ORIGINAL ARTICLE

EXPRESSION FEATURES OF HUMAN PAPILLOMA VIRUS TYPE 16 AND ANTI-EPSTEIN-BARR VIRUS IN PLEOMORPHIC ADENOMA, SURROUNDING AND INTACT SALIVARY GLAND

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ABSTRACT

The aim is to reveal the expression features of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus in the pleomorphic adenoma, surrounding and intact salivary gland.

Materials and methods: It was used surgical and biopsy material from 30 patients, represented by pleomorphic adenomas with surrounding to tumor tissue of the salivary gland and intact tissue of the salivary gland (the distance between the tumor and the intact salivary gland – 10 mm). Immunohistochemical study was performed using mouse monoclonal antibody (MCA) to human papilloma virus type 16 (clone CAMVIR-1, «Diagnostic BioSystems», USA) and anti-Epstein-Barr virus (LMP, clone CS. 1-4, «Dako», Denmark). Visualization was performed, using an EnVision™ FLEX detection system (Dako, Denmark). Antigen unmasking was carried out in citrate buffer pH 6.0 at 95°C. Primary antibodies were incubated at room temperature for 30 minutes, secondary antibodies – 20 minutes. Sections were counterstained with Gill hematoxylin. We assessed the immunohistochemical reaction by a semi-quantitative method by counting the percentage of positively stained cells in the field of view of a microscope × 400. Microspecimens were studied and photoarchived on an Olympus BX-41 microscope (Japan).

Results: In this study it was detected a positive immunohistochemical reaction with MCA to human papilloma virus type 16 and anti-Epstein-Barr virus, respectively, in 26 (86.7%) and 8 (26.7%) cases. Epithelial, mixed and mesenchymal variants of pleomorphic adenoma of the salivary glands are characterized, respectively, by the severely expressed, moderately expressed and minimally expressed of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus. The parenchymal component of pleomorphic adenoma is characterized by more marked expression of these markers as compared to the stromal component.

The epithelial cells of the salivary glands, surrounding the pleomorphic adenoma, as well as intact salivary glands, express MCA to human papilloma virus type 16 and anti-Epstein-Barr virus. The severity of the expression of these markers in the salivary gland is determined by the histological variant of the tumor (severely expressed in the epithelial variant, moderately expressed in the mixed variant, and minimally expressed in the mesenchymal variant).

Conclusions: The immunohistochemical study has shown that the Epstein-Barr virus and, especially, human papilloma virus type 16 can act as exogenous trigger factors involved in the development of pleomorphic adenoma of the salivary glands. The revealed immunohistochemical features of MCA expression to human papilloma virus type 16 and anti-Epstein-Barr virus in the salivary gland surrounding the pleomorphic adenoma and in the intact tissue of the salivary gland make it possible to recommend the extracapsular dissection of the tumor with resection of the adjacent intact tissue of the salivary gland at a distance of 10 mm in patients with pleomorphic adenoma.

KEY WORDS: anti-Epstein-Barr virus, human papilloma virus type 16, immunohistochemistry, pleomorphic adenoma, surrounding the tumor and intact salivary gland

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INTRODUCTION

The problem of virus-associated oncogenesis is extremely relevant and is of scientific and practical interest for doctors of various specialties. Prevailing virus-associated tumors varies greatly across the world [1]. The study of viral nature of human tumors has continued for many years. However, today there are many controversial and unresolved issues related to the search for causal relationships and significant correlations between viral infection and the tumors manifestation [2].

Virus-associated tumors include pleomorphic adenoma of the salivary glands. The latter is the most common benign salivary glands neoplasm, accounting for 33.2% to 68.4% of all cases [3]. This tumor most commonly occurs in the parotid gland but it may involve submandibular, lingual and minor salivary glands, too [4].

The human papilloma virus type 16 and the Epstein-Barr virus play a significant role in the genesis of pleomorphic adenomas of the salivary glands development, showed in our earlier morphological studies [5] and the works of other scientists [6]. Immunohistochemical research methods using monoclonal antibodies (MCA) play an important role in establishing the viral genesis of pleomorphic adenomas development. To date, the question of

MCA expression peculiarities to anti-Epstein-Barr virus in various histological variants of pleomorphic adenomas remains controversial. There are no data on the research of expression of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus in the salivary gland adjacent to the pleomorphic adenoma, as well as in the intact salivary gland in the available domestic and foreign literature. All of the above actualizes this study.

THE AIM

The aim is to reveal the expression features of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus in the pleomorphic adenoma, surrounding and intact salivary gland.

MATERIALS AND METHODS

In this study, we used surgical and biopsy material from 30 patients, represented by pleomorphic adenomas with surrounding to tumor tissue of the salivary gland and intact tissue of the salivary gland (the distance between the tumor and the intact salivary gland – 10 mm). Among all cases of pleomorphic adenomas, we found a mesenchymal variant in 15 cases, a mixed variant in 10 cases, and an epithelial variant in 5 cases.

The cut pieces were placed in cassettes. With the help of a cassette holder they were placed in a container for 24-36 hours for fixation in a buffered 10% formalin solution with a pH of 7.4. Conduction and filling the tissue with paraffin was performed using a histoprocessor «HISTOS-5» («Milestone», Italy) on the program for the operating material. After completion of the paraffin impregnation program, the cassettes were removed from the histoprocessor paraffin unit, and at the «HESTION TEC-2800 Embedding Center», the tissue pieces were filled with molten paraffin into molds, followed by solidification on the «HESTION TEC-2800 Cryo Console» refrigeration module. From the obtained paraffin blocks histological sections 3-4 μm thick were made, using a microtome «MICROM HM 325» («Thermo Fisher Scientific», Germany).

Immunohistochemical study was performed using mouse monoclonal antibody (MCA) to human papilloma virus type 16 (clone CAMVIR-1, «Diagnostic BioSystems», USA) and anti-Epstein-Barr virus (LMP, clone CS. 1-4, «Dako», Denmark). Visualization was performed, using an EnVision™ FLEX detection system (Dako, Denmark). Antigen unmasking was carried out in citrate buffer pH 6.0 at 95°C. Primary antibodies were incubated at room temperature for 30 minutes, secondary antibodies – 20 minutes. Sections were counterstained with Gill hematoxylin. We assessed the immunohistochemical reaction by a semi-quantitative method by counting the percentage of positively stained cells in the field of view of a microscope × 400. Microspecimens were studied and photoarchived on an Olympus BX-41 microscope (Japan).

The obtained digital data were statistically processed using the Statistica 10.0 program. The average values were compared, using the nonparametric Mann-Whitney U test. Differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

In this study, we detected a positive immunohistochemical reaction with MCA to human papilloma virus type 16 and anti-Epstein-Barr virus, respectively, in 26 (86.7%) and 8 (26.7%) cases.

Analysis of the immunohistochemical reaction with MCA to human papilloma virus type 16 revealed its nuclear expression in the parenchymal and stromal components of pleomorphic adenomas (fig. 1). In the tumor parenchyma, expression was in the nests and cords that formed epithelial cells, as well as in solid, trabecular, cystic, glandular, ductal, and tubular structures. Some myoepithelial cells also expressed this MCA. Fibroblastic cells, immune cells, vascular endotheliocytes, as well as cellular elements of myxoid and mucoid zones expressed this marker in the tumor stroma. Moreover, the parenchymal component of pleomorphic adenomas was more expressed than the stromal component.

In a reaction with MCA to human papilloma virus type 16 among all histological variants of pleomorphic adenomas, the indicator of the relative number of positively stained cells had the maximum, moderate and minimum values ($p<0.05$), respectively, in epithelial, mixed and mesenchymal variants of the tumor (table I).

We have earlier published the obtained analysis results of the MCA expression to human papilloma virus type 16 in various histological variants of pleomorphic adenomas [5].

It is interesting to note that the expression of MCA to human papilloma virus type 16 was determined not only in pleomorphic adenomas, but also in the surrounding (fig. 2)

Table I. The average value of the relative number of positively stained cells (%) in the reaction with MCA to human papilloma virus type 16.

Pleomorphic adenoma, histological variant	Salivary gland tissue	
Surrounding the tumor	Intact	
Mesenchymal	27.2±3.22 [1,3,4,5]	14.8±2.65 [1,3,4]	5.6±0.75 [1,3,5]
Mixed	56.9±2.87 [2,3,4,5]	31.4±1.92 [2,3,4]	19.3±2.15 [2,3,5]
Epithelial	75.8±4.12 [1,2,4,5]	45.3±3.42 [1,2,4]	30.7±2.76 [1,2,5]

Note: [1] – significant differences compared with the indicators in the mixed variant of pleomorphic adenoma, [2] – significant differences compared with the indicators in the mesenchymal variant of pleomorphic adenoma, [3] – significant differences compared with the indicators in the epithelial variant of pleomorphic adenoma, [4] – significant differences compared with the indicator in the salivary gland intact tissue, [5] – significant differences compared with the indicator in the tissue of the salivary gland surrounding the pleomorphic adenoma.

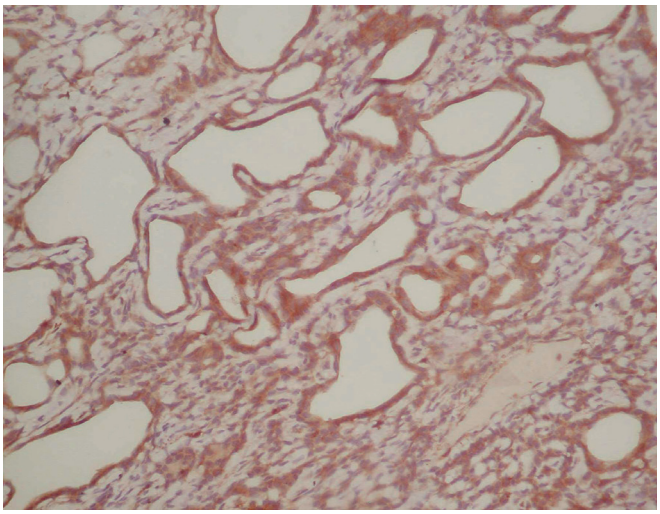

Fig. 1. The expression of MCA to human papilloma virus type 16 in pleomorphic adenoma. Immunohistochemical reaction with MCA to human papilloma virus type 16, × 200.

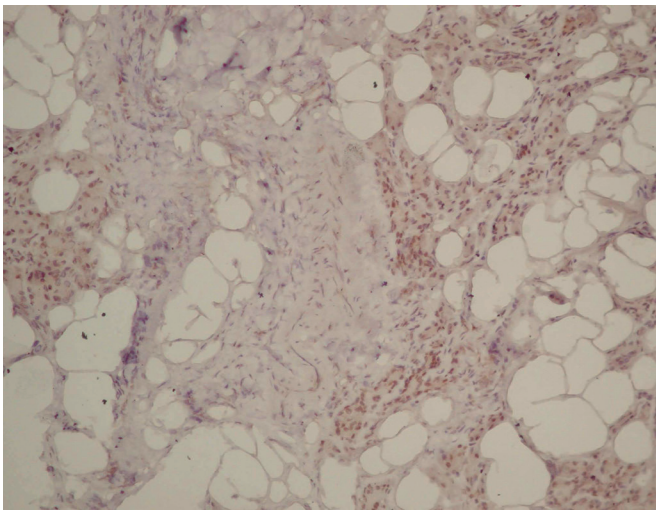

Fig. 2. The expression of MCA to human papilloma virus type 16 in the salivary gland tissue surrounding the pleomorphic adenoma. Immunohistochemical reaction with MCA to human papilloma virus type 16, × 200.

and intact tissue of the salivary gland. The expression of this MCA was detected in the parenchyma of the salivary glands by epithelial structures and myoepithelial cells.

The indicators values of the relative number of positively stained cells both in the tissue of the salivary gland surrounding the tumor and in the intact tissue of the salivary gland were determined by the histological variant of pleomorphic adenoma (table I). These indicators were taken as maximum, moderate and minimum values ($p<0.05$), respectively, in epithelial, mixed and mesenchymal variants of the tumor. In all histological variants of pleomorphic adenomas, the indicators of the relative number of positively stained cells were high ($p<0.05$) in the salivary gland tissue surrounding the tumor as compared to the intact salivary gland tissue.

A comparative analysis of the MCA expression features to human papilloma virus type 16 in the tissue of pleomorphic adenomas, surrounding the tumor and intact tissue of the salivary gland revealed an interesting pattern. Thus, there was a decrease ($p<0.05$) in the expression of this marker in the direction from the tumor tissue to the intact tissue of the salivary gland, as evidenced by survey microscopy and counting the relative number of positively stained cells (table I).

Analysis of the immunohistochemical reaction with MCA to anti-Epstein-Barr virus revealed its membrane-cytoplasmic expression by the cellular elements of the parenchyma and stroma of pleomorphic adenomas (fig. 3). The parenchymal component of the tumor was more expressed in comparison with the stromal component. This MCA expressed the same structures as MCA to human papilloma virus type 16. When counting the positively stained cells, we revealed the maximum, moderate and minimum values ($p<0.05$) of this indicator, respectively, in epithelial, mixed and mesenchymal variants of pleomorphic adenoma (table II).

In the tissue of the salivary gland surrounding the pleomorphic adenoma, as well as in the intact tissue of the salivary gland (fig. 4) we determined a positive immunohistochemical reaction with MCA to anti-Epstein-Barr virus. Positive membrane-cytoplasmic expression of this marker was found in the epithelial structures and myoepithelial cells of the salivary glands. In all histological variants of pleomorphic adenoma, the relative number of positively stained cells in the intact tissue of the salivary gland was lower ($p<0.05$) compared with the tissue of the salivary gland surrounding the tumor (table II). The indicators of the relative number of positively stained cells in the tissue of the salivary gland (intact and surrounding the tumor) had maximum, moderate and minimum values ($p<0.05$), respectively, in epithelial, mixed and mesenchymal variants of pleomorphic adenoma (table II). The indicator of the relative number of positively stained cells decreased ($p<0.05$) in the direction from the tumor tissue to the tissue of the salivary gland surrounding the tumor and especially the intact tissue of the salivary gland.

Today, viruses are the cause of 15% of tumors development worldwide. The main evidence of the viral etiology of a tumor is an immunohistochemical study, determining the virus or viral particles in tumor cells, as well as viral oncogenes detection, the appearance of which in the cell genome promotes tumor initiation [7].

The immunohistochemical study has shown that the Epstein-Barr virus and, especially, human papilloma virus type 16 can act as exogenous trigger factors involved in the development of pleomorphic adenoma of the salivary glands in humans. This fact is consistent with the research results of other scientists [8]. Our analysis revealed that human papilloma virus type 16, in comparison with Epstein-Barr virus, significantly contributes to the development of pleomorphic adenomas of the salivary glands.

A few studies revealed that there was no causal relationship between the infection of an individual with the human papilloma virus, the Epstein-Barr virus and the development of salivary gland tumors in him [9].

Table II. The average value of the relative number of positively stained cells (%) in the reaction with MCA to anti-Epstein-Barr virus.

Pleomorphic adenoma, histological variant	Salivary gland tissue		
Surrounding the tumor	Intact		
Mesenchymal	17.9±2.77 [1,3,4,5]	9.4±1.96 [1,3,4]	1.9±1.05 [1,3,5]
Mixed	31.4±3.07 [2,3,4,5]	18.1±1.52 [2,3,4]	10.2±1.18 [2,3,5]
Epithelial	46.2±3.93 [1,2,4,5]	29.7±2.49 [1,2,4]	17.5±2.55 [1,2,5]

Note: [1] – significant differences compared with the indicators in the mixed variant of pleomorphic adenoma, [2] – significant differences compared with the indicators in the mesenchymal variant of pleomorphic adenoma, [3] – significant differences compared with the indicators in the epithelial variant of pleomorphic adenoma, [4] – significant differences compared with the indicator in the salivary gland intact tissue, [5] – significant differences compared with the indicator in the tissue of the salivary gland surrounding the pleomorphic adenoma.

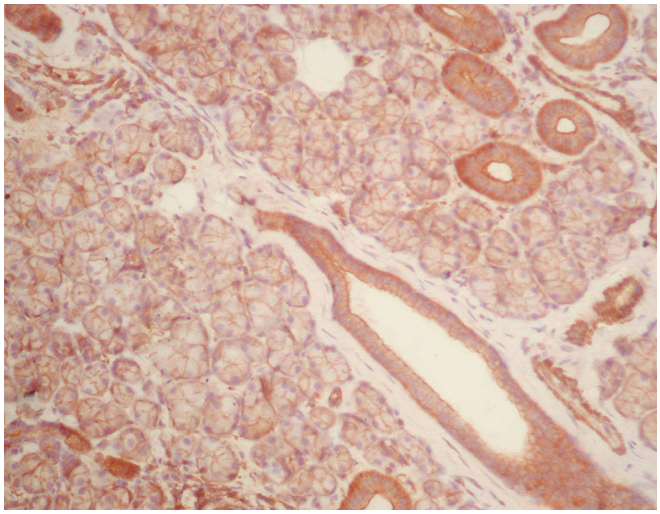

Fig. 3. The expression of MCA to anti-Epstein-Barr virus in pleomorphic adenoma of salivary gland. Immunohistochemical reaction with MCA to anti-Epstein-Barr virus, × 200.

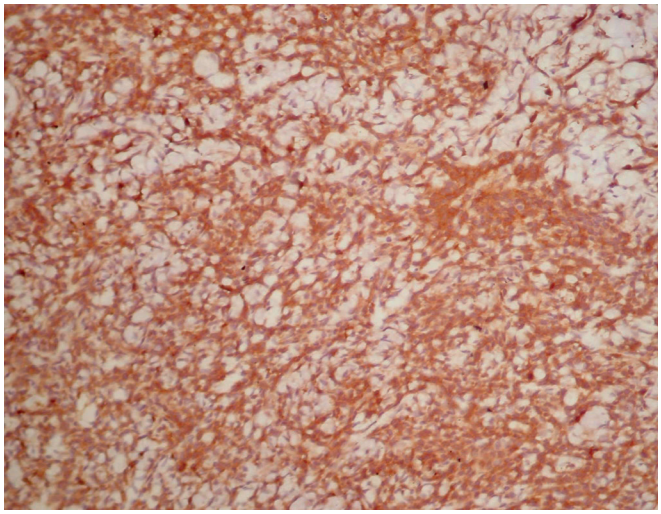

Fig. 4. The expression of MCA to anti-Epstein-Barr virus in intact tissue of the salivary gland. Immunohistochemical reaction with MCA to anti-Epstein-Barr virus, × 200.

The study identified 8 cases when a combination of both markers expression (MCA to human papilloma virus type 16 and anti-Epstein-Barr virus) of varying severity was determined in patients with pleomorphic adenoma. In these cases, from our point of view, the synergistic effect of both viruses may increase the risk of tumor recurrence after surgical treatment or the development of a malignant tumor (carcinoma ex pleomorphic adenoma) [10].

Co-infection with viruses and virus/virus direct or indirect interactions have been found in some malignancies, and a typical example for a malignant tumor due to virus/virus interactions is Kaposi's sarcoma-associated *herpes virus* (KSHV)-induced Kaposi sarcoma in patients with acquired immunodeficiency syndrome [11].

During the study, the authors revealed a statistically significant difference between the expression in pleomorphic adenomas of MCA to human papilloma virus type 16, anti-Epstein-Barr virus and the histological variant of the tumor. It has been proved that epithelial, mixed and mesenchymal variants of the tumor were characterized, respectively, by the severely expressed, moderately expressed and minimally expressed the above-mentioned MCA.

Bartkowiak E. et al. showed that human papilloma virus type 16 overexpression was connected with pleomorphic adenoma proliferation and subsequent malignant transformation to carcinoma (carcinoma ex pleomorphic adenoma) [12].

In the course of the study, the authors also revealed the expression of MCA to human papilloma virus type 16, anti-Epstein-Barr virus not only in pleomorphic adenomas, but also in the surrounding tissue of the salivary gland, as well as in the tissue of the intact salivary gland. Interestingly, the expression of these markers decreased in the direction from the tumor tissue to the surrounding and especially intact salivary gland tissue.

In the intact salivary gland, as well as in the salivary gland surrounding the pleomorphic adenoma, the expression of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus was found in the parenchymal component in epithelial structures and myoepithelial cells. The latter were epithelial cells in origin, and functionally they were contractile elements that resemble muscle cells. The expression features, revealed by the authors, indicate the tropism of human papilloma virus type 16 and Epstein-Barr virus specifically to the epithelial cells of the salivary gland. The severity of expression of the above-mentioned markers in the salivary gland was determined by the histological variant of the tumor (the most pronounced in the epithelial variant, moderately pronounced in the mixed variant, minimally pronounced in the mesenchymal variant).

The expression of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus, detected by the authors in the tissue of the salivary gland surrounding the pleomorphic adenoma, as well as in the intact tissue of the salivary gland, is of great

therapeutic importance in terms of choosing the correct tactics of surgical intervention in this category of patients. This fact, as well as the results of the previous genetic study [13], allows us to recommend the extracapsular tumor dissection in patients with pleomorphic adenoma with resection of the adjacent intact tissue of the salivary gland at a distance of 10 mm [14].

CONCLUSIONS

1. Epithelial, mixed and mesenchymal variants of pleomorphic adenoma of the salivary glands are characterized, respectively, by the severely expressed, moderately expressed and minimally expressed of MCA to human papilloma virus type 16 and anti-Epstein-Barr virus. The parenchymal component of pleomorphic adenoma is characterized by more marked expression of these markers as compared to the stromal component.
2. The epithelial cells of the salivary glands, surrounding the pleomorphic adenoma, as well as intact salivary glands, express MCA to human papilloma virus type 16 and anti-Epstein-Barr virus. The severity of the expression of these markers in the salivary gland is determined by the histological variant of the tumor (severely expressed in the epithelial variant, moderately expressed in the mixed variant, and minimally expressed in the mesenchymal variant).
3. The revealed immunohistochemical features of MCA expression to human papilloma virus type 16 and anti-Epstein-Barr virus in the salivary gland surrounding the pleomorphic adenoma and in the intact tissue of the salivary gland make it possible to recommend the extracapsular dissection of the tumor with resection of the adjacent intact tissue of the salivary gland at a distance of 10 mm in patients with pleomorphic adenoma.

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Conflict of interest:

The Authors declare no conflict of interest.

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