV positive and negative cases did not show statistical significance (p>0.05).

Table 42. Comparison of the intensity of MMP-9 and EBV antigen expression in SGC

		MMP-9 Expression				Total
		Negative	Weak	Moderate	Strong	
EBV Expression	Negative	48 (80,0%)	11 (18,3%)	0 (0,0%)	1 (1,7%)	60 (100%)
	Positive	23 (71,9%)	6 (18,8%)	2 (6,2%)	1 (3,1%)	32 (100%)
Total		71 (77,2%)	17 (18,5%)	2 (2,2%)	2 (2,2%)	92 (100%)
Linear-by-Linear Association Value= 1.619, p=0.222						

The presence of both CMV and EBV viruses in the same SGC case and the expression of MMP-9 were also analyzed (Table 43; Chart 5). Out of 16 cases that did not show expression of neither CMV nor EBV, only 3 showed MMP-9 positivity. In 22 cases where both CMV and EBV were present, 8 showed MMP-9 positivity. These results did not show a statistical significance ($p>0.05$).

Table 43. Comparison of MMP-9 and both CMV and EBV antigen expression in SGC

	MMP-9 Expression		Total
	Negative	Positive	
CMV-, EBV-	13 (81.2%)	3 (18.8%)	16 (100,0%)
CMV+ EBV-	35 (79,5%)	9 (20,5%)	44 (100,0%)
CMV- EBV+	8 (80,0%)	2 (20,0%)	10 (100,0%)
CMV+ EBV+	14 (63.6%)	8 (36.4%)	22 (100,0%)
Total	71 (77,2%)	21 (22.8%)	92 (100,0%)
Pearson Chi-Square Value= 8.706, p=0.083			

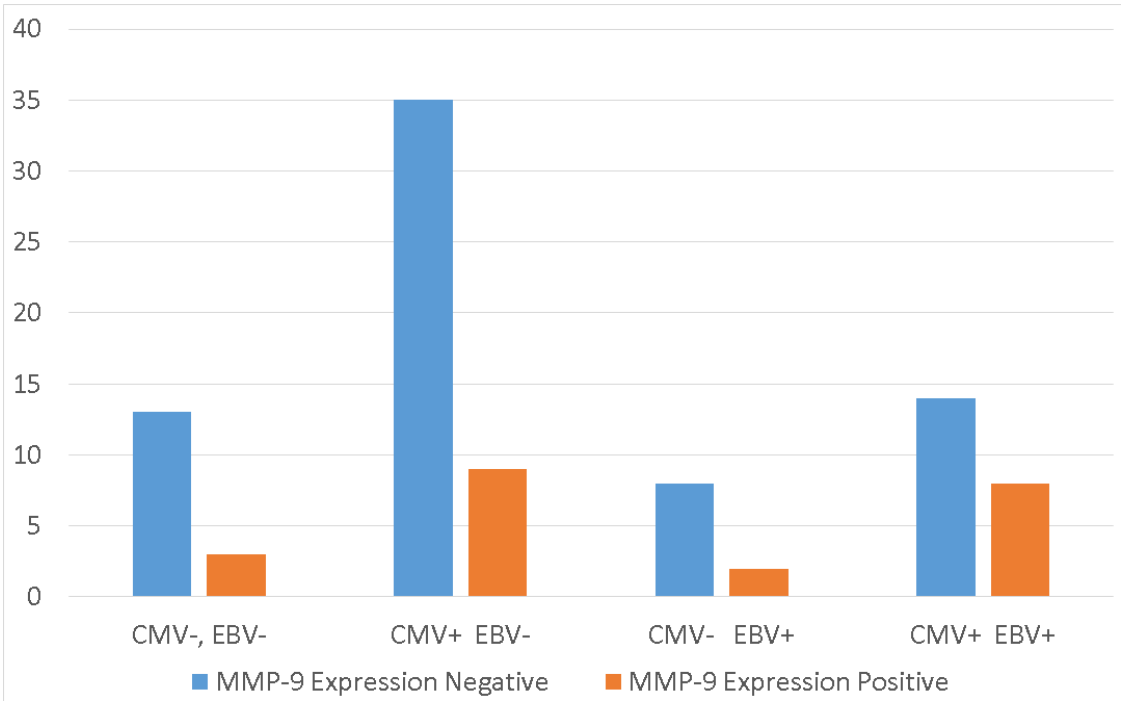

Chart 5. Comparison of MMP-9 and both CMV and EBV antigen expression in SGC

4.4 The genotype and allele frequencies of IL-6, MMP-2, and MMP-9 polymorphisms

The analysis concerning DNA polymorphisms was performed on all 93 SGC cases. The control group consisted of buccal swabs from 100 healthy volunteers, matching the study group in sex and age. Observed and expected frequencies for all 4 polymorphic cites met the Hardy-Weinberg equilibrium (HWE) criteria.

4.4.1 The genotype and allele frequencies of IL6 -174G>C polymorphism

A significant difference in genotype frequencies was also found between the SGC group and controls for the IL6 -174G>C polymorphism (Table 44). Heterozygous carriers of the variant allele C, as well as the homozygotes of the C allele have an increased risk of SGC development compared to wild type homozygotes (CC). ($p<0.001$). This increase in susceptibility was also confirmed using the dominant model (OR 3.77, 95% CI 1.91-7.44, $p<0.001$for the CT+TT genotypes).

Table 44: Genotype distribution and allelic frequencies (p (%) and q (%)) and logistic regression analysis data for IL-6 -174G>C

IL-6 -174G>C	SGC (Σ=93)	Controls (Σ=100)	OR	95% CI	P
GG	15	42	REF		
GC	44	32	3.85	1.83-8.11	<0.001*
CC	34	26	3.66	1.68-7.99	<0.001*
GC+CC	78	58	3.77	1.91-7.44	<0.001*
p (%)	40	58	REF		
q (%)	60	42	2.07	1.18-3.64	0.01*
OR – odds ratio; 95% CI – 95% confidence interval; p – Pearson χ^2 or Fisher exact test two-sided value, * significant at <0.05					

4.4.2 The genotype and allele frequencies of MMP-2 -1306 C>T polymorphism

Genotype and allele frequencies along with risk estimates of MMP-2 -1306 C>T polymorphism are shown in Table 45. The T allele for the MMP-2 -1306 C>T polymorphism exhibits its effect in heterozygous carriers, leading to increased risk of SGC development (OR 1.98, 95% CI 1.07-3.65, P=0.03). In addition, according to the dominant model, individuals with CT+TT genotypes had a 2-fold increased risk of developing the SGC (p=0.02).

Table 45. Genotype distribution and allelic frequencies (p (%) and q (%)) and logistic regression analysis data for MMP-2 -1306C>T

MMP-2 -1306 C>T	SGC (Σ=93)	Controls (Σ=100)	OR	95% CI	P
CC	35	55	REF		
CT	44	35	1.98	1.07-3.65	0.03*
TT	14	10	2.2	0.88-5.50	0.09
CT+TT	58	45	2.03	1.14-3.60	0.02*
p (%)	61	73	REF		
q (%)	39	27	1.73	0.95-3.14	0.07

OR – odds ratio; 95% CI – 95% confidence interval; p – Pearson χ^2 or Fisher exact test two-sided value, * significant at <0.05

4.4.3 The genotype and allele frequencies of MMP2 -1575 G>A polymorphism

Genotype and allele frequencies along with risk estimates of MMP2 -1575 G>A polymorphism are shown in Table 46. When the dominant model is applied for the MMP2 -1575 G>A, individuals with GA+AA genotypes exhibited a 1.77-fold increase in cancer risk, but only with borderline significance (P=0.049).

Table 46. Genotype distribution and allelic frequencies (p (%) and q (%)) and logistic regression analysis data for MMP-2 -1575 G>A

MMP-2 -1575 G>A	SGC (Σ=93)	Controls (Σ=100)	OR	95% CI	P
GG	38	55	REF		
GA	39	35	1.61	0.87-2.99	0.13
AA	16	10	2.3	0.95-5.65	0.06
GA+AA	55	45	1.77	1.00-3.13	0.049*
p (%)	62	74	REF		
q (%)	38	26	1.74	0.96-3.19	0.07

OR – odds ratio; 95% CI – 95% confidence interval; p – Pearson $\chi 2$ or Fisher exact test two-sided value, * significant at <0.05

4.4.4 The genotype and allele frequencies of MMP-9 -1562 C>T polymorphism

Genotype and allele frequencies along with risk estimates of MMP-9 -1562 C>T polymorphism are shown in Table 47. A significant difference in genotype frequencies was also found between the SGC group and controls for the MMP-9 -1562 C>T polymorphism. Heterozygous carriers of the variant allele T had roughly a two-fold increase in susceptibility for SGC compared to wild type homozygotes (CC). (p=0.02). This increase in susceptibility was also confirmed using the dominant model (OR 2.04, 95% CI 1.12-3.73, p=0.02 for the CT+TT genotypes)

Table 47. Genotype distribution and allelic frequencies (p (%) and q (%)) and logistic regression analysis data for MMP-9 -1562C>T polymorphism

MMP-9 -1562 C>T	SGC (Σ=93)	Controls (Σ=100)	OR	95% CI	P
CC	53	73	REF		
CT	37	25	2.04	1.10-3.78	0.02*
TT	3	2	2.07	0.33-12.80	0.65
CT+TT	40	27	2.04	1.12-3.73	0.02*
p (%)	77	86	REF		
q (%)	23	14	1.83	0.88-3.82	0.10

OR – odds ratio; 95% CI – 95% confidence interval; p – Pearson χ^2 or Fisher exact test two-sided value, * significant at <0.05

4.5 Comparison between the genotype and protein expression for IL-6, MMP-2 and MMP9

The prevalence and percentage of each genotype for the IL-6 -174G>C polymorphism in the group of IL-6 positive and IL-6 negative cases is given in Table 48 and Chart 6. The results show that there is no association between IL-6 -174G>C genotypes and IL-6 expression.

Table 48.IL-6 -174G>C genotype distribution compared to IL-6 expression

		IL-6 expression		Total
		Negative	Positive	
IL-6 -174G>C	GG	6 (40,0%)	9 (60,0%)	15 (100,0%)
	GC	12 (27,9%)	31 (72,1%)	43 (100,0%)
	CC	9 (26,5%)	25 (73,5%)	34 (100,0%)
Total		27 (29,3%)	65 (70,7%)	92 (100,0%)
Pearson Chi-Square Value= 1.000, p=0.607				

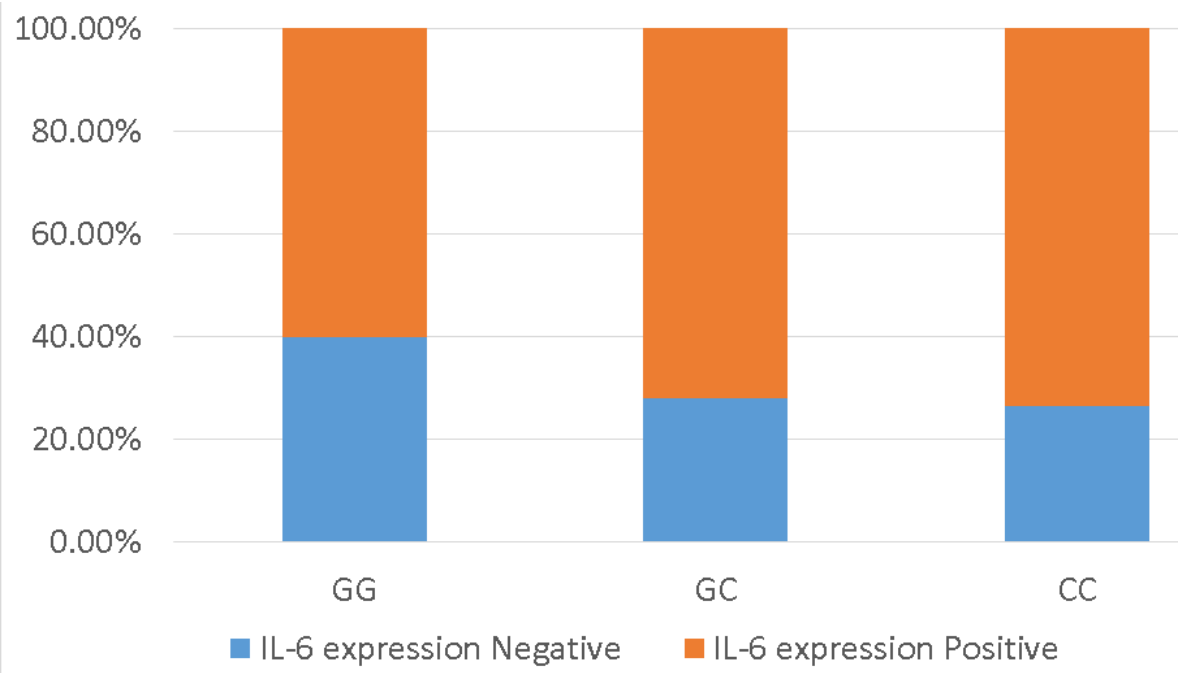

Chart 6. IL -174G>C genotype distribution compared to IL-6 expression

The prevalence and percentage of each genotype for the MMP-2 -1306C>T polymorphism in the group of MMP-2 positive and MMP-2 negative cases is given in Table 49 and Chart 7. The results show that there is no association between MMP-2 -1306C>T genotypes and MMP-2 expression.

Table 49. MMP2 -1306C>T genotype distribution compared to MMP-2 expression

		MMP-2 expression		Total
		Negative	Positive	
MMP2 -1306C>T	CC	24 (70,6%)	10 (29,4%)	34 (100,0%)
	CT	32 (72,7%)	12 (27,3%)	44 (100,0%)
	TT	9 (64,3%)	5 (35,7%)	14 (100,0%)
Total		65 (70,7%)	27 (29,3%)	92 (100,0%)
Pearson Chi-Square Value= 0.365, p=0.833				

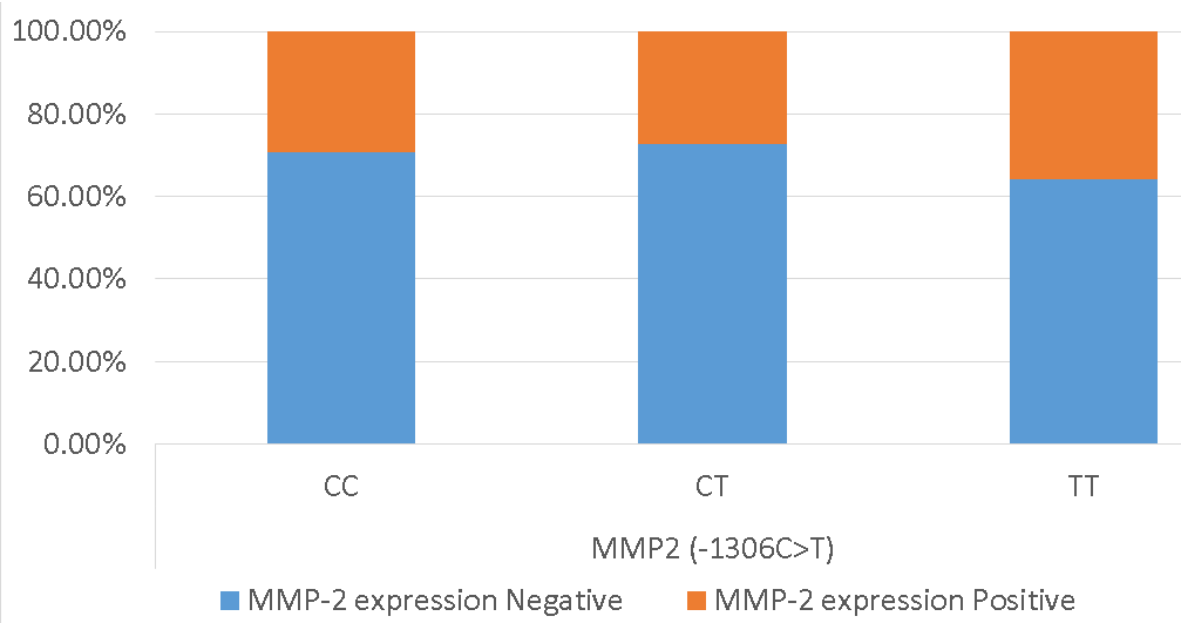

Chart 7. MMP2 -1306C>T genotype distribution compared to MMP-2 expression

The prevalence and percentage of each genotype for the MMP-2 -1575G>A polymorphism in the group of MMP-2 positive and MMP-2 negative cases is given in Table 50 and Chart 8. The results show that there is no association between MMP-2 -1575G>A genotypes and MMP-2 expression.

Table 50. MMP2 -1575G>A genotype distribution compared to MMP-2 expression

		MMP-2 expression		Total
		Negative	Positive	
MMP2 -1575G>A	GG	26 (68,4%)	12 (31,6%)	38 (100,0%)
	GA	27 (71,1%)	11 (28,9%)	38 (100,0%)
	AA	12 (75,0%)	4 (25,0%(	16 (100,0%)
Total		65 (70,7%)	27 (29,3%)	92 (100,0%)
Pearson Chi-Square Value= 0.240, p=0.887				

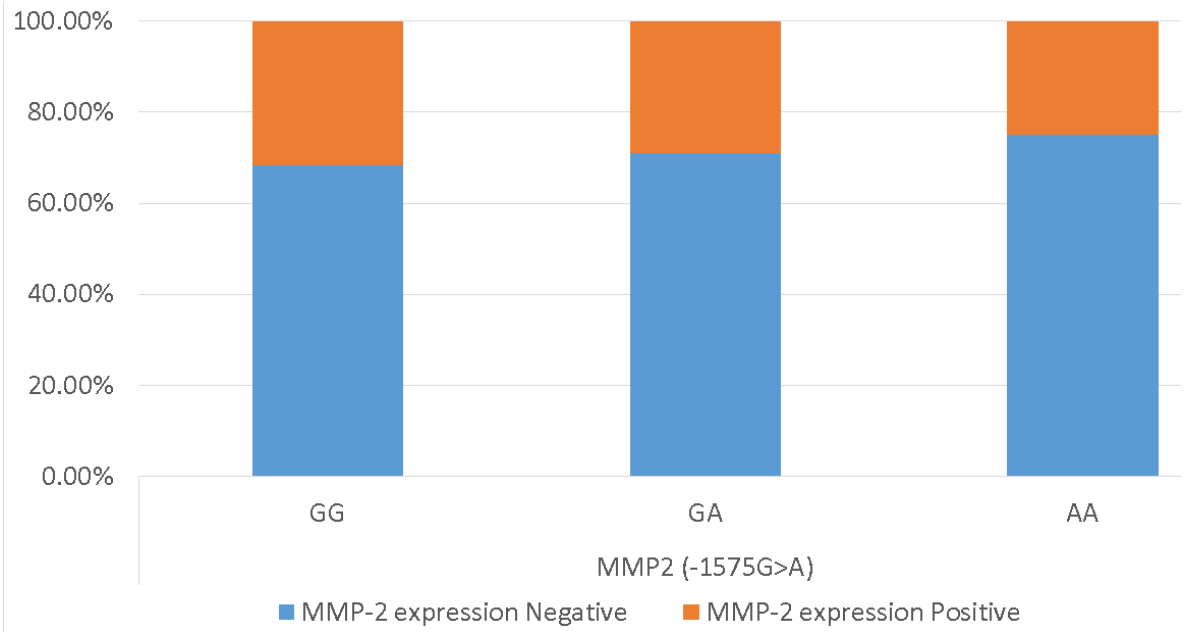

Chart 8. MMP2 -1575G>A genotype distribution compared to MMP-2 expression

The prevalence and percentage of each genotype for the MMP-9 -1562C>T polymorphism in the group of MMP-9 positive and MMP-9 negative cases is given in Table 51 and Chart 9. The results show that the CC genotype has a higher expression of MMP-9 than the other genotypes, however the difference shows only borderline significance (p= 0.49)

Table 47. MMP-9 -1562C>T genotype distribution compared to MMP-9 expression

		MMP-9 expression		Total
		Negative	Positive	
MMP-9 -1562C>T	CC	37 (68,5%)	17 (31,5%)	54 (100,0%)
	CT	31 (88,6%)	4 (11,4%)	35 (100,0%)
	TT	3 (100,0%)	0 (0,0%)	3 (100,0%)
Total		71 (77,2%)	21 (22,8%)	92 (100,0%)
Pearson Chi-Square Value= 5,765, p=0.049				

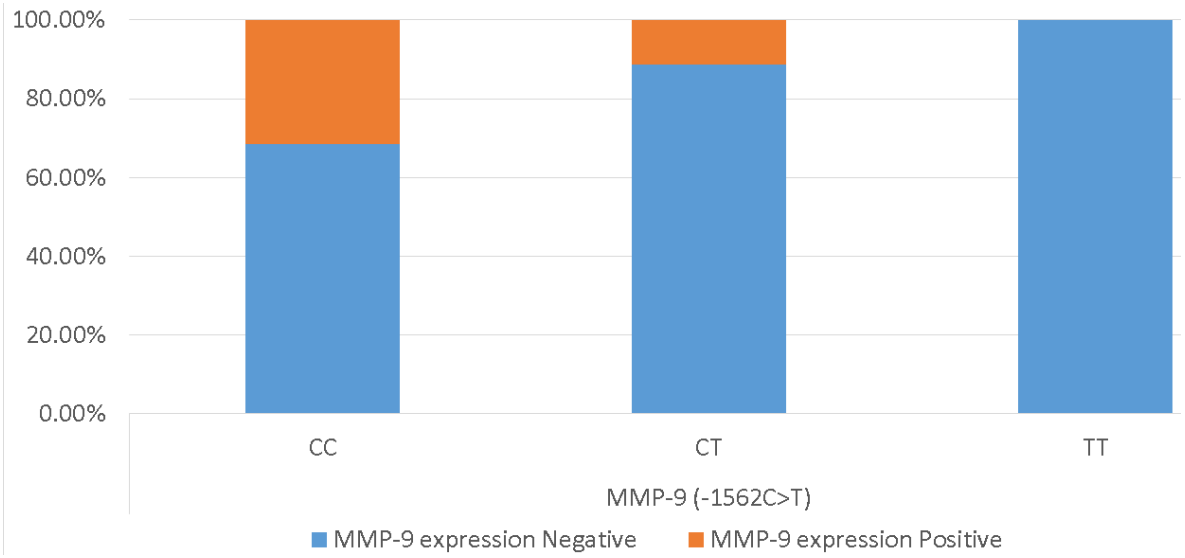

Chart 9. MMP9 -1562C>T genotype distribution compared to MMP-9 expression

5. DISCUSSION

Salivary gland cancer (SGC) is rare, however in the last couple of decades the incidence shows an increased trend, particularly among men where an annual increase in incidence of 1.1% is noticed (Horn-Ross, 1997). In our biopsy material, obtained from at the Clinic of Otorhinolaryngology and Maxillofacial Surgery of the Clinical Center of Serbia in a 10 year period, we have diagnosed only 93 cases of SGC. Even though epidemiological data suggests that females are affected more often than males, in our study we have found a slightly higher percentage of male patients 52.7%.

The average age of SGC patients was 60 years, and the majority of cases were in the seventh decade, which is higher than the global average age of 47 years. However, in our population the most common histologic type with a prevalence of 38% is adenoid cystic carcinoma, which occurs more often in patients over 60 years, as opposed to mucoepidermoid cancer, which is reported to be the most common histologic type of SGC in majority of studies (Barnes, 2005). In our study, mucoepidermoid cancer was the fifth most common with a prevalence of 8,7%, after adenoid cystic carcinoma, myoepithelial carcinoma, polymorphous low-grade adenocarcinoma, and adenocarcinoma NOS. Some histological types are extremely rare in our population. We have registered only one case of low-grade cribriform cystadenocarcinoma, mucinous adenocarcinoma and oncocytic carcinoma in this 10 year period, and some types we did not diagnose at all.

The most common site where SGC occurred in our study was the parotid gland (41,3%), which corresponds to the epidemiological data from other studies. The second most common localization of SGC in other studies was reported to be the submandibular gland (Ellis, Auclair and Gnepp, 1991). However, in our population we have noticed a higher prevalence of SGC in minor salivary glands in the epipharynx (32,6%) and oral cavity (18,5%) than in submandibular gland (7,6%).

The etiology of SGC is still unknown, although a correlation has been shown between the occurrence of SGC and some environmental factors, such as ionizing radiation (Metso, et al., 2007), direct contact with asbestos (Johnson, 2011), rubber during manufacturing (Mancuso and Brennan, 1970) or nickel compounds (Horn-Ross, Ljung and Morrow, 1997).

The role of CMV and EBV in SGC

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 gastric cancer or cervical cancer. Even 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 (Colotta, 2009) (Grivennikov, 2010). It is known that infectious agents, primarily viruses, are directly involved in the development of several head and neck malignancies. High-risk human papillomaviruses (HPVs) have been detected as etiologic agents in head and neck squamous cell carcinomas (Gillison, et al., 2000), and EBV is found to cause undifferentiated carcinoma of nasopharyngeal type in Asian population (Shanmugaratnam and Sobin, 1978) (Chien, et al., 2001).

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 (Bishop, Valle Oseguera and Spencer, 2015) (Richardson, et al., 2015) (Söderberg-Nauclér and Johnsen, 2015) (Cobbs, Matlaf and Harkins, 2014) (Arastefar, et al., 2015) (Samanta, et al., 2003) (Giuliani, Jaxmar and Casadio, 2007). 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 (Soroceanu and Cobbs, 2011) (Slinger, et al., 2011).

After primary exposure and infection, CMV establishes a latent infection and a lifelong persistence. During latency, the viral genome is episomal form in the nucleus, and the majority of the viral genes are not expressed. 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 enhancer/promoter is regulated by coordinated expression of various cis-acting elements (Yuan, et al., 2009), which can cause changes in the cell cycle and favors cell proliferation.

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 (Nichols and Boeckh, 2000), as well as the fact that inflammation is always present in cancer (Colotta, 2009), 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 cancerogenesis. In this study, we showed that the CMV antigens are present in different histological types of SGC.

Protein products of several CMV genes have the ability to disrupt mechanisms involved in mutagenesis, apoptosis, cell cycle, angiogenesis, cell invasion and host antitumor response (Scheurer, et al., 2008). 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 (Scheurer, et al., 2008). 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 (Jarvis, et al., 1999). Another *in vivo* study of colorectal cancer and its precancerous lesions showed that CMV IE1-72 protein was always found in cancerous 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 (Harkins, et al., 2002). In our study we also found that CMV was absent in normal gland tissue, both in

healthy controls and in unchanged surrounding peritumorous tissue, however in tumor tissue the expression of CMV antigens was detected.

A detailed investigation of the role of CMV in salivary gland tumors was performed by Melnick et al (Melnick, et al., 2012). However they focused their research only on one histological type of SGC, mucoepidermoid carcinoma (MEC). They found that CMV antigens were present in 38/39 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 an upregulation and activation of the oncogenic COX-2/AREG/EGFR/ERK signaling pathway (Melnick, et al., 2013).

In our study we used two different methods, immunohistochemistry and nested PCR, in order to determine the presence of CMV in SGC. Although, for the detection of CMV from fresh tissue, PCR can be the method of choice, its utility is unknown for formalin fixed, paraffin embedded (FFPE) tissues. The process of paraffinization is aggressive and could damage the viral DNA, and make it undetectable by the standard PCR method. In our study we decided to perform nested PCR in order to increase sensitivity. The gold standard for CMV detection in FFPE tissue is immunohistochemistry (IHC). This is why we used the results of IHC detection of CMV antigens to analyze the association of CMV presence with the expression of prognostic markers. We decided to use the commercially available CCH2 and DDG9 clones that detect products of immediate-early (IE) and early (E) CMV genes, since these products are thought to be able to disrupt the cell cycle and the normal processes in the infected cell.

We have found CMV protein expression in 66 of the 92 SGC cases (71.7%). The expression did not differ with respect to gender, salivary gland size or localization. Similar to the findings of Melnick et al, CMV expression was positive in all 8 cases of MEC, 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), 73,3% in myoepithelial carcinoma (11/15 cases), 55,6% in adenocarcinoma NOS (5/9 cases), in all 3 cases of salivary duct carcinoma. Unfortunately, in this 10 years 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 (Scheurer, et al., 2008).

Even though CMV is extremely frequently present in SG ducts, SGC remains a rather rare malignancy. On the other hand, positive expression 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 MEC, adenoid cyctic carcinoma and acinic cell carcinoma. We suspect that some environmental or intrinsic pathological agents can alter the salivary gland cells and make them more susceptible to CMV infection, allowing CMV products to further disrupt cell mechanisms and lead to cancer formation and propagation. Although much is known about the processes that CMV gene products cause in the cell, the role of CMV in the development of cancer is still uncertain.

When comparing the nested PCR to the IHC results, we have noticed that the results coincide in a high percentage (94%). The Cohen's Kappa value that measures inter-rater agreement, or in our case the agreement between two methods used, was high (0.846) with a high statistical significance ($p<0.01$). In only 1 case nested PCR gave a positive result, while it was negative in the IHC analysis. In 5 cases nested PCR did not detect the viral genome, while IHC showed a positive expression of CMV antigens. It is interesting that in all 5 cases the IHC expression was marked as weak. Reports from other authors concerning the comparison of the two methods are contradictory. In the past, PCR was shown to be less sensitive than IHC (Ozono, et al., 1997) (Kandiel and Lashner, 2007). However, with the development of new techniques such as nested PCR or real-time PCR the sensitivity of the DNA detecting methods from FFPE tissues of various origins increased significantly (Muir, et al., 1998) (Mills, et al., 2013).

Another virus whose role in the development of various epithelial cancers is causing interest is Epstein Barr virus (EBV). EBV is associated with the development of lymphomas, including Burkitt's lymphoma, classical Hodgkin lymphoma (cHL), diffuse large B-cell lymphoma (DLBCL), and natural killer (NK)/T-cell lymphoma (Vockerodt, et al., 2015). Besides its role in many lymphoid cancers, EBV is thought to be involved in the development

of some epithelial cancers. The most described association is between EBV and certain types of gastric cancer and nasopharyngeal carcinoma (NPC), especially in the Asian population. It has been shown that EBV latent gene products are consistently expressed in these cancers (Pittaluga, et al., 1992). Recently, the presence and role of EBV has been investigated in many other epithelial malignancies, such as breast, lung, prostate and oral squamous cell cancer.

EBV latency is thought to play an important role in cancerogenesis. The selective expression of only certain latency genes, corresponding to the type II latency program, is believed to disrupt certain cellular processes and signaling pathways, and lead to malignant transformation of epithelial cells (Wang, et al., 2014). For example EBV latency is rarely seen in normal pharyngeal epithelium, but is detected in NPC, as well as in precancerous lesions. It is thought that the establishment of latency in premalignant epithelial cells is a crucial step in malignant transformation. The products of latency genes that are expressed in epithelial cells include EBNA1, LMP1, LMP2A and EBERs (Tsao, et al., 20012).

Epstein-Barr nuclear antigen 1 (EBNA1) plays an important role in cell survival after DNA damage, by disrupting the process of DNA repair and p53 activation. It has been shown that, in both NPC and gastric cancer, EBNA1 disrupts the promyelocytic leukaemia (PML) nuclear bodies that contain proteins important for many cellular processes. The disruption of PML nuclear bodies leads to the activation of CK2 kinase or ubiquitin-specific protease 7, which degrades p53 (Sivachandran, et al., 2010). Therefore, the role of EBNA1 could be to promote the survival of cells with DNA damage. Beside this role, EBNA1 has been shown to up-regulate oxidative stress response proteins SOD1 and Prx1 (Cao, Mansouri and Frappier, 2012), as well as to modulate several signaling pathways, such as suppressing TGF-β1 signaling and enhancing the nuclear accumulation of NFκB (Chung, et al., 2013).

BARTs are a family of rightward transcripts from the EBV region *Bam*HI A. A much higher expression of BARTs was found in NPC and gastric cancer than in lymphoid malignancies (Marquitz and Raab-Traub, 2012).There is still no proof of the existence of BART encoded proteins, therefor BARTs are non-coding RNA that regulate the cell cycle.

The higher expression of BARTs might be due to interferon regulatory factors (IRF5 and 7), and NFκB (Chen, et al., 2005). This implies the importance of local infection and inflammation for the increased expression of BARTs.

Latent membrane protein 1 (LMP1) shows many oncogenic properties including cell proliferation, resistance to apoptosis, invasion and angiogenesis (Dawson, Port and Young, 2012). LMP1 is an agonist of TNFα. This is a transmembrane protein that acts as a constitutively activated TNF receptor 1, and therefore has the ability to activate many signaling pathways, including NFκB, PI3K-AKT and ERK-MAPK. The angiogenesis induction by LMP1 can be explained by the reduction of hypoxia inducible factor-1α (HIF-1α) and by the induction of vascular endothelial growth factor (VEGF) expression (Wakisaka, et al., 2004). LMP-1 also has a role in cancer inflammatory processes. In NPC the expression of LMP1 activates the NFκB signaling pathway and up-regulates inflammatory cytokines. Another study in NPC showed that LMP1 enhances the IL-6-STAT3 signaling pathway, which in turn leads to a positive feedback and an even higher LMP1 expression (Zhang, et al., 2013). For this reason we decided to analyze the IHC expression of LMP1 antigen, and to compare it to the expression of IL-6 and NFκB in SGC.

We have found EBV LMP1 protein expression in 32 of the 92 SGC cases (34.8%). The expression did not differ with respect to gender, salivary gland size or localization. We found a high EBV positivity percentage of 54.3% in adenoid cystic carcinoma (19/35 cases) and 55,6% in adenocarcinoma NOS (5/9 cases). In other histologic types, EBV antigens were found less frequently, 20% in polymorphous low-grade adenocarcinoma (2/10), 25% in mucoepidermoid carcinoma (2/8 cases), 13.3% in myoepithelial carcinoma (2/15 cases). We found only 1 positive case of salivary duct carcinoma and mucinous adenocarcinoma. Several histological types showed no cases that were EBV positive. Unfortunately, in this 10 years period, some histological types were very rare, and therefore accurate statistical evaluation that could be performed was to compare the prevalence of EBV positivity in adenoid cystic carcinoma, with the highest number of positive cases, and in the other SGC histologic types. It occurs that EBV is found much more often in adenoid cystic carcinoma than in other histologic types.

When comparing the nested PCR to the IHC results, we have noticed that the results coincide in an even higher percentage than CMV (94,6%). The Cohen's Kappa value that measures inter-rater agreement, or in our case the agreement between two methods used, was high (0.883) with a high statistical significance ($p<0.01$). In only 1 case IHC analysis gave a positive result, while it was negative in nested PCR analysis. In 4 cases nested PCR detected the viral genome, while IHC showed no expression of EBV antigens. This might be explained by the fact that nested PCR does not differentiate between infections in different cells. It is possible that in these 4 cases EBV developed a latent infection in some B lymphocyte that was in the cancer or surrounding peri-tumorous tissue. Reports from other authors concerning the comparison of the two methods are contradictory. Bonnet et al. obtained similar results by PCR and IHC from FFPE samples of breast cancer (Bonnet, et al., 1999). Liu et al. also found no significant difference among the results of PCR, IHC, and in situ hybridization (ISH) when detecting EBV in 130 cases of colorectal tumors (Liu, et al., 2003). Suh et al. compared PCR and IHC detection of EBV from FFPE samples of hepatitis, and obtained significantly more positive results by PCR (Suh, et al., 2007). Actually, in that study, 6/8 cases of hepatitis were found EBV positive by PCR, while there were no cases of IHC positivity. We believe that for the IHC detection of viral antigens in carcinomas, the sections should be slightly thicker than what is necessary for other markers, and also the concentration of the primary antibody should be higher in order to detect viral presence.

The presence of EBV latency proteins in the malignant cells of NPC and gastric cancer has been undeniably proven. The percentage of positive cases is especially high in the Asian population. Previously, LMP-1 was reported to be expressed in 20-40% of NPC, however, by using a more sensitive IHC staining, it has been shown that almost 100% of primary NPC samples are LMP-1 positive (Tsao, et al., 2002) (Dawson, Port and Young, 2012). In primary gastric cancer LMP-1 expression was found by high-coverage transcriptome sequencing, despite the absence in previous reports (Strong, et al., 2013).

The presence of EBV in other epithelial cancers is still controversial. Several studies have been performed to determine the presence of EBV in breast cancer. Glaser et al. did not

detect EBER-1 transcripts in any case of breast cancer (Glaser, et al., 1998). In another IHC analysis on 60 invasive breast cancer cases LMP-1 was not detected (Chu, Chen and Chang, 1998). In a PCR study, EBV was present in about 40% of breast tumors (Labrecque, et al., 1995). Another study, that used 2 methods of virus detection, IHC and Southern blot, also found that EBV was present in over 50% of invasive breast cancers (Bonnet, et al., 1999). A study on cervical cancer found EBV to be present in 36% of cervical squamous cell carcinoma, but also in the pre-neoplastic lesions (Kim, et al., 2005). In a study of colorectal tumors, EBV was found in 26 out of 130 cases (Liu, et al., 2003).

The few studies performed on SGC confirmed the presence of EBV only in the Asian population (Kuo and Tsang, 2001), while the studies in Caucasian population were negative (Pollock, et al., 1999). In our study we have detected EBV in more than 30% of the SGC cases. Very similar results were obtained when 2 methods were applied, detection of LMP-1 antigen by IHC, and the nested PCR detection of LMP-1 gene, which speaks in favor of the credibility of our results.

The role of IL-6 in SGC

Interleukin 6 (IL-6) is a pleiotropic cytokine that regulates gene expression in various organs, interacts with B and T lymphocytes, modulates the synthesis of acute phase reactants in the liver, stimulates the resorption of bone by osteoclasts, regulates the hypothalamic-pituitary axis, and acts as a colony-stimulating and megakaryopoetic factor in the bone marrow. Its role as a growth factor in several malignant diseases has also been noticed. The serum and tissue levels of IL-6 are increased in patients with renal cell carcinoma (RCC). Also, the IL-6 expression was higher in the higher stages of RCC (Yoshida, et al., 2002). In prostate cancer and prostatic pre-cancerous lesions, IL-6 levels were found to be higher than in normal prostate tissue (Hobisch, et al., 2000). Another in vitro study on prostate cancer demonstrated that IL-6 undergos a functional transition from paracrine growth inhibitor to autocrine growth stimulator during progression of prostate cancer cells to the hormone-refractory phenotype (Chung, et al., 1999). John et al. found that levels of IL-6 are elevated in the saliva and serum of patients with oropharyngeal squamous cell carcinoma (John, et al.

2004). There are contradictory reports about the presence and role of IL-6 in breast cancer. Green et al. found no significant difference in IL-6 mRNA expression between normal breast tissues and breast tumors (Green, et al., 1997). There are some reports indicating that IL-6 is a positive prognosticator. In a study by Karczewska et al. IL-6 mRNA expression is found in 57% of breast carcinoma tissues, and expression was strongly correlated with earlier stages of the disease (Karczewska, et al., 2000) The role of IL-6 as a positive prognosticator was also proven by Fontanini et al (Fontanini, et al., 1999). However, there are reports stating that IL-6 is a negative prognosticator in breast cancer (Purohit, et al., 1995) (Garcia-Tunon, et al., 2005). They have suggested that breast cancer cells produce more IL-6 than normal breast epithelial cells, but also the response on the tumor cells this interleukin is greater. Furthermore, they reason that high expression of IL-6 and its receptors in breast tumors might be related to the enhanced cell proliferation occurring in breast cancer.

There are few reports on the expression of IL-6 in salivary gland cancers. Gandour-Edwards et al. report that increased levels of IL-6 are found in both benign and malignant salivary gland tumors compared to normal tissue (Gandour-Edwards, et al., 1995). Leivo et al. found IL-6 overexpression in mucoepidermoid carcinoma of the salivary gland (Leivo, et al., 2005), while Mochizuki et al. found that the inhibition of IL-6 in mucoepidermoud carcinoma has an anti-tumor effect (Mochizuki, et al., 2015). In our study we found a significantly high association between the level and intensity of IL-6 expression and SGC. IL-6 expression was positive in 65 out of 92 SGC cases (70.7%), and only 4 control cases (20%). While in the cases of control tissues, the intensity of positive reaction is always weak, out of the 65 positive cases 35 (53.8%) showed a moderate to strong expression of IL-6. These results indicate IL-6 to be a positive prognosticator in SGC.

We also assessed the individual role of the IL-6 -174G>C single nucleotide polymorphism (SNP) implicated as a potential modifier for the predisposition to SGC. The results from literature about this polymorphism are also conflicting. A study showed that this SNP is not involved in susceptibility to neuroblastoma development. However, the results also show that a low frequency of genotype CC is significantly associated with a low overall survival, advanced stage, and high-risk phenotype. Interestingly, in that study the CC

genotype was correlated with increased level of IL-6 expression (Totaro, et al., 2013). A meta-analysis study of this SNP showed that the GG genotype was associated with a higher risk of hepatocellular carcinoma (Liu, et al., 2014). A meta-analysis study found no significant association between this SNP and prostate cancer risk in the Caucasian population, however in the African-American population the CC genotype was associated with a higher risk of prostate cancer (Yang, Li and Li, 2014) Another study on non-small cell lung cancer found that smokers with the -174C allele were found to be significantly associated with the cancer (Bhat, et al., 2015). In our study, the C allele was also found to be associated with SGC, when both the dominant (CC) and recessive model (CC+GC) were applied. Our results showed high statistical significance. We also compared the IL-6 -174G>C polymorphism to the IHC expression of IL-6 in SGC cases. We found that there was no difference in the expression of IL-6 among all of the IL-6 -174G>C genotypes.

There are reports that indicate that the reactivation of latent CMV and the expression of major immediate-early (MIE) genes lead to the increased production of IL-6 (Iwamoto and Konicek, 1997) (Rott, et al., 2003) (Rahbar, et al., 2003). Melnick et al. found a strong connection between CMV infection of the salivary gland tumors and the increased production of IL-6 in an animal model (Melnick, et al., 2013). We also investigated the difference in IL-6 expressions in CMV positive and negative cases of SGC. IL-6 expression was positive in 31.5% cases of CMV negative SGC. On the other hand, 83.4% of CMV positive cases also showed IL-6 expression. These results showed a high statistical significance ($p<0.01$). When analyzing the intensity of IL-6 expression, we have noticed that only 2 cases of CMV negative SGC showed moderate and strong positivity, compared to 33 cases of SGC where CMV was detected ($p<0.01$). On the other hand, there was no difference neither in the prevalence, nor the intensity of IL-6 expression in EBV positive and negative cases ($p>0.05$).

The role of NF-κB in SGC

Inflammation has an important role in the development of cancer. Among various signaling pathways activated by inflammation and infection, nuclear factor kappa B (NF-κB) might be the most important tumor-promoting component. For this reason we analyzed NF-

κB expression in SGC. The ability of NF-κB to promote cancer is mostly due to the realization that NF-κB is a major activator of anti-apoptotic gene expression. These findings were first explained through TNF-α signaling (Liu, et al., 1996) (Van Antwerp, et al., 1996). When TNF-α binds to its receptor TNFR1, it activates a signaling pathway that could either lead to cell death, or has an anti-apoptotic effect. In order for cell death to occur, TNFR1 has to be combined with certain inhibitors of RNA or protein synthesis, otherwise the anti-apoptotic signaling pathway will be activated, mostly the NF-κB signaling pathway. Before the cell is stimulated, NF-κB is present in the cytoplasm in its inactive form, forming a bond to the inhibitors of NF-κB (IκBs). Cell stimulation activates IκB kinases (IKK) that phosphorylate IκBs, which leads to their degradation by the ubiquitin system (Karin, 2006). Once NF-κB is freed from its inhibitor, it moves into the nucleus and coordinates the activation of several hundred genes that have an anti-apoptotic effect. In our study we have seen that the NF-κB positivity in SGC is mostly both cytoplasmic and nuclear, implying that the NF-κB is in its active form. Elevated levels of NF-κB have been found in many cancers including skin cancer (Lind, et al., 2004), breast cancer (Chua, et al., 2007), lung cancer (Tew, et al., 2008), colon cancer (Scartozzi, et al., 2007), pancreatic cancer (Weichert, et al., 2007) and many others. We have also found a very high percentage of NF-κB positive cases of SGC (92.4%), compared to 10% of positive cases of normal salivary gland. While the NF-κB expression in the normal salivary gland was weak, in SGC cases the expression in over 60% was moderate to strong, with a clear nuclear positivity, which indicates the presence of the active form.

CMV is believed to be able to up-regulate and activate NF-κB in many cells. An increase in the secretion of tumor promoting, M2-type cytokines was found following HCMV virus infection, resulting from activation of the NF-κB transcription factor and the phosphoinositol-3-kinase (PI3K) signaling pathway (Chan, et al., 2009). Similarly, CMV was shown to infect tumor associated fibroblasts and dermal fibroblasts, resulting in stimulation of the NF-κB signaling pathway and secretion of inflammatory cytokines (Juckem, et al., 2008). A study has showed that CMV enhances NF-κB signaling in inflammatory breast cancer (El-Shinawi, et al., 2013). In our study we investigated the difference in NF-κB

expressions in CMV positive and negative cases of SGC. NF-κB expression was positive in 97% of the CMV positive SGC cases. In SGC cases where CMV was negative, NF-κB had a positive expression in 80%.This difference showed a high statistical significance ($p<0.01$). When analyzing the intensity of NF-κB expression, we have noticed that most CMV negative SGC cases weak positivity, while more than 60% of CMV positive cases showed moderate to strong NF-κB positivity.

There are also reports from in vitro and in vivo studies that report that the EBV LMP1 protein induces NF-κB activity (Eliopoulos, et al., 1997) (Kaye, et al., 1996). However, we have found no difference neither in the prevalence, nor the intensity of NF-κB expression in EBV positive and negative cases ($p>0.05$). When comparing the SGC cases that were both EBV and CMV positive, to the cases that were only CMV positive, and EBV negative, it was seen that NF-κB expression was higher in the co-infected cases, than in cases where just CMV was present, however this value did not reach statistical signigicance.

The role of MMPs in SGC

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are capable of degrading the extracelluar matrix (ECM). Since they can degrade essentially all components of ECM including collagens, elastin, proteoglycans, laminin and fibronectin, their role is crucial in the invasion and metastasis of most malignancies (Freije, et al., 2003). It has been shown that MMPs could be involved in several steps of cancer development, such as cancer cell growth, differentiation, apoptosis, migration, invasion, and metastasis (Bellayr, Mu and Li, 2009). They also cleave several non-matrix proteins, including growth factors, cytokines, chemokines, and their receptors, in this manner regulating cell growth and inflammation. Elevated levels of MMPs have been associated with the invasive properties of cancer and their role as prognostic factor has been studied widely in different cancer types (Ala-aho and Kahari, 2005) (Wiercinska, et al., 2011).

Although many MMPs are thought to have a role in carcinogenesis, most attention has focused on MMP-2 and MMP-9, members of the gelatinase protein family. MMP-2 and MMP-9 are capable of degrading type IV collagen, the most abundant component of the

basement membrane, that provides structural support for cells and influences cell signaling and polarity. Therefore, the destabilization of the basement membrane is an essential step for both the local and metastatic spread of most cancers. These molecules are overexpressed in a variety of malignant tumors and their expression and activity are often associated with tumor aggressiveness and a poor prognosis (Bauvois, 2012). Since their role in the carcinogenesis of many malignant tumors is proven, we investigated the role of MMP-2 and MMP-9 in SGC. The presence of these MMPs was analyzed in SGC tissue compared to normal salivary gland tissue. Since the deregulation of MMP-2 and MMP-9 expression can be due to single nucleotide polymorphisms (SNPs) in the gene promoter region, we analyzed the significance of three SNPs (MMP-2 -1306 C>T, MMP-2 -1575G>A and MMP-9 -1562 C>T) in SGC, by comparing the presence of the genotypes in the SGC population and healthy controls, but also by comparing the MMP-2 and MMP-9 expression with the genotypes. Finally, we examined the difference of MMP-2 and MMP-9 expression in SGC infected and non-infected with CMV and EBV.

Elevated levels of MMP-2 and/or MMP-9 are found in many malignant tumors, such as breast, brain, ovarian, pancreas, colorectal, bladder, prostate and lung cancers and melanoma (Klein and Bischoff, 2011) (Roy, Yang and Moses, 2009)(Rydlova, et al., 2008). However, when we compared the expression of MMP-2 in SGC and healthy control tissue, no significant difference was found ($p=0.618$). We also analyzed whether the intensity of MMP-2 expression is stronger in SGC. In 7 SGC cases we found moderate to strong expression of MMP-2, while in the control group, MMP-2 expression, when positive, was always weak. However, due to small sample sizes, this difference did not reach statistical significance ($p=0.396$). We have also noticed that in several SGC cases even when there was no expression of MMP-2 in cancer tissue, there was a strong MMP-2 expression in the inflammatory cells in surrounding nonmalignant tissue. Also in several SGC cases that had an over-all weak expression of MMP-2, the expression of MMP-2 was stronger in the peripheral areas than in the rest of the tumor. The results of MMP-9 expression were similar to MMP-2. There was no difference in the prevalence of MMP-9 positivity between SGC and healthy control cases ($p>0.05$). Also here, the only 4 cases that showed moderate to

strong expression of MMP-9 belonged to SGC group, while MMP-9 expression in the control group was always weak. Also here, due to the small sample size, this result did not show statistical significance ($p>0.05$). In cases where MMP-9 expression was positive, MMP-9 was located either only in the peripheral zones of the cancer, or it showed a diffuse distribution, but the intensity of the expression was stronger in the peripheral zones of the tumor. In five cases we found that MMP-9 was expressed only in a thin marginal zone, however, since in the marking criteria we stated that for a sample to be marked as positive, it has to express immunoreactivity in more than 10% of the cells, which it did not in these cases, these cases were marked negative. This could imply that both MMP-2 and MMP-9 are overexpressed only in certain areas where the tumor invades the surrounding tissue, and also that they are not always present in the malignant tissue, but only in periods of most intensive growth and invasion.

The deregulation of MMP-2 and MMP-9 expression can be due to single nucleotide polymorphisms (SNPs) in the gene promoter region. We assessed the individual role of 3 functional SNPs in two genes (MMP-2 and MMP-9) implicated as potential modifiers for the predisposition to SGC. Our results showed that MMP-2 -1306 CT, MMP-2 -1306 CT+TT genotypes and MMP-9 -1562 CT and MMP-9 -1562 CT+TT genotypes were significantly associated with an increased risk of SGC. However, there was little association of MMP-2 -1575 G>A with SGC risk.

The MMP- 2 -1306 C> T transition in a core recognition sequence of Sp1 (CCACC box), abolishes the Sp1-binding site and also diminishes promoter activity. Transient transfection experiments showed that reporter gene expression driven by the C allele was significantly greater than reporter gene expression driven by the T allele both in epithelial cells and macrophages (Price, et al., 2001). This SNP has an interactive effect on MMP-2 transcription. To date, several studies have evaluated this genetic variation in MMP-2 in relation to cancer susceptibility but the results are conflicting. A large study showed that the MMP-2 -1306 C>T polymorphism is associated with increased risk of breast cancer but the effect was not significant (Beeghly-Fadiel, et al., 2009). However, a meta-analysis study from the Asian population suggest that CC genotype of MMP-2 -1306 C>T polymorphism may

contribute to head and neck cancer susceptibility (Zhang, et al., 2011) and another meta-analysis from the Asian population revealed that -1306T allele act as a protective factor in digestive tract cancers (Zhand and Ren, 2011). On the other hand, a recent study from India showed that the T allele increased the risk of gull bladder cancer (Sharma, et al., 2012). Also, another study showed that genotypes MMP- 2 -1306 CT and MMP- 2 -1306 TT increase the risk of prostate cancer (Srivastava, et al., 2012). Until now there is no report on MMP-2 polymorphisms in SGC, and the present study showed increased risk for SGC owing to variant containing MMP- 2 -1306 CT and CT+TT genotypes of these SNPs. This could be explained by the possibility that MMP-2 polymorphism influences the inflammatory response by acting on alternate substrates as MMP-2 was shown to contribute to inflammation by being an alternative activator of pro-interleukin 1-b in the absence of the cytokines' favored activator caspace-1 (Schonbeck, et al., 1998).

The MMP-2 -1575G>A variant was located immediately 5' to a half-palindromic potential estrogen receptor binding site and -1575G allele functioned as an enhancer, whereas the -1575A allele lost its transcription activation (Morgan, et al., 2011). This polymorphism has been found to play an important role in the development of certain diseases such as various metabolic disorders or cardiovascular diseases (Yadav, et al., 2014) (Pérez-Hernández, et al., 2012) (Saracini, et al., 2012). The significance of this polymorphism in carcinogenesis is yet to be determined. The results of our study did not implicate this polymorphism as a risk factor for SGC, since the increase in susceptibility, with borderline significance, was only found when dominant model is applied (GA + AA vs. GG).

MMP-9 has also been implicated in tumor invasion and metastasis. However, rare work has been carried out on MMP-9 and SGC. Our study indicates that the heterozygous carriers of the variant allele have a 2-fold increase in risk of developing salivary gland carcinomas. The variant T allele is associated with higher promoter activity compared to the more common C allele (Zhang, et al., 1999). In line with this, a previous study on Serbian population reported a 4-fold increase in risk for pleomorphic salivary gland adenoma in heterozygous carriers of the variant allele T (Nikolic, et al., 2013). Several studies showed the association between the -1562T allele and vascular disease, gastric cancer, head and neck

squamous cell carcinoma (Zhang, et al., 2014) (Lee, et al., 2013). Contrary to those reports, Park et al. (Park, et al., 2011) found that the MMP-9 -1562CC genotype was more common in patients with colorectal cancer. However, the risk of lymph node metastasis of colorectal cancer is higher in patients with -1562T allele.

In conclusion, our findings suggest MMP-2 -1306 C>T and MMP-9 -1562 C>T polymorphisms seem to influence the development of SGC, whereas MMP-2 -1575 G>A polymorphism seems to be of a minor importance.

We also compared the MMP-2 and MMP-9 polymorphisms to the IHC expression of these markers in SGC cases. We found that there was no difference in the expression of MMP-2 among all of the MMP-2 -1306 C>T, and MMP-2 -1575 G>A genotypes. When comparing the MMP-9 -1562 C>T polymorphism, the CC genotype is associated to higher positivity, but only with borderline significance (p=0.49). These results are in agreement with the results of a study in non-small cell lung cancer, where cases with different MMP-2 -1306C>T and MMP-9 -1562 C>T genotypes had similar levels of MMP-2 and MMP-9 mRNA, respectively (Rollin, et al., 2006). Another study on breast cancer also confirmed that expression of the MMP-9 gene does not appear to be influenced by the −1562C>T SNP (Przybylowska, et al., 2006).

Recently, researchers have been investigating the relationship between viruses and the presence and activity of certain MMPs. Most research focuses on the effect CMV has on different MMPs in the placenta tissues. Also, a connection was found between the disregulation of certain MMPs in blood vessels, which leads to the development of atherosclerosis, and the presence of CMV. However, the results of these researches are contradictory. Lui et al. (Lui, et al., 2011) investigated the effect of CMV on MMP-2 and MMP-9 in early pregnant extravillous cytotrophoblast, and discovered that both MMP-2 and 9 levels were significantly decreased in the CMV positive group compared to control. Another study focused on the effect of CMV on human macrophages. In the presence of CMV, decreased levels of MMP-9 were found, however there was no difference between the levels of MMP-2 in CMV positive and negative samples (Straat, et al., 2009). On the other

hand, another research investigated the role of CMV in the pathogenesis of atherosclerosis. It was shown that CMV upregulated MMP-2 protein and activity in smooth muscle cells, which led to the reduction of extracellular matrix (Reinhardt, et al., 2006). CMV was also shown to increase MMP-2 levels in renal tubular epithelial cells (Shimamura, et al., 2010). We also investigated the difference in MMP-2 and 9 expressions in CMV positive and negative SGC. Out of 26 cases of CMV negative SGC only 1 showed MMP-2 expression. Also, 39.4% of CMV positive cases also showed MMP-2 expression. These results showed a high statistical significance ($p<0.01$). Also, with 7 cases that showed moderate and strong positivity, the intensity of MMP-2 expression in CMV positive cases was significantly stronger then in CMV negative cases ($P<0.01$). On the other hand, there was no difference neither in the prevalence, nor the intensity of MMP-9 expression in CMV positive and negative cases ($p>0.05$).

Besides CMV, EBV is also thought to be able to alter the production of MMPs. There are several reports stating that EBV increases the production of MMP-9 in nasopharyngeal cancer (Chew, et al., 2010) (Lan, et al., 2013). We, however, did not find any difference in the prevalence and intensity of MMP-2 or MMP-9 expression in CMV positive and negative cases ($p>0.05$). However, the role of EBV cannot be easily dismissed. MMP-2 was positive in 50% of SGC cases that were both CMV and EBV positive, compared to 34% in SCG cases that were only infected with CMV, but not EBV. This difference showed a statistical significance. Therefore, it is possible that some CMV and EBV products work synergistically, leading to a higher expression of MMP-2. This type of synergism was not seen when analyzing the expression of MMP-9.

6. CONCLUSIONS

1. When analyzing the prevalence of CMV and EBV in salivary gland cancer and normal salivary gland tissues it can be concluded that:
    - Normal salivary gland tissues showed a prominent CMV antigen expression in ductal epithelial cells, while the there was no positivity in the gland tissue. On the contrary, in SGC tissues, CMV antigens were detected in 71.7% of the SGC cases.
    - EBV antigens were expressed only in 1 case of and normal salivary gland, while they were detected in 34.8% of the SGC cases.
    - In 22 SGC cases we have found a dual infection of both CMV and EBV.

2. When comparing the nested PCR to the IHC results, we have noticed that the results coincide in a high percentage for both CMV (93.5%) and EBV (94.6%). It can be concluded that both methods can be used in the detection of viruses from FFPE tissues.

3. When analyzing viral protein expression in different histologic types of SGC it can be concluded that :
    - CMV was found in all cases of MEC, and in a high percentage in adenoid cyctic carcinoma, acinic cell carcinoma and myoepithelial carcinoma, however the sample sizes were too small to be able to do precise statistical analysis
    - EBV is found much more often in adenoid cystic carcinoma than in other histologic types.

4. When analyzing the immunohistochemical expression of IL6, MMP2, MMP9 and NFκB it can be concluded that:
    - IL-6 and NFκB expressions were found more often in SCG than in normal salivary gland tissue. The results showed high statistical significance ($p<0.01$)
    - While, if detected, the intensity of IL-6 and NFκB expression in normal controls was weak, in SGC tissues more than half of the cases showed moderate to strong expression. The difference in intensity showed high statistical significance ($p<0.01$)
    - MMP-2 and MMP-9 expressions did not show a significant difference between normal salivary gland tissues and SGC tissues.

- If detected, the intensity of MMP-2 and MMP-9 expression in normal tissues was always weak, while in several SGC cases (7 and 4 respectively) there was moderate to strong expression. However, due to the small number of cases, the difference did not reach statistical significance.
- Both MMP-2 and MMP-9 expression was more prominent in the peripheral zones of SGC tissues, which demonstrated its role in tumor infiltration of normal tissue and spreading.

5. Analyzing the association of the presence of CMV and EBV and the immunohistochemical expression of IL6, MMP2, MMP9 and NFκB SGC it can be concluded that:
    - MMP-2, IL-6 and NFκB expression was found more often in SCG cases infected with CMV then cases that didn't show CMV presence ($p<0.01$).
    - The intensity of MMP-2, IL-6 and NFκB expression was stronger in CMV infected SGC tissue than cases that didn't show CMV presence ($p<0.01$).
    - MMP-2, IL-6 and NFκB expression did not show a significant difference in the prevalence or intensity between EBV infected and non-infected SGC cases.
    - Cases that were co-infected with both CMV and EBV showed a higher and more intense expression of MMP-2, IL-6 and NFκB compared to cases that were infected only with CMV. This shows that EBV also contributes to the increase in MMP-2, IL-6 and NFκB expression and carcinogenesis.
    - MMP-9 expression did not show a significant difference in the prevalence or intensity between EBV infected and non-infected SGC cases.

6. Analyzing the polymorphisms of IL6, MMP2 and MMP9 genes and investigating the association of the polymorphisms and the corresponding protein expression it can be concluded that:
    - IL-6 -174G>C, MMP-2 -1306 C>T and MMP-9 -1562 C>T polymorphisms seem to influence the development of SGC, whereas MMP-2 -1575 G>A polymorphism seems to be of a minor importance.
    - IL-6 -174G>C, MMP-2 -1306 C>T and MMP-2 -1575 G>A polymorphisms do not seem to have an influence on their protein product expression, while MMP-9 -1562 CC genotype shows a higher MMP-9 expression, however this result shows a borderline significance

7. LITERATURE

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