Research Article

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Complex immunohistochemical evaluation of pleomorphic adenomas of the salivary glands

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Background: Diagnostic methods are important in modern medicine. Among the most well-known methods are histological and immunohistochemical. Each of the listed methods is used for diagnostics of various human neoplasms, in particular, tumours of the salivary glands. Thus, most sources and scientific articles attempt to evaluate and carry out the diagnosis of tumours of the salivary glands (pleomorphic adenomas) by only one immunohistochemical criterion, while a complex approach is used only in individual cases. The purpose of the research was to conduct a comprehensive immunohistochemical evaluation of pleomorphic adenomas of the salivary glands.

Materials and Methods: Diagnostic methods are important in modern medicine. Among the most well-known methods are histological and immunohistochemical. Each of the listed methods is used for diagnostics of various human neoplasms, in particular, tumours of the salivary glands. Thus, most sources and scientific articles attempt to evaluate and carry out the diagnosis of tumours of the salivary glands (pleomorphic adenomas) by only one immunohistochemical criterion, while a complex approach is used only in individual cases. The purpose of the research was to conduct a comprehensive immunohistochemical evaluation of pleomorphic adenomas of the salivary glands.

Results: The analyses of the research showed that the parotid salivary gland was affected in most of the patients with pleomorphic adenomas. Among the viruses that were present-19 (67.85%) patients had HPV type 16, and 8 (28.57%) patients had the Epstein-Barr virus dominating. Among the hormones, the distribution was as follows: 3 (10.7%)-progesterone, 2 (7.14%)-oestrogen. PLAG1 had the highest expression in the parotid salivary gland tissues-24 (88.88%).

Conclusion: The complex evaluation of the immunohistochemical parameters of the pleomorphic adenomas of the salivary glands (mainly in the parotid salivary glands) has established the presence of 5 markers: ER (oestrogen), PR (progesterone), HPV type 16, EBV, and PLAG1.

Keywords: Salivary gland tumours, Pleomorphic adenoma, Immunohistochemistry, Oestrogen (ER), Progesterone (PR), Human papillomavirus (HPV) type 16, Epstein-Barr virus (EBV), PLAG1

Introduction

Diagnostic methods are important in modern medicine. They allow to clarifying the clinical diagnosis, choose the best method of treatment, and carry out preventive measures [1]. Among the most well-known methods are X-ray (CT and MRI with and without contrast, scintigraphic studies), ultrasound (ultrasonography), various biopsies (incisional, excisional, precision, needle, FNA-fine-needle aspiration biopsy with or without additional ultrasound), immunological, histological, and immunohistochemical [2]. Each of the listed methods is used for the diagnostics of various human neoplasms, in particular, tumours of the salivary glands. They make up to 1 to 5% of all human tumours [3]. One of the most common benign tumours of the salivary glands is pleomorphic adenoma (61-90%) [4].

Clinical peculiarities of pleomorphic adenomas are sufficiently studied and do not pose any specific difficulties among specialists when establishing a preliminary diagnosis. However, the issues of aetiology, morphology, and the immunohistochemical verification of this nosology are constantly debated and supplemented by new data.

Thus, the influence of persistent viral infections on the development of various types of tumours and their endocrine dependence has already been sufficiently studied. In particular, it is known of the role of human papilloma virus (types 6, 8, 16, 18) in the development of cervical cancer [5,6], as well as the effect of hyperestrogenemia on the development of tumours in the mammary glands [7].

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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It is also known that the accuracy of morphological verification, as a rule, is no more than 85%. In order to increase the percentages of morphological verification, a modern immunohistochemical analysis is used with paraffin blocks produced from the tumour tissues. This allows for establishing the nature of the tumours (benign or malignant), and their differentiation, nature of growth, together with most of any additional signs of tumours, in order to obtain a final diagnosis [5,6]. One of the most prominent immunohistochemical markers for the diagnosis of pleomorphic adenoma is PLAG1. The accuracy of the diagnosis when using this indicator is not less than 94% [8-11].

Thus, most sources and scientific articles attempt to evaluate and carry out the diagnosis of tumours of the salivary glands (pleomorphic adenomas) by only one immunohistochemical criterion, while the complex approach is only used in individual cases. Therefore, the purpose of this research was to conduct a comprehensive immunohistochemical evaluation of pleomorphic adenomas of the salivary glands.

Materials and Methods

The histological typing of the salivary gland tumours was performed using routine staining with hematoxylin and eosin, and immunohistochemistry. The study was conducted based on excisional biopsies of the pleomorphic adenomas of the salivary glands in 28 patients. For the study, a 10 × 10 tumour of up to 4 mm in thickness was collected from the removed parotid and the submandibular salivary glands. The cut tumour pieces were inserted into cassettes, then placed into the cassette holder, which was moved to a fixation container with 10% formalin buffered with pH 7.4 for 16 hours. The material was condensed in paraffin using a Histos-5 histoprocessor (Milestone, Italy), according to the program for surgical material 4 mm. After completing the paraffin-condensing program, the cassettes were removed from the paraffin block of the histoprocessor and moved to the filling station of the HESTION TEC-2800 Embedding Centre, where they were filled with molten paraffin, followed by freezing in the refrigerator module of the HESTION TEC-2800 Cryo Console. From the obtained paraffin blocks, histological sections were made of 5 microns thick using a Microm HM 325 (Thermo Scientific, Germany) microtome. The sections were stained with hematoxylin and eosin for the histopathological examinations of the tumour, the morphometry, and for the calculations of the volume of viable tumour tissue.

The immunohistochemistry was performed with PLAG1 Monoclonal Antibody (M02) clone 3B7, China; Monoclonal Mouse Anti-Epstein Barr Virus LMP Clones CS. 1-4 (Dako IS 753, Denmark); Polyclonal Rabbit Antibody p16 (CDKN2A) (Thermo Scientific PAL. 16662); Monoclonal Rabbit Anti-Human Oestrogen Receptor a Clone SPI (Dako IS 151, Denmark); Mouse Anti-Human Progesterone Receptor Clone PgR 636 (Dako IR068, Denmark); with the use of an EnVision TM FLEX detection system, Dako, Denmark. The antigen masking was carried out in a citrate buffer pH 6.0 at 95°C. The primary antibodies were incubated at room temperature for 30 min, the secondary antibodies for 20 min. The sections were stained with Gill hematoxylin. The tissue samples with a definite positive reactivity were used for the positive control, and for the negative control, the procedure without the use of primary antibodies was performed.

The obtained histological tissue samples were studied and photographed using an OLYMPUS CX 41 microscope with a Quick PHOTO MICRO 2.3 camera and software under standardized conditions.

Results

The analyses of Table 1 showed that the parotid salivary gland was affected in most patients with pleomorphic adenomas. Among the viruses that were present in the tumour tissues of the parotid salivary glands, 19 (67.85%) patients had HPV type 16 and 8 (28.57%), with the Epstein–Barr virus dominating. Among the hormones, the distribution was as follows: 3 (10.7%)- progesterone, 2 (7.14%)-oestrogen. PLAG1 had the highest expression in the parotid salivary gland tissues 24 (88.88%).

When calculating the immunohistochemical parameters of the pleomorphic adenomas in the different types of salivary glands, an increase of the HPV type 16 parameter to 75% (Figures 1-4) was observed Tables 2 and 3.

The analyses of the data showed that 12 (44.44%) patients (or biopsy samples) had the marker intensity of "++"; in 9 (33.33%) patients, it was "+++"; and in 6 (22.22%) patients-"+" (Figures 5-7).

Table 1

Immunohistochemical parameters among the pleomorphic adenomas of different types of salivary glands (n=28)

Immunohistochemical parameters	Parotid salivary glands	Submandibular salivary glands	Sublingual salivary glands	Minor salivary glands
Epstein-Barr virus (EBV)	8 (28.57%)	-	-	-
Human papillomavirus type 16 (HPV)	19 (67.85%)	1 (3.57%)	-	1 (3.57%)
Oestrogen (ER)	2 (7.14%)	-	-	-
Progesterone (PR)	3 (10.7%)	-	-	-
PLAG1	24 (88.88%)	2 (7.4%)	-	1 (3.7%)
Total number of examined patients with adenomas (28)	Positive in 27 patients			
The indicator was negative in one patient!!!				

When calculating, the researchers took into account that one patient had several positive immunohistochemical parameters at once

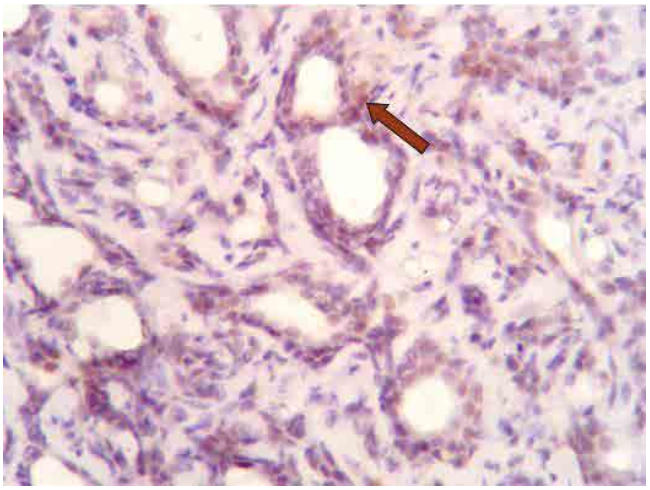

Figure 1: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient P., 23-years-old. Magnification 200x, weak positive nuclear immunohistochemical reaction to ER (indicated by an arrow).

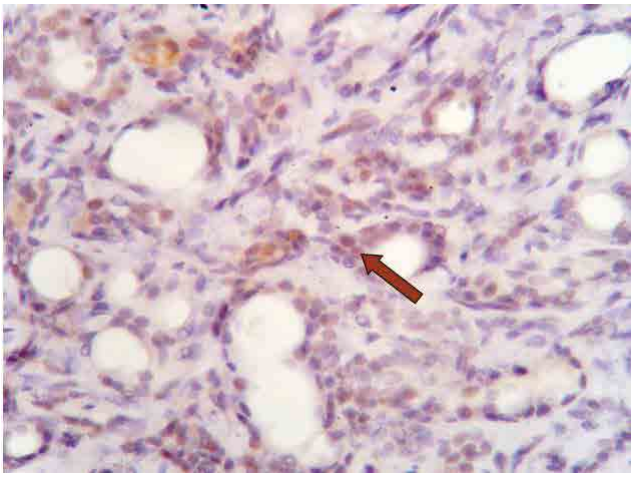

Figure 2: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient P., 23-years-old. Magnification 200x, moderately positive nuclear immunohistochemical reaction to PR (indicated by an arrow).

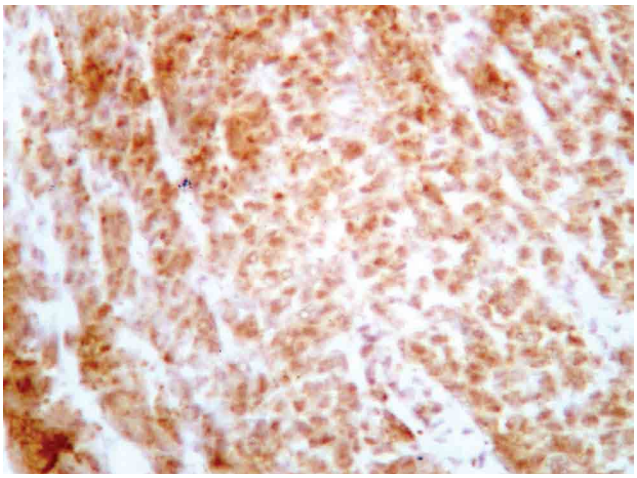

Figure 3: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient L., 33-years-old. Magnification 200x, positive nuclear-cytoplasmic immunohistochemical reaction to HPV type 16.

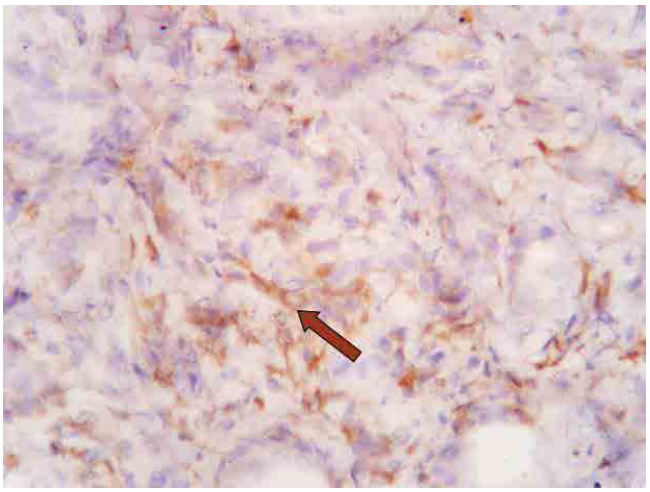

Figure 4: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient P., 23-years-old. Magnification 200x, positive membrane cytoplasmic immunohistochemical reaction to EBV (indicated by an arrow)..

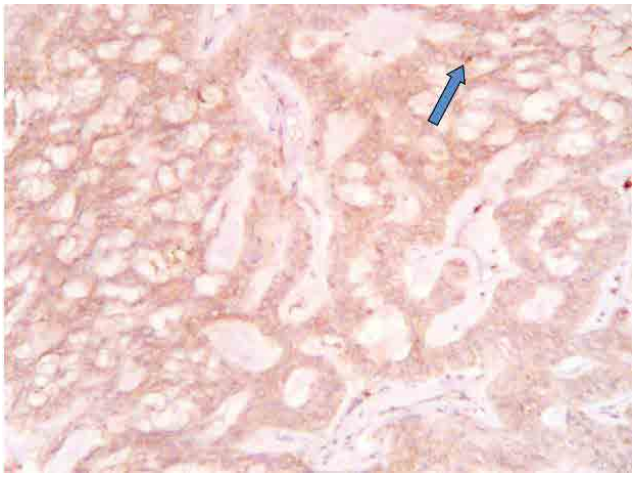

Figure 5: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient B., 46-years-old. Magnification 200x, weak positive (+) nuclear-cytoplasmic immunohistochemical reaction to PLAG1 (indicated by an arrow).

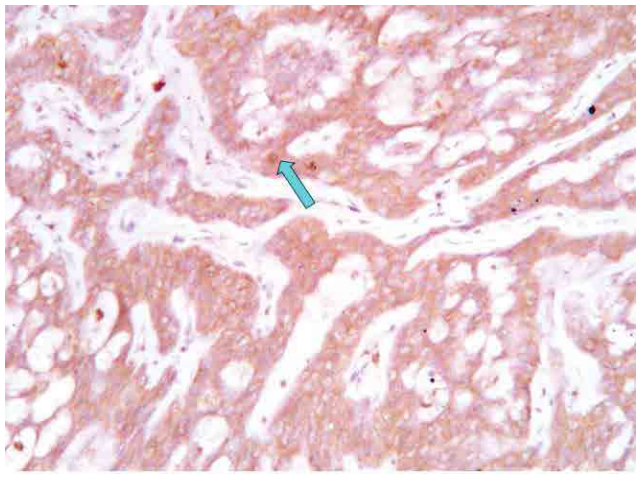

Figure 6: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient F., 18-years-old. Magnification 200x, moderately positive (++) nuclear-cytoplasmic immunohistochemical reaction to PLAG1 (indicated by an arrow).

Table 2

Immunohistochemistry parameters for the pleomorphic adenomas of the salivary glands (n=28).

Immunohistochemical parameters	Absolute quantity	Relative quantity, %
Oestrogen (ER)	2	7.14%
Progesterone (PR)	3	10.70%
Epstein-Barr virus (EBV)	8	28.57%
Human papillomavirus type 16 (HPV)	21	75%
PLAG1	27	96.40%
Total number of examined patients with adenomas	28	100%

When calculating, the researchers took into account that one patient had several positive immunohistochemical parameters at once

Table 3

Immunohistochemical marker PLAG1. Characteristics of the intensity of staining for the pleomorphic adenomas of the salivary glands.

Immunohistochemical markers	+	++	+++
PLAG1	6 (22.22 %)	12 (44.44%)	9 (33.33%)
Total number of examined patients with adenomas (n=28)	Positive in 27 patients (96.4%)		
The indicator was negative in one patient!!!			

The calculations in percentages were based on the number of patients with a positive reaction

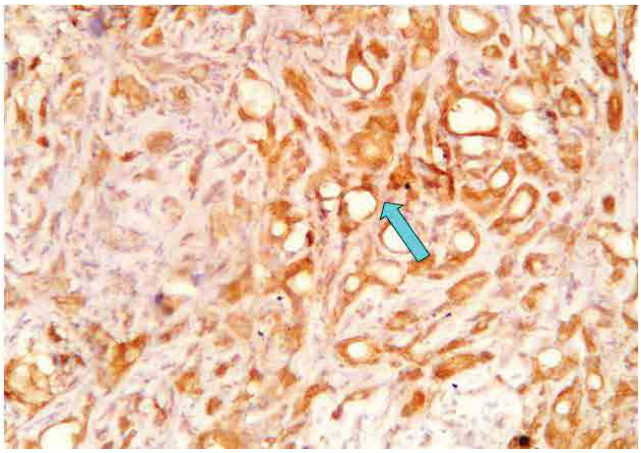

Figure 7: Photo of a histological tissue sample of pleomorphic adenoma of the parotid salivary gland of patient B., 68-years-old. Magnification 200x, strong positive (+++) nuclear-cytoplasmic immunohistochemical reaction to PLAG1.

Discussion

The influence of viral infections on tumour development is already well known. Most of them play a significant role in the aetiology of both benign and malignant tumours. Among the most prominent viral agents are human papillomavirus, Epstein–Barr, cytomegalovirus, hepatitis B, hepatitis C, and various types of herpesvirus. Human papillomavirus, cytomegalovirus, and the herpes simplex virus play a significant role in the development of cervical cancer [12]. Human papillomavirus causes the development of various types of oropharyngeal, hypo- and nasopharyngeal carcinomas, as well as non-melanoma skin cancer. Hepatitis B, Hepatitis C, and Kaposi's sarcoma-associated herpesviruses are involved in the development of various types of hepatocellular carcinomas [13]. The Epstein–Barr virus, in most cases, causes the development of the majority of lymphocyte-associated tumours, in particular, lymphoma [14]. In addition, the relationship between some viruses (retrovirus is activated by Kaposi's sarcoma-associated herpesvirus and the Epstein–Barr virus) and their ability to mutually activate and enhance each other's activity, thereby, promoting the development of tumours, with subsequent carcinogenesis [15].

The salivary glands, as an organ that, in addition to the salivation function, perform the endocrine and immune functions too; they often suffer defeat from various viruses, with the further development of tumour processes. Most researchers have described the immunohistochemical studies of pleomorphic adenomas, adenolymphomas, and adenocarcinomas, whereby the HPV types 16 and 18, as well as EBV [12,13], were the major viral agents. Thus, 16-24 (33-39%) pleomorphic adenomas have a positive reaction to HPV type 16, and 2 (5-7%) have a positive reaction to EBV [8]. There are studies, which indicate the absence of HPV and EBV in the tumour tissues and in the salivary glands, or in an insignificant and statistically insignificant percentage of observations, which calls into question the influence of these types of viral pathogens on the development of salivary gland tumours [14].

In the literary sources, there has been a long-time discussion about the importance of the influence of certain types of hormones on tumour genesis. Thus, among the main ones are androgen, oestrogen, and progesterone-hormones that act through specific receptors and that play an important role in the growth and the development of several tumours, including breast, endometrial, and prostate carcinoma [8]. Actis indicated a certain connection and a similarity of histopathological types between the tumours of the salivary glands, breast, and prostate tumours. Thus, the structure of pleomorphic adenoma is very similar to a mucocellular tumour of the breast gland [16].

When analysing most immunohistochemical studies of salivary gland tumour tissues, it is evident that the expression of ER, AR, and PR is 7-30% for benign tumours, and 30-50% for malignant tumours [15,17,18]. In this case, some authors argue in their studies that the expressions of ER, AR, and PR do not play a significant role in the tumour genesis of the salivary glands [19-21].

The conducted immunohistochemical studies of the paraffin blocks from the removed tumours of the salivary glands (pleomorphic adenomas) regarding the expressions of the oestrogen (ER) and progesterone (PR) receptors have shown the following results for PA (pleomorphic adenomas): ER-2 (7.14%) and PR-3 (10.14%) at (n=28).

Thus, this current data suggests a rather low hormonal dependence in the pleomorphic adenomas (ER-7.14% and PR-10.14%).

The researcher's immunohistochemical studies of the paraffin blocks from the removed tumours of the salivary glands (pleomorphic adenomas) have indicated that two viruses, HPV type 16-21 (75%) and EBV-8 (28.57%) were dominant for the pleomorphic adenomas. By comparing the data obtained with available scientific sources, it can be concluded that the increase (prevalence) of HPV type 16 and EBV in the tumour tissues of the salivary glands and, therefore, their role in tumour development is beyond doubt.

Hence, it can be reliably asserted that when comparing the role of viruses and hormones in the possible development of tumour processes in the salivary gland tissues, the primary influence is shown by HPV type 16 and EBV. The presence of oestrogen (ER) and progesterone (PR) receptors in the tumour tissues of the salivary glands can further increase the tumour growth, but only in some cases. The importance of immunohistochemistry in the diagnosis of benign tumours is also beyond doubt. The ability for malignancy and the relapse for some of them, like the pleomorphic adenomas of the salivary glands, prompt a search for more modern and targeted immunohistochemical tumour markers [22].

It is well known that chromosomal translocations in control points 8q12, 3p21, and 12q13-15, which correspond to the PLAG1, b-catenin 8, and HMGIC9 genes, are one of the factors for the development of pleomorphic adenoma [11]. PLAG1 is currently the main and most reliable immunohistochemical marker that is used to confirm the diagnosis of pleomorphic adenoma of the salivary glands.

PLAG1 (pleomorphic adenoma gene 1) is a proto-oncogene, which according to some reports, occurs in 93% of cases of pleomorphic adenomas, and in 94% of cases with a relapse of pleomorphic adenoma. It is expressed in the epithelial cells, but it is more frequent in the myoepithelial cells. It plays a certain role in embryogenesis and foetal development and it reduces its expression during morphosis. In addition to pleomorphic adenomas, the PLAG1 expression has also been described in lipoblastomas, hepatoblastomas, leiomyomas, leiomyosarcomas, and in some cases of acute myeloid leukaemia. The PLAG1 protein enhances the expression of several gene growth factors, not only significantly contributing to tumour genes, but also to suppressing the genes that inhibit cell proliferation. Therefore, PLAG1 may play a double role in tumour formations, and this has been confirmed in various studies of benign tumours and low-differentiated carcinomas [6].

The current researchers' study has shown that 27 (96.4%) patients with a morphological diagnosis of "pleomorphic adenoma of the salivary glands" showed a positive reaction to the immunohistochemical marker PLAG1. That is, the accuracy of the immunohistochemical verification was 96.4%. At the same time, the intensity of staining by this marker was different. In most cases, in 12 (44.44%) patients, the intensity was "++". In 24 (88.88%) patients, the process was localized in the parotid salivary gland. Thus, the use of PLAG1 in the diagnosis of pleomorphic adenoma of the salivary gland can further increase the morphological and immunohistochemical verification of the diagnosis to 96.4%.

Conclusion

The study's complex evaluation of the immunohistochemical parameters of pleomorphic adenomas of the salivary glands (mainly in the parotid salivary glands) has established the presence of 5 markers: ER (oestrogen)-3 (10.7%), PR (progesterone)-2 (7.14%), HPV type 16-21 (75%), EBV-8 (28.57%), and PLAG1-27 (96.4%) [23]. The immunohistochemical marker PLAG1 had a positive reaction in 96.4% of the biopsies of the pleomorphic adenomas of the salivary glands, with a different intensity of staining by the recommended scale: "++" in 12 (44.44%), "+++" in 9 (33.33%), and "+" in 6 (22.22%) patients.

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