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MOLECULAR BIOLOGICAL DIAGNOSIS OF ORAL CAVITY AND OROPHARYNGEAL CANCER AND METHODS OF THEIR TREATMENT

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Abstract. The analysis of the study results demonstrated the significance of determining the serum concentration of vascular endothelial growth factor (VEGF) in patients with oropharyngeal cancer. In combination with immunological indicators, this marker helps diagnose severe forms of the disease process and is also important in predicting the risk of recurrence. A comprehensive approach to immunodiagnostics, virological diagnostics, treatment, and prognosis of disease progression was developed depending on immune status indicators, virological characteristics of Epstein–Barr virus, and the level 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.

Relevance of the Study

The study of the role of herpesviruses in the development of human tumors is an important aspect of modern oncology research, since members of the herpesvirus family possess neuropathogenic and lymphoproliferative properties and act as

immunodepressants at the systemic level [1, 2, 9, 12, 24]. Human herpesviruses are capable of persisting in the body for decades or remaining in a latent form; upon reactivation, they can cause severe clinical manifestations, including meningoencephalitis, hepatitis, pancreatitis, keratitis, or thyroiditis, which may lead to fatal outcomes. In moderate or mild cases, infection may occur as a chronic recurrent or asymptomatic chronic process. Therefore, herpesviruses exert a lifelong and cumulative impact on the immune system, inevitably affecting the functional state of immunoreactivity [4, 10, 16, 20, 21, 25].

Although all herpesviruses are potentially pathogenic, during evolution alongside other species and their ancestors over more than one million years, they have reached a balance in the "virus–host" relationship, the stability of which critically depends on host immune control. Most herpesviruses are widespread in the population and often remain silent or manifest only mildly during primary infection in childhood, after which they persist throughout life in a state of asymptomatic latent infection [11, 14, 15, 16, 26].

Epstein–Barr virus (EBV) has unique properties: like all herpesviruses, it can cause cytolysis or persist chronically in cells, but it is also capable of transforming B lymphocytes, endothelial cells, and epithelial cells of the host. Therefore, EBV is a latent immunotropic virus that induces profound immunodeficiency, against the background of which a malignant proliferative process may develop [1, 18, 19, 22, 23, 27, 32].

Thus, the main aim of this study was to investigate the state of immune reactivity by determining the parameters of the immune system and identifying Epstein–Barr virus activity through the detection of serological viral markers in blood serum, as well as quantitative determination of the virus in blood plasma and saliva in these patients.

Materials and Methods

The study included examination results of 45 patients with oral cavity and oropharyngeal cancer. These patients underwent serological, immunological, and molecular genetic investigations to clarify the pathogenetic mechanisms of virological and immunological factors in the development and course of oropharyngeal cancer variants. At the Tashkent Regional Branch of the Republican Specialized Scientific and Practical Medical Center of Oncology and Radiology, from 2010 to 2019, 1,043 patients with oral cavity and oropharyngeal cancer underwent combined and complex treatment. All patients were diagnosed with oral cavity and oropharyngeal cancer based

on morphological examination. A total of 405 patients who underwent combined and complex treatment were included in the study. Among them, 33 patients were operated on at the Republican Specialized Scientific and Practical Medical Center of Oncology and Radiology. The patients were divided into two groups: the main group (n = 143), who received standard treatment including mandibulotomy with orostomy, and the control group (n = 262), in whom this type of surgery was not performed.

The main inclusion criterion was clinically and morphologically confirmed advanced oral cavity and oropharyngeal cancer. Patients with early-stage oral cavity cancer, 231 individuals (22.1%), were not included in the study. Therefore, only locally advanced forms (T3, T4) of oral cavity and oropharyngeal cancer were considered. In addition, 361 patients (35.3%) who already had distant organ metastases at the time of initial presentation were also excluded. The sex distribution was as follows: in the main group, men accounted for 59.4% (85) and women for 40.5% (58); in the control group, men accounted for 58.3% (153) and women for 41.6% (109), respectively (Table 1).

The distribution of patients by sex and age showed a predominance of men: 238 (58.7%) compared with 167 women (41.2%), with a ratio of 1.45:1. The mean age was 59.0±1.4 years for men and 59.6±2.08 years for women, with an age range from 19 to 85 years. Advanced oral cavity and oropharyngeal cancer was found at any age; however, two thirds of patients with stage IV disease were in the 50–70-year age group.

The serological method for identifying EBV markers was based on the detection of antibodies to EBV antigens using enzyme-linked immunosorbent assay (ELISA). The sensitivity of the method was 2.7 ng/ml. “Vector-Best” test systems (Russia) were used. Polymerase chain reaction (PCR) is an in vitro DNA amplification method that allows a specific DNA sequence to be isolated and amplified billions of times within several hours. The principle of the method lies in repeated copying (amplification) in a test tube of relatively short RNA or DNA fragments ranging from several dozen to several hundred nucleotide pairs during repeated temperature cycles.

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

All patients underwent immunological examinations of peripheral blood serum at the time of first admission, after diagnosis and before treatment, in order to identify the most vulnerable immunological markers that are important for diagnosis, treatment dynamics, and prognosis of oncological diseases.

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

Immunological studies included examination of cellular and humoral immune parameters, as well as the activation marker CD95+, using monoclonal antibodies in accordance with methodological recommendations developed by the Institute of Immunology of the Ministry of Health of the Russian Federation and the Institute of Immunology of the Academy of Sciences of the Republic of Uzbekistan (2009). Accordingly, CD4+ T helpers, CD8+ cytotoxic T lymphocytes, CD95+ lymphocytes with receptors for physiological apoptosis, and CDHLA-DR+ MHC class II molecules were determined.

Circulating immune complexes (CIC) of different sizes were determined by ELISA using a Stat-Fax analyzer (USA) in the immunocytokine laboratory of the Institute of Immunology of the Academy of Sciences of the Republic of Uzbekistan. CIC size was determined using different concentrations of polyethylene glycol (PEG). Low PEG concentrations precipitate large CIC with antigen predominance, while high concentrations precipitate small CIC with antibody predominance [17, 31].

Table 1. Distribution of patients by sex in the main and control groups with oropharyngeal cancer

No.	Sex	Main group (abs.)	Main group (%)	Control group (abs.)	Control group (%)	Total
1	Men	85	59.4%	153	58.3%	238
2	Women	58	40.5%	109	41.6%	167
	Total	**143**	**100%**	**262**	**100%**	**405**

Study Results

To detect Epstein–Barr virus in patients with oropharyngeal cancer, enzyme immunoassay and molecular genetic methods were used. First, we determined serum-specific immunoglobulins of classes M and G to various EBV antigens: capsid (VCA), nuclear (NA), and early (EA) proteins. In the group of patients with oropharyngeal cancer, no class M antibodies to the above antigens were found, which made it possible to exclude an acute infectious process caused by EBV.

Table 2. Frequency of detection of specific antibodies to various EBV antigens in oropharyngeal cancer

Combination of detected antibodies to EBV antigens	Number of patients (abs.)	Number of patients (%)
IgG to capsid (VCA) + IgG to nuclear (NA) antigens	25	55.5%
IgG to capsid (VCA) + IgG to nuclear (NA) + IgG to early (EA) antigens	3	6.6%
IgG to nuclear (NA) antigen	7	15.5%
IgG to capsid (VCA) antigen	10	22.2%
Total	**45**	**100%**

Among the examined patients, 25 individuals with oral cavity and oropharyngeal cancer most frequently showed a combination of class G antibodies to capsid and nuclear antigens (VCA + NA), observed in 55.5% of cases, which confirms long-term viral persistence in the body. It is important to note that, according to a number of authors, this combination of specific antibodies is associated with herpesvirus-related oncological conditions [28]. In 3 patients (6.6%), antibodies to all EBV antigens were detected simultaneously. Detection of antibodies to all proteins in serum reflected activation of the acquired process. Detection of antibodies only to the capsid antigen in 10 patients (22.2%) can be explained by the fact that these patients had already undergone an active EBV infection, after which antibodies to the nuclear antigen were no longer produced. Detection of all these antigens in patient serum confirms the significant role of EBV in the proliferative process [26].

When analyzing the frequency of EBV antigen detection in both isolated and combined variants, it was found that class G antibodies to VCA and NA were detected significantly more often ($p < 0.001$) than antibodies to other antigens, which is considered proof of EBV infection. In the examined patients, IgG antibodies to VCA were detected in 56% of cases, while antibodies to EBV NA were found in 44% of patients; these serological markers were the most frequently identified in patients with oropharyngeal cancer. The frequency of IgG detection to EA was significantly lower and amounted to only 9%.

Given that serological diagnostics revealed antibodies to various viral antigens, indicating the presence of EBV in the body, the next stage of our study was aimed at investigating viral replicative activity by detecting viral RNA in biological fluids using PCR. According to current international literature, PCR analysis is a decisive criterion for diagnosis and treatment strategy based on detection of viral activity. However, it remained necessary to determine in which biological fluid it was more promising to assess EBV replicative activity, since the virus may be present in various biological fluids. Considering that, according to the literature, the main site of lytic EBV replication is the oropharynx, where the virus is detected in saliva, we performed PCR studies not only in plasma but also in saliva.

According to the results of molecular genetic studies with qualitative determination of EBV RNA in patients with oropharyngeal cancer, EBV replicative activity was detected in 87.4% of patients (PCR plasma “+” and/or PCR saliva “+”), while in 12.6% of the examined patients, no active EBV replication was detected either in plasma or in saliva.

Among patients with positive PCR results for EBV, analysis of the frequency of viral replication in saliva and plasma showed that in almost 94.8% of these patients, viral replicative activity was detected in saliva, whereas PCR in plasma was negative (PCR plasma “–”; PCR saliva “+”), reflecting pronounced viral tropism toward epithelial cells of the salivary glands. Only in 3.8% of patients with positive PCR results was viral replicative activity found both in saliva and plasma (PCR plasma “+”; PCR saliva “+”), and only in 1.4% of patients was replicative activity detected only in plasma (PCR plasma “+”; PCR saliva “–”). Thus, in most patients with oropharyngeal cancer, local EBV replicative activity was observed in saliva, which allows us to assert the significant role of this infection in the development of the proliferative process in this region.

According to our data, patients with oral cavity and oropharyngeal cancer showed a significant increase in the absolute leukocyte count in peripheral blood compared with the control group ($p < 0.05$). In patients, the leukocyte level in peripheral blood was 5977±154.5 per μl, whereas in the control group it was 4690±126.8 per μl. Study of the relative lymphocyte content between the groups showed some decrease in lymphocytes in the patient group ($p > 0.05$). Thus, the relative lymphocyte count was suppressed: 18.7±2.8% in the patient group versus 32.1±1.38% in the control group.

Results of T-lymphocyte immunophenotyping in patients with oral cavity and oropharyngeal cancer revealed a significant decrease in CD3+ lymphocytes compared with the control group ($p < 0.05$). We assumed that the decrease in the total number of T lymphocytes with CD3+ receptors was due to reduced expression of CD4+ T helpers. In the examined patients, the relative number of CD4+ T helpers was 26.3±2.2%, while in the control group it was 30.8±1.15%. Cytotoxic CD8+ T lymphocytes tended to increase to 25.4±3.3% compared with 18.7±0.61% in the control group ($p > 0.05$).

The immunoregulatory index (IRI), defined as the ratio of CD4+ T helpers to CD8+ cytotoxic T lymphocytes, is normally about 1.5 in healthy individuals. In the examined patients with oropharyngeal cancer, this indicator was 0.89±0.02, which was significantly lower than in the control group, 1.5±0.05 ($p < 0.05$). This decrease appears to be related to the reduction in CD4+ T helpers against the background of a slight increase in CD8+ T lymphocytes. Thus, patients with oral cavity and oropharyngeal cancer showed dysregulation of the cellular immune response, since a decrease in the immunoregulatory index is an indicator of pronounced T-cell immunodeficiency, which is often observed in viral and oncological processes, especially in latent chronic viral infections.

Another immunological indicator that attracted our attention was the activation marker of apoptosis of peripheral blood lymphocytes, CD95+. In the examined patients, CD95+ amounted to 33.4±1.53%, whereas in the control group it was 21.9±0.48%. Increased expression of the CD95+ activation marker, observed in 65% of patients, indicates pathological activation and death of immune cells, and their death, in turn, aggravates the existing cellular immunodeficiency in the oncological process associated with EBV infection.

To study the humoral arm of immunity in patients with oropharyngeal cancer, we examined circulating immune complexes (CIC). In the examined patients, increased levels of both large CIC (3%) and small CIC (4%) were identified, with values 2–3 times higher than in the control group. Since small circulating immune complexes have greater pathological potential, this indicator is especially important. A high level of small CIC reflects active immune struggle against viral antigens on the one hand, and impaired viral elimination mechanisms on the other hand, which plays an important role in the formation and maintenance of the malignant process.

For a deeper study of certain stages of neoplasm pathogenesis, we determined the serum VEGF level in newly diagnosed patients with oral cavity and oropharyngeal cancer (T3–4N0M0 stages), as well as in a control group of healthy individuals. In the control group, the level of vascular endothelial growth factor was 35.4±0.43 ng/ml, whereas in patients with newly diagnosed oral cavity and oropharyngeal cancer this value was significantly higher ($p < 0.05$), averaging 531.21±58.3 ng/ml. In patients with metastases, VEGF levels (874.21±67.3 ng/ml) were approximately three times higher than in patients without metastases (362.21±53.1 ng/ml).

On postoperative day 14, the VEGF level averaged 308.18±43.1 ng/ml, which was still significantly higher than in the control group but almost two times lower than before surgery. Retrospective follow-up revealed that among patients who had high VEGF levels after tumor removal, the number of recurrences was 2.1 times greater than in patients with lower postoperative VEGF.

A correlation analysis revealed a direct relationship between serum VEGF and patient age ($r = 0.51$; $p = 0.041$), as well as a significant direct correlation between VEGF and the apoptosis marker CD95+ ($r = 0.75$; $p = 0.004$) and an inverse correlation with the immunoregulatory index (IRI) ($r = −0.487$; $p = 0.021$). In patients with T4N0M0 disease, 89% of cases showed a high VEGF level (above 531.21±58.3 ng/ml), increased CD95+ expression (above 33.4±1.53%), and a reduced immunoregulatory index (below 1.0), or at least a combination of two of these three indicators. In contrast, such values in T3N0M0 disease were found in only 58% of cases.

Thus, the combined determination of VEGF, the apoptosis marker CD95+, and the immunoregulatory index makes it possible to predict a more severe course of oral cavity and oropharyngeal cancer. Combined determination of these markers in complex

clinical cases improves diagnosis and allows timely prediction of a more severe course of oral cavity and oropharyngeal cancer, helping to avoid unjustified biopsies or sectoral resections.

Conclusion

Thus, the analysis of the study results showed the significance of determining the serum concentration of vascular endothelial growth factor (VEGF) in patients with oral cavity and oropharyngeal cancer. In combination with immunological indicators, this marker helps diagnose severe forms of the process and is also important for predicting recurrence risk. A comprehensive approach to immunodiagnostics, virological diagnostics, treatment, and prognosis of disease course was developed depending on immune status indicators, virological characteristics of Epstein–Barr virus, and the level of vascular endothelial growth factor.

Conclusions

1. Patients with oral cavity and oropharyngeal cancer demonstrated a T-cell imbalance manifested by a deficiency of CD4+ T helpers with an increased number of CD8+ cytotoxic T lymphocytes, which may lead to inadequate immune responses.
2. An increase in the activation marker of apoptosis, CD95+, was identified, confirming the development of cellular immunodeficiency.
3. Activation of the humoral arm of the immune system was observed, manifested by increased levels of circulating immune complexes of both large and small size.

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