



THE ROLE OF MOLECULAR BIOLOGICAL AND IMMUNOLOGICAL MARKERS IN THE DIAGNOSTICS AND TREATMENT OF PATIENTS WITH OROPHARYNGEAL CANCER

6395

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ABSTRACT

The analysis of the results of the study showed the significance of determining the serum concentration of vascular endothelial growth factor (VEGF) in patients with oropharyngeal cancer, which, in combination with immunological parameters, helps in the diagnosis of severe forms of the process, and is also significant in predicting the risk of recurrence. An integrated approach to immunodiagnosics, virological diagnostics, treatment, and prognosis of the course of the disease has been developed depending on the parameters of the immune status, virological characteristics of the Epstein-Barr virus, and the index of vascular endothelial growth factor.

Keywords: oncology, dentistry, cancer of the oral cavity and oropharynx, diagnostics, Epstein-Barr virus, molecular biological and immunological markers, treatment, prognosis.

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INTRODUCTION

The study of the role of herpesviruses in the occurrence of human tumors is an important aspect of modern research in the field of oncology since members of the herpesvirus family have neuropathogenic, lymphoproliferative properties, are immunosuppressants that act at the system level [1, 2, 5, 6, 9, 12, 14, 16].

Human herpesviruses can persist in the body for decades or proceed in a latent form, and when reactivated, they can cause violent clinical manifestations, possibly before the development of meningoencephalitis, hepatitis, pancreatitis, keratitis, or thyroiditis, which can often lead to death, and in moderate or mild - can occur as a chronic recurrent or asymptomatic chronic infection. Therefore, herpesviruses throughout a person's life have a constant increasing effect on the immune system, which cannot but affect the

functional state of the body's immunoreactivity [3, 4, 7, 8, 10, 11, 13, 15].

Thus, the main goal of this study was to study the state of the body's immunoreactivity by determining the parameters of the immune system and identifying the activity of the Epstein-Barr virus by detecting serological markers of the virus in the blood serum, as well as quantifying the virus in the blood plasma and saliva of these patients.

MATERIALS AND METHODS

The results of a survey of 65 patients with oropharyngeal cancer were included in the study. Patients underwent serological, immunological, and molecular genetic studies to clarify the pathogenetic mechanisms of virological and immunological factors in the development and course of oropharyngeal cancer variants. In the Tashkent regional branch of the Republican



Specialized Scientific and Practical Medical Center of Oncology and Radiology (TRB RSPMCMCOR) from 2008 to 2017 (over the past 10 years), 1021 patients with oropharyngeal cancer (OPRC) underwent combined and complex treatment. According to the morphological study, all patients were diagnosed with OPR. Of these, the study included 427 patients who underwent combined and complex treatment. The study included 33 patients operated on at the Rostov Cancer Research Institute (RCRI). The patients were divided into 2 groups: the main group (n=149), who underwent standard treatment, including mandibulotomy with urostomy, and the control group (n=278), who did not undergo this type of operation.

The main criteria for inclusion in the study were clinically and morphologically established widespread OPR. Patients with early cancer - oropharyngeal zone (OPD) - 233 (22.8%) patients were not included in the study. As a result, in our work, we consider locally common (T3, T4) forms of OPR. Those 361 (35.3%) patients who had distant organ metastases by the time of the initial visit were also not included in the study. The distribution of patients by gender was as follows: in the main group - men 60.4% (90), women - 39.6% (59); in the control - 58.6% (163); 41.4% (115).

The serological method for identifying EBV markers is based on the detection of antibodies to EBV antigens, based on the method of enzyme immunoassay (ELISA). The sensitivity of the method was 2.7 ng/ml. Test systems "Vector-Best", Russia were used. Polymerase chain reaction (PCR) is an in vitro DNA amplification method that can isolate and multiply a certain DNA sequence billions of times within a few hours. The principle of the method consists of repeated copying (amplification) in a test tube of certain, relatively small sections of RNA, tDNA with a length of several tens to several hundred base pairs in the process of repeated temperature cycles.

Active virus replication in patients was confirmed by semi-quantitative PCR in serum. All patients after PCR - detection of EBV RNA in peripheral blood serum were determined by the level of viral load by ELISA using commercial test systems of International Reagents Corporation (Japan). The sensitivity of the method was 0.8 pg/ml.

All patients underwent immunological studies in peripheral blood serum at the initial admission after the diagnosis was established before treatment to identify the values of the most vulnerable immunological markers that play an important role in the diagnosis, dynamics of treatment, and prognosis of oncological diseases.

We have studied cellular and humoral parameters of immunity in patients with oropharyngeal cancer (main group) and, for comparison, in practically healthy individuals (control group). Cellular immunity parameters were determined by the content of leukocytes, lymphocytes, the total pool of T-lymphocytes (CD3+), T-helpers/inducers (CD4+), and T-suppressors/cytotoxic lymphocytes (CD8+), immunoregulatory index - IRI (CD4+/CD8+ ratio).

6396

RESULTS AND DISCUSSION

To detect the Epstein-Barr virus in patients with oropharyngeal cancer, we used enzyme immunoassay and molecular genetic research methods.

First, in the blood serum of patients, we determined specific immunoglobulins of class M and G to various EBV antigens: capsid (VCA), nuclear (NA), and early (EA) proteins. In the group of patients with oropharyngeal cancer, class M antibodies to the above antigens were not detected, which makes it possible to exclude an acute infectious process caused by EBV. The frequency of detection of specific antibodies to various EBV proteins in oropharyngeal cancer is shown in Table. 2.

Table 2

The frequency of detection of specific antibodies to various antigens in oropharyngeal cancer

The combination of detected antibodies to antigens EBV infections	Several patients, abs.	Number of patients, %



IgG to capsid (VCA) + IgG to nuclear (NA) antigens	31	48%
IgG to capsid (VCA) + IgG to nuclear (NA) + IgG to early (EA) antigens	4	6%
IgG to nuclear (NA) antigen	9	14%
IgG to capsid (VCA) antigen	21	32%
TOTAL	65	100%

Among the examined patients, in 31 patients with oropharyngeal cancer, a combination of class G antibodies to capsid and nuclear antigens (VC + NA) was detected significantly more often (in 48% of cases), which confirms the long-term presence of the virus in the body. It is important to note that, according to the results of studies by several authors, this combination of specific antibodies is associated with herpesvirus-associated oncological conditions. In 4 patients (6%), antibodies to all EBV antigens were immediately detected. The detection of antibodies to all proteins in the blood serum of patients reflected the process of activation of the acquired process. The detection of antibodies only to the capsid antigen in 21 patients (32% of cases) can be explained by the fact that these patients have already suffered an active infectious process caused by EBV, as a result of which antibodies to the nuclear antigen of the virus are no longer

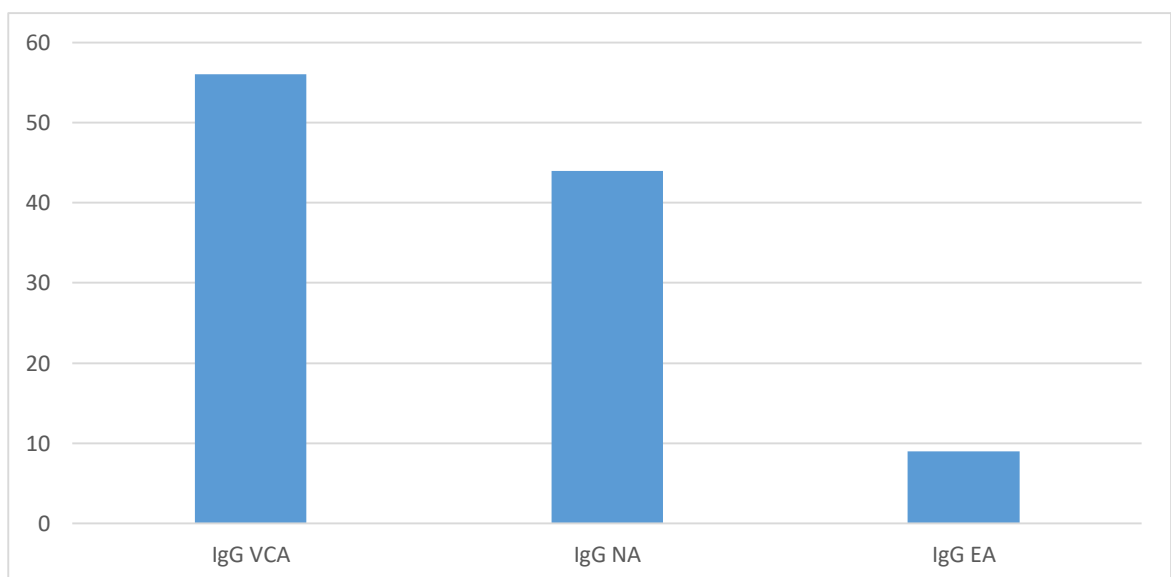
produced. The detection of all these antigens in the serum of patients confirms the essential role of EBV in the proliferative process.

When considering the frequency of detection of EBV infection antigens, both in isolated and combined variants, the following data were revealed (Fig. 1).

It was revealed that in the examined patients significantly ($p < 0.001$) in more cases, class G antibodies developed to VCA and NA were detected than to other antigens which are considered evidence of the presence of EBV infection.

In the examined patients, IgG antibodies to VCA were detected in 56% of cases, and to EBV NA in 44% of patients, and antibodies to these antigens were serological markers more often detected in patients with oropharyngeal cancer. The frequency of detection of IgG to EA was significantly lower than IgG to NA and EA and was only 9%.

6397



* - the difference in the frequency of detection of IgG to VCA and NA is significant ($p < 0.01$).



Fig. 1. Detection of IgG to antigens of Epstein-Barr virus infection in patients with oropharyngeal cancer

Considering that the performed serological diagnostics revealed antibodies to various antigens of the virus, which indicates the presence of EBV in the body, the next stage of our research was aimed at studying the replicative activity of the virus by detecting virus RNA in biological fluids by polymerase chain reaction (PCR). According to the latest data in the world literature, it is known that PCR analysis is a decisive criterion for making a diagnosis and choosing a treatment strategy for patients based on the detection of virus activity. However, it was necessary to decide in which biological fluid it would be more promising to determine the replicative activity of EBV since it is already known that the virus can be found in any biological fluid. Elevated levels of circulating DNA of the virus in the blood plasma of cancer

patients, according to the observations of some researchers, have a high diagnostic significance. And given that, according to the literature, the main site of EBV lytic replication is in the oropharynx, where the virus is found in saliva, we conducted PCR studies not only in the blood plasma of patients but also in their saliva.

According to the results of molecular genetic studies, with a qualitative determination of EBV infection RNA in patients with oropharyngeal cancer, the replication activity of EBV infection was detected in 87.4% of patients (PCR plasma "+" and/or PCR saliva "+"), and in 12.6% of the patients we examined, active EBV replication was not detected either in blood plasma or salivary fluid (Fig. 2).

6398

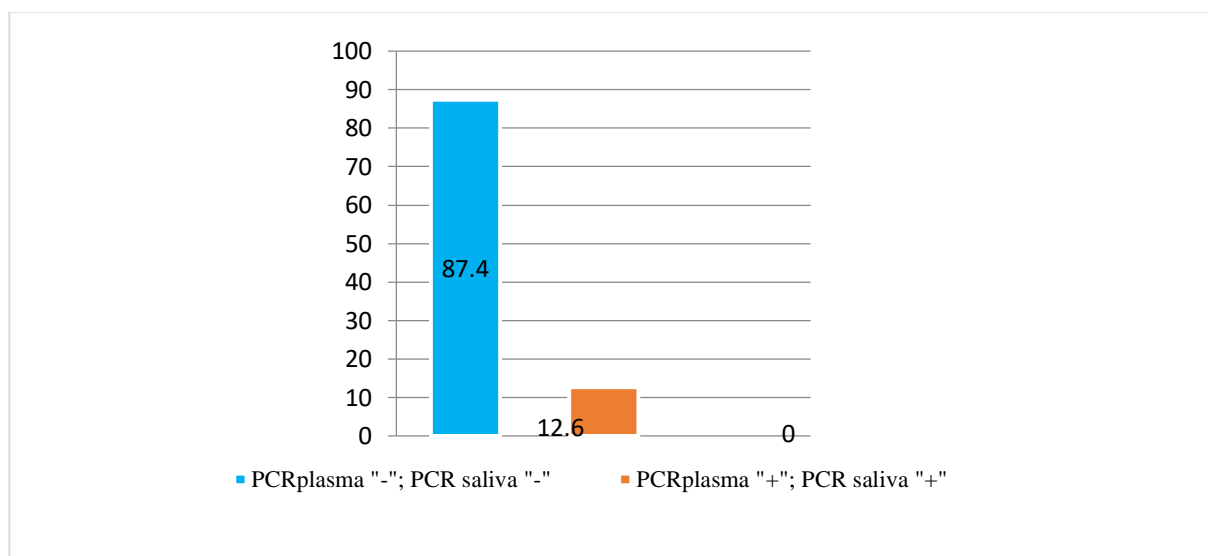


Fig. 2. Results of a PCR study for EBV in plasma and saliva of patients with oropharyngeal cancer

When considering these patients with a positive PCR test result for EBV in terms of the frequency of occurrence of replication in saliva and blood plasma, it was found that in almost 94.8% of these patients, the replication activity of the virus was detected in the salivary fluid of the body, while in plasma blood PCR study gave a negative result (PCR plasma "-"; PCR saliva "+"), which reflects the pronounced tropism of the virus to the epithelial cells of the salivary glands. And only in 3.8% of patients with positive results of PCR studies, the replication

activity of the virus was observed both in the salivary fluid and in the blood plasma of patients (PCR plasma "+"; PCR saliva "+"), and only in 1.4% patients, replicative activity was detected only in saliva (PCR plasma "+"; PCR saliva "-") (Fig. 3). That is, in most patients with oropharyngeal cancer, local (in saliva) replicative activity of EBV is noted, which allows us to assert a significant role of this infection in the development of the proliferative process in this zone.



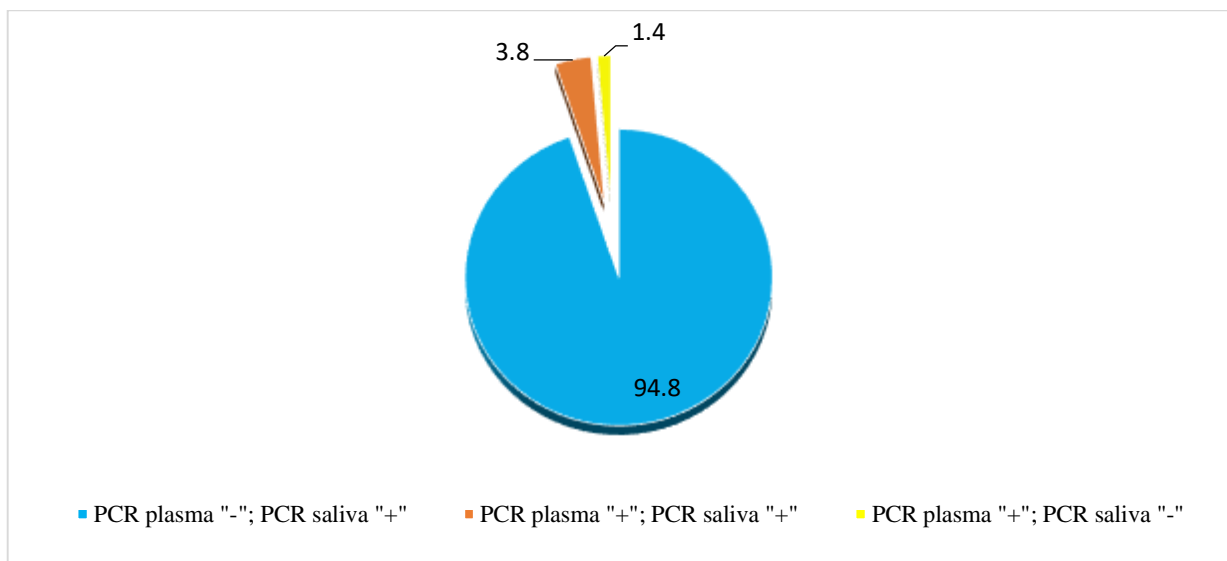


Fig. 3. Distribution of EBV RNA detection in plasma and saliva of patients with oropharyngeal cancer

Thus, as a result of complex studies of serological and molecular genetic parameters, it was determined that patients with oropharyngeal cancer were characterized by more frequent (56%) detection of antibodies to the capsular antigen (VCA) and more often in combination with antibodies to the nuclear antigen (NA) of the Epstein-Barr virus, which is an indicator of chronic persistence of the virus, as well as the frequent detection of viral DNA in the saliva of these patients in the absence of replication in the blood plasma, which gives reason to use these indicators as predictive criteria for the possible development of oropharyngeal cancer.

According to our research, in oropharyngeal cancer, there was a significant increase in the absolute number of leukocytes in the peripheral blood relative to those in the control group ($p < 0.05$). Thus, in patients with oropharyngeal cancer, the level of leukocytes in the peripheral blood was 5977 ± 154.5 per μl , while in the control group it was 4690 ± 126.8 per μl .

The study of the relative content of lymphocytes between the studied groups revealed a slight decrease in lymphocytes in the group of patients ($p > 0.05$). That is, the relative number of lymphocytes was suppressed - $18.7 \pm 2.8\%$ of lymphocytes in the group of

patients with the indicator in the control group - $32.1 \pm 1.38\%$.

CD3+, CD4+, CD8+ differentiation markers are the main immunophenotypic receptors of T-lymphocytes. The presence of CD3+ receptors on the membrane of thymus-dependent lymphocytes shows its function and makes it possible to identify the total number of T-lymphocytes involved in the body's immune response at the moment. The results of studies of the immunophenotype of T-lymphocytes in the group of patients with oropharyngeal cancer revealed a significant decrease in the number of CD3+ lymphocytes compared with the values of the control group ($p < 0.05$). We hypothesized that a decrease in the total number of T-lymphocytes with the CD3+ receptor is observed as a result of a decrease in the expression of CD4+ T-helpers. The CD4+ T-cell response is an important defense mechanism of the body against infections, in particular against viruses, since lymphocytes carrying the CD4+ receptor increase the production of antibodies by B-lymphocytes and increase the activity of lymphocytes with the CD8+ receptor. In the patients with oropharyngeal cancer examined by us, a significant decrease in the expression of CD4+ T-lymphocytes was observed compared with the values of the control group ($p < 0.05$). Thus, in the group of patients, the relative number of



CD4+T-helpers was $26.3 \pm 2.2\%$, while in the control it was $30.8 \pm 1.15\%$.

We need to suppress the T-cell immune response, which has the most pronounced antiviral protective properties.

Cytotoxic CD8+T-lymphocytes play a leading role in the pathogenesis of diseases caused by various viral infections. The leading function of these cells is considered to be the detection of antigens on the cell surface in combination with class 1 MHC molecules. And given their presence on all nuclear substances of the body, each cell carrying MHC class 1 molecules in combination with an antigenic peptide can activate a clone of cytotoxic T-lymphocytes. The evolutionary purpose of the provided activation is the removal of mutated or infected cells. CD8+T-lymphocytes play a key role in the removal of the pathogen which is justified, on the one hand, by their ability to destroy infected cells expressing the appropriate peptides presented by MHC class 1 molecule, and, on the other hand, by their ability to produce substances with antiviral activity. (pro-inflammatory cytokines - IFN- α , TNF- α , etc.).

The study of the expression of CD8+T-lymphocytes in the studied groups revealed that in patients with oropharyngeal cancer there is a tendency to increase the percentage of CD8+T-lymphocytes - $25.4 \pm 3.3\%$, compared with the data of the control group - $18.7 \pm 0.61\%$ ($p > 0.05$). This was the result of the long-term chronic persistence of EBV in the body of patients. Literature data show that EBV can persist even in the presence of a high pool of CD8+ T-lymphocytes, which becomes one of the mechanisms for the progression of viral damage.

Particular attention in viral and oncological processes is attracted by the immunoregulatory index (IRI), which is determined by the ratio of the number of CD4 + T-helpers to the number of CD8 + T-cytotoxic lymphocytes, and normally IRI in healthy people is about 1.5. In the patients with oropharyngeal cancer examined by us, this indicator was equal to 0.89 ± 0.02 this significant decrease compared to the control group 1.5 ± 0.05 ($p < 0.05$), is associated with a decrease in the number of

CD4+T -helpers against the background of some increase in the number of CD8 + T-lymphocytes.

Thus, in patients with oropharyngeal cancer, dysregulation of the cellular immune response was revealed, since a decrease in the immunoregulation index is an indicator of the severity of T-cell immunodeficiency, which is often observed in viral and oncological processes, especially characteristic of infection with latent chronic viruses.

Another immunological indicator that attracted our attention was the activation marker of apoptosis of peripheral blood lymphocytes - CD95+. In the patients with oropharyngeal cancer examined by us, the CD95+ index was $33.4 \pm 1.53\%$, while the value in the control group was $21.9 \pm 0.48\%$. Increased apoptosis in oropharyngeal cancer against the background of chronic EBV persistence in combination with dysregulation of T-cell immunity contributes to the progression of the underlying disease. An increase in the expression of the activation marker of CD95+ lymphocytes, which was observed in 65% of patients, indicates pathological activation and cell death of immune cells, the death of which, in turn, exacerbates the existing cellular immunodeficiency in the oncological process associated with EBV infection. Based on the data obtained by us, data on the immunophenotype of T-lymphocytes confirm the importance of lymphocyte activation markers in the immune response in chronic persistent viral infections.

Thus, the analysis of the results of the study showed the significance of determining the serum concentration of vascular endothelial growth factor (VEGF) in patients with oropharyngeal cancer, which, in combination with immunological parameters, helps in the diagnosis of severe forms of the process, and is also significant in predicting the risk of recurrence. An integrated approach to immunodiagnosics, virological diagnostics, treatment, and prognosis of the course of the disease has been developed depending on the parameters of the immune status, virological characteristics of the Epstein-Barr virus, and the index of vascular endothelial growth factor.

CONCLUSION



1. In patients with oropharyngeal cancer revealed T-cell imbalance, which is manifested by a lack of CD4+T-helpers with an increased number of CD8+T-cytotoxic lymphocytes, which can lead to an inadequate immune response.

2. An increase in the activation marker CD95+, a marker of apoptosis, was revealed, which confirms the formation of cellular immunodeficiency.

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