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ORIGINAL ARTICLE

DYNAMICS OF CHANGES IN EXPRESSION OF VEGF NEOANGIOGENIC FACTOR IN TUMOR TISSUE BIOPTATES IN PATIENTS WITH SQUAMOUS CELL CARCINOMA OF ORAL MUCOSA RECEIVING CETUXIMAB TREATMENT AND CHEMOTHERAPY

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ABSTRACT

Purpose of the study. An analysis of changes in the expression of the VEGF neoangiogenic factor in the tumor tissue of patients with squamous cell carcinoma of the oral mucosa receiving targeted therapy with cetuximab and chemotherapy.
Patients and methods. We performed an immunohistochemical study of tumor samples obtained from 60 patients with squamous cell carcinoma of the oral mucosa T3-4N0-1M0. The main group comprised 30 patients who received therapy with cisplatin and fluoruracil plus cetuximab. The control group included 30 patients receiving standard chemotherapy without targeted therapy. Each group was divided into two subgroups with different treatment efficacy: patients sensitive to treatment (n = 17 in the group with cetuximab and n = 12 in the group without cetuximab) and resistant to treatment (n = 13 in the group with targeted therapy and n = 18 in the group with standard chemotherapy).
Results. Quantification of the VEGF expression demonstrated minimal numbers of vessels stained positively for this marker in the field of view in patients of the main group sensitive to chemotherapy and cetuximab. The value was 5.3 times lower than initial values, and 4.3 times lower than in the subgroup of patients resistant to the treatment (the data were statistically significant, p = 0.0132 and p = 0.0455, respectively). In the control group, patients who were sensitive to the treatment showed 1.4 times lower values than initially (p = 0.921), and patients who were resistant to the treatment had 1.1 times lower values than initial values (p = 0.936). The data were not statistically significant.
Conclusions. The study showed that the number of microvessels in patients resistant to chemotherapy and cetuximab was 4.3 times higher than in patients with effective targeted therapy (p = 0.0455). The differences in the control group were not statistically significant.

Keywords:
oral squamous cell cancer, sensitivity, resistance, VEGF, neoangiogenesis, targeted therapy, cetuximab

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ДИНАМИКА ИЗМЕНЕНИЯ ЭКСПРЕССИИ ФАКТОРА НЕОАНГИОГЕНЕЗА VEGF В БИОПТАТАХ ОПУХОЛЕВОЙ ТКАНИ У БОЛЬНЫХ ПЛОСКОКЛЕТОЧНЫМ РАКОМ СЛИЗИСТОЙ ОБОЛОЧКИ ПОЛОСТИ РТА ПРИ ПРОВЕДЕНИИ ТЕРАПИИ ЦЕТУКСИМАБОМ И ХИМИОТЕРАПИИ

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РЕЗЮМЕ

Цель исследования. Изучить изменение экспрессии фактора неоангиогенеза VEGF в ткани опухоли при проведении таргетной терапии цетуксимабом и химиотерапии у больных плоскоклеточным раком слизистой оболочки полости рта.
Пациенты и методы. Было проведено иммуногистохимическое исследование образцов опухолевой ткани, полученных от 60 больных с плоскоклеточным раком слизистой оболочки полости рта T3-4N0-1M0. Основную группу составили 30 пациентов, которым была проведена лекарственная терапия препаратами цисплатин и фторурацил с добавлением цетуксимаба. Контрольную группу также составили 30 больных, которые подвергались стандартной химиотерапии без таргетной терапии. По степени эффективности каждая из исследуемых групп была поделена на две подгруппы: по чувствительности к лечению (n = 17 в группе с цетуксимабом и n = 12 в группе без цетуксимаба) и по резистентности к лечению (n = 13 в группе с таргетной терапией и n = 18 в группе со стандартной химиотерапией).
Результаты. При количественной оценке экспрессии VEGF было выявлено, что минимальное количество сосудов, эндотелий которых окрашен данным маркером в поле зрения, наблюдалось у пациентов основной группы с чувствительностью к ХТ и цетуксимабу. Данный показатель был ниже в 5,3 раза по сравнению с исходными значениями и в 4,3 раза по сравнению с подгруппой пациентов, у которых наблюдалась резистентность к данному лечению (данные статистически значимы p = 0,0132 и p = 0,0455, соответственно). Что касается контрольной группы, то было отмечено, что при чувствительности к проводимому лечению значения были в 1,4 раза ниже исходных цифр (p = 0,921), а при резистентности – в 1,1 раза ниже относительно исходных цифр (p = 0,936). Данные оказались статистически не значимы.
Заключение. В ходе исследования нами выявлено, что при наличии резистентности к химиотерапии и цетуксимабу количество сосудов в микроциркуляторном русле было в 4,3 раза выше по сравнению с больными, у которых наблюдался эффект от таргетной терапии (p = 0,0455). В контрольной группе значения оказались статистически незначимыми.

Ключевые слова:
плоскоклеточный рак слизистой оболочки полости рта, чувствительность, резистентность, VEGF, неоангиогенез, таргетная терапия, цетуксимаб

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RELEVANCE

Oncological diseases are one of the causes of mortality and disability worldwide.

Head and neck cancer ranks sixth in prevalence among all malignant neoplasms worldwide. Cancer of the oral mucosa is one of the urgent problems in oncology. About 630,000 new cases are diagnosed every year, and more than 350,000 patients with this pathology die every year. More than 90 % of head and neck cancers are squamous cell carcinoma, which mainly occurs in the oral cavity and oropharynx [1; 2].

Squamous cell carcinoma of the oral mucosa is characterized by an aggressive course, early metastasis and accounts for 90–95 % of all malignant neoplasms of the oral cavity [3]. Most of them are tumors of the tongue and the bottom of the oral cavity. Even after radical surgical intervention followed by adjuvant chemoradiotherapy, the survival rate of such patients remains extremely low due to the development of early recurrence and regional metastasis [4–6].

Vascular endothelial growth factor (VEGF) is a signaling protein best known for its role in the development of a pathological vascular network. It is a key mediator of angiogenesis (formation of new blood vessels) and binds two VEGF receptors (VEGF-1 receptor and VEGF-2 receptor), which are expressed on vascular endothelial cells. The production of VEGF and other growth factors by the tumor leads to an "angiogenic switch", where a new vascular network is formed inside and around the tumor, which allows it to grow exponentially [7; 8]. Therefore, it is very important to understand the basic cell biology of such tumors.

The term angiogenesis was first applied in 1971 by Folkman. The researchers reported that tumors can grow, forming new blood vessels from the existing vascular system, and that angiogenesis is closely related not only to tumors, but also to various other diseases, such as proliferative retinopathy, etc. [9; 10]. Various numerous interactions occur between cells, mediated by autocrine pathways that contribute to neoangiogenesis, uncontrolled tumor proliferation and metastasis [11].

It is known that cetuximab in malignant tumors is able to block not only the EGFR signaling pathway, but also indirectly affect the secretion of VEGF-A, thereby suppressing neoangiogenesis

Vascular endothelial growth factor (VEGF) is overexpressed in squamous cell carcinoma of the oral cavity and is in the focus of attention when creating new targeted drugs that are under development because increased cell proliferation and a rich vascular network are directly involved in the progression of the tumor.

The complexity of mechanisms in the development and progression of a malignant tumor requires a different approach to improve diagnosis, therapeutic decision-making and monitoring of the disease in personalized oncology. Although survival rates for squamous cell carcinoma of the oral cavity have improved over the past two decades, the prognosis still remains unfavorable compared to the development of therapy and the success achieved for other types of malignant tumors. Prognostic factors are numerous, and their interactions are complex and still unclear [12–14].

The main achievements in the treatment of oncological diseases are mainly associated with the

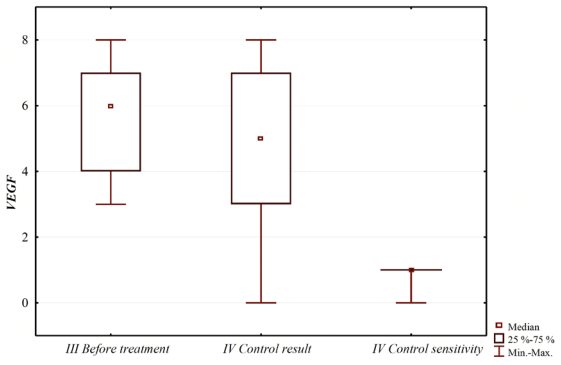

Fig. 1. Box-and-whiskers plot of VEGF expression in tumor cell vessels in patients of the main group.

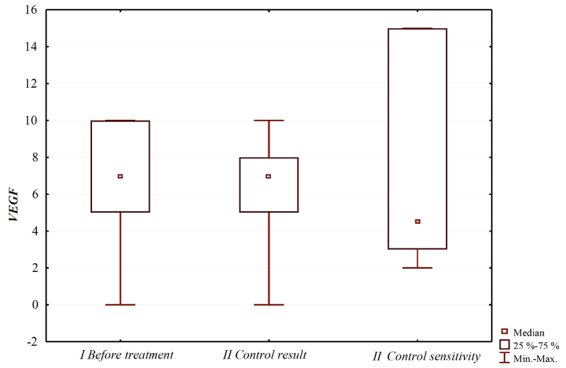

Fig. 2. Box-and-whiskers plot of VEGF expression in tumor cell vessels in patients of the control group.

appearance of molecular-targeted drugs, namely, acting on various molecules, cyclin-dependent kinases, VEGF growth factors, epidermal growth factor EGF, as well as on molecules suppressing apaptosis bcl-2, P53 cell cycle regulators, etc.

Cetuximab is a monoclonal antibody of immunoglobulin G1 that binds the epidermal growth factor receptor (EGFR), which is activated in about 90 % of patients with squamous cell carcinoma of the head and neck. Monoclonal antibodies against EGFR, such as cetuximab, compete with natural ligands, thereby preventing their binding to the receptor and, consequently, blocking the induction of cell growth signals and inhibiting the RAS signaling pathway and activation of ERK. Cetuximab binds to the extracellular domain of EGFR with higher affinity than natural ligands, blocking the tyrosine kinase-dependent signal transmission pathway induced by activation of the intracellular domain. Consequently, the antitumor effect of cetuximab is partly due to direct oncogenic signaling stress, which blocks cell survival, induces apoptosis and reduces the production of matrix metalloproteinase and vascular endothelial growth factor [15–17]. Understanding the molecular basis of the carcinogenesis of oral tumors and identifying potential molecular markers that may affect the prognosis and survival of patients with oral cancer is an urgent task of modern oncology. Neoangiogenesis stimulates tumor growth and promotes the occurrence of relapses and metastases due to a violation of the balance of proangiogenic and antiangiogenic factors. Vascular endothelial growth factor (VEGF) is the main regulator of angiogenesis, activating pro-angiogenic signaling pathways and regulating the formation of new blood vessels by binding to its main receptor. Angiogenesis plays a key role in the

Table 1. Expression with the VEGF medium in tumor cells in the studied groups (average numbers of vessels in a field of view)

Marker of angiogenesis	Main group (*n* = 30)		
	Initial values (*n* = 30)	Sensitivity (*n* = 17)	Resistance (*n* = 13)
VEGF	5.86 ± 0.72	1.1 ± 0.28* **↓ * *p* = 0.0132 ** *p* = 0.0455	4.8 ± 1.3
	Main group (*n* = 30)		
	Initial values (*n* = 30)	Sensitivity (*n* = 12)	Resistance (*n* = 18)
VEGF	6.5 ± 1.52	4.5 ± 1.71	6.1 ± 1.5

Note: * – statistically significant differences related to background values,
** – statistically significant differences related to subgroup with resistance.

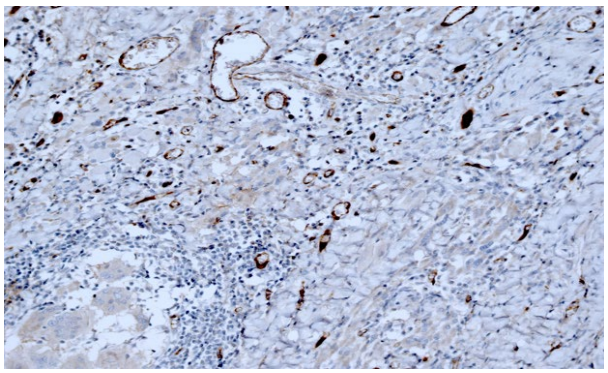

Fig. 3. VEGF-stained vessels in tumor cells of patients in the studied groups. Magnification × 200.

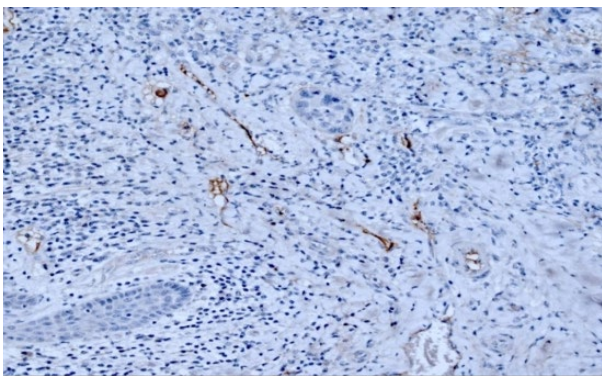

Fig. 4. Solitary VEGF-stained vessels in tumor cells of patients in the studied groups. Magnification × 200.

progression of the disease and mediates resistance to treatment. Thus, understanding neoangiogenesis, especially the VEGF pathway, is essential for risk stratification in patients with oral cancer and the development of new therapeutic targets [18].

The purpose of the study was to investigate changes in the expression of the neoangiogenesis VEGF in tumor tissue during targeted therapy with cetuximab and chemotherapy in patients with squamous cell carcinoma of the oral mucosa

PATIENTS AND METHODS

The study analyzed data on 60 patients with cancer of the oral mucosa, with the degree of prevalence of the tumor process T3-4N0-1M0. The surveyed groups were dominated by males (in the main group there were 22 men (73.3 %) and 8 women (26.7 %); in the control group – 19 men (63.3 %) and 11 (36.7 %) women). Most of the patients had moderate-grade squamous cell carcinoma (76.6 % in the main 23 patients) and 83.3 % in 25 control patients). The average age of patients in the main group was 62 years, and in the control group – 60 years. According to the stage of the tumor process in the main group, 17 patients were with stage III of the disease and 13 patients with stage IV; in the control group, the figures were distributed as follows: 15 patients with stage III and 15 patients with stage IV of the disease. Thus, we see that the patients in both groups were comparable in gender, age, degree of differentiation of the tumor, stage of the disease. The main group consisted of 30 patients who underwent chemotherapy with platinum cisplatin and fluorouracil with targeted EGFR blocker therapy: cisplatin 100 mg/m^2, intravenously, day 1, 5-fluorouracil 1000 mg/m^2/day, intravenously, 96-hour continuous infusion in combination with targeted therapy (cetuximab 400 mg/m^2 on day 1 in a loading dose, then 250 mg/m^2 on days 8 and 15). The control group also included 30 patients who underwent courses of standard chemotherapy without cetuximab – cisplatin 100 mg/m^2, intravenously, day 1, 5-fluorouracil 1000 mg/m^2/day, intravenously with 96-hour continuous infusion in 1–4 days. According to the degree of effectiveness, both the main and control groups were divided into two subgroups: with sensitivity and resistance. In the main group, the effect of treatment with cetuximab was observed in 17 patients, and resistance in 13 people. In the control group, 12 people were sensitive to treatment, and 18 were resistant to chemotherapy, respectively [19].

A solution of 10 % neutral buffered formalin was used to fix the material. Next, standard wiring and paraffinization of the fabric was performed. The next step was to prepare sections 3–5 microns thick, which were stained with hematoxylin and eosin (Accu-Cut SRM 200, Sakura (Japan)). The slices were applied to highly adhesive glasses and dried vertically in a thermostat at 37 °C overnight or at 60 °C for 1 hour. IHC was performed on sections from paraffin blocks intended for standard morphological examination. Primary monoclonal mouse and rabbit antibodies were used in the work. The degree of VEGF expression was determined by counting the number of vessels in each field of vision using 40 lenses (antibody VG1 Diagnostic BioSystems, dilution 1:200, 10 mMTris, buffer for "unmasking antigens" – 1 mMEDTA (pH 9.0). The calculation was carried out in 10 fields of view, then the average amount in the preparation was calculated [19].

The Statistica 10.0 application program was used to process statistical data [20]. The studied data were checked for compliance with the normal distribution according to the Shapiro-Wilk criterion. The data of the tables are presented in the form of M ± m, where M is the arithmetic mean, m is the standard error of the mean, $p \leq 0.05$ was taken as the level of reliability or statistical significance. If the distribution turned out to be far from normal, the comparison of groups was carried out using the nonparametric Mann-Whitney criterion (U-criterion) [19].

RESEARCH RESULTS AND DISCUSSION

Tumor biopsy was performed in all patients prior to treatment with further immunohistochemical examination of tumor tissue biopsies. During the processing of the obtained results, no fundamental differences in the initial values were revealed between the groups, and therefore, when describing the results, it was decided not to divide the background values of the studied markers into groups of patients depending on sensitivity, but to consider them as background in a single group. The analysis of the results of treatment of patients according to the response to the treatment according to the RECIST 1.1 criteria was carried out and compared with the VEGF level for each patient.

In the main group after cetuximab therapy, in the presence of efficacy from the treatment, the vascular spread was from 0 to 1, in the group without cetuximab, in the presence of sensitivity, these figures ranged from 2 to 15 vessels. In the absence of effectiveness from cetuximab, the spread of the number of vessels was up to 0 to 8 vessels in the field of view, in the group where cetuximab was not used – from 0 to 10 vessels [19].

The data is graphically shown in figures 1 and 2.

The average number of vessels whose endothelium is stained with the VEGF marker in the field of view is shown in the table.

When quantifying VEGF expression, it was revealed that in the main group of patients who underwent chemotherapy with targeted therapy with cetuximab in the presence of sensitivity to the treatment, there was a minimum number of vessels whose endothelium was stained with a VEGF marker. Compared with the initial values, this indicator was 5.3 times lower. And in the presence of resistance to chemotherapy and cetuximab, this indicator was 4.3 times lower than the baseline values (the data are statistically significant $p = 0.0132$ and $p = 0.0455$, respectively). In the chemotherapy group without cetuximab, the following indicators were noted: in the presence of sensitivity to chemotherapy, the minimum number of vessels stained with VEGF was 1.4 times lower relative to the initial figures ($p = 0.921$), and in the presence of resistance in this group – 1.1 times ($p = 0.936$). The data were not statistically significant [19].

When comparing the data, we obtained the following results: in the main group where chemotherapy with targeted therapy with cetuximab was used, in the presence of sensitivity to treatment, the number of vessels stained with VEGF marker in tumor cells was 4.1 times statistically lower compared to the control group of patients who also had sensitivity to the treatment ($p = 0.0035$). When resistance to chemotherapy and cetuximab appeared in the main group, the number of vessels stained with VEGF marker in tumor cells was 1.3 times lower compared to patients who also had resistance to the treatment ($p = 0.3699$) [19].

A statistical analysis of the effect of targeted therapy (comparison of control and main groups) revealed a statistically significant effect of cetuximab on this marker ($p = 0.028$).

Variants of VEGF expression in patients' tumor cells are shown in Figures 3 and 4.

Vascular endothelial growth factor (VEGF) and its receptors play an important role in both physiological and pathological angiogenesis. VEGF-A is widely expressed in almost all malignant tumors and is considered the most important factor in tumor angiogenesis. VEGF-A signaling also plays an important role in the development of diseases associated with angiogenesis, especially in malignant neoplasms.

VEGF causes deep angiogenesis during tumor formation. In this study, we tracked how the density of vessels stained with VEGF marker changed in the tumor cells of patients of the studied groups. (moved from the beginning of this section according to the reviewer's comment)

Multiple growth factors/cytokines and their signaling receptors often coexist in the same tumor microenvironment and collectively modulate tumor growth, invasiveness and metastasis. Among all known angiogenic factors, vascular endothelial growth factor A (VEGF-A), which modulates angiogenesis, vascular permeability, vascular survival and vascular remodeling, is probably the best characterized.

Transcription of the VEGF gene is activated under hypoxia by factor HIF1a (Hypoxia-inducible factor 1a). Hypoxia is one of the main causes of VEGF signaling activation in tissues. VEGF increases the level of VEGFR2 receptor expression by endotheliocytes of tumor microvessels, which activates cell growth and proliferation of endothelial cells [20]. Recent advances in molecular biology have revealed multiple gene changes in carcinogenesis in oral cancer that cause aberrant expression and function of proteins in a number of cellular processes, including angiogenesis.

Angiogenesis – the formation of new vessels from pre-existing ones, is crucial for tumor growth, invasion and metastasis of solid tumors. As the tumor grows, the cells in the tumor mass are deprived of oxygen due to their distance from the nearest blood vessels. The generation of a hypoxic state in tumors induces the production of vascular endothelial growth factor VEGF, a key mediator of angiogenesis. VEGF is overexpressed in a large number of human carcinomas, including squamous cell carcinoma of the head and neck, in particular in cancer of the oral mucosa [21; 22].

Cancer metastasis is the cause of mortality in cancer patients and involves complex interactions

modulated by various factors and cytokines between malignant cells and host cells. Vascular structures in solid tumors are crucial for intravasation of tumor cells into the bloodstream [23].

Although genetic changes in malignant cells determine the internal characteristics of invasiveness, cellular and molecular components of the host may play a predominant role in the invasion and metastasis of cancer [24].

For example, the vascular network of a tumor is necessary for tumor growth and metastasis, and blocking tumor angiogenesis is successfully used to treat cancer in animals and humans.

The subsequent formation of metastatic niches and the repeated growth of metastatic nodes to clinically detectable masses depend on angiogenesis and vascular remodeling. Tumors often express angiogenic factors at high levels, causing neovascularization.

A search for relevant studies was conducted in electronic databases. A meta-analysis of studies was conducted in which the relationship between overexpression of VEGF and survival of patients with oral cancer was quantified. Survival data were quantified. The results of these studies suggest that overexpression of VEGF has an adverse effect on overall survival and progression-free survival in patients with squamous cell carcinoma of the oral cavity, in patients with adenocystic cancer and mucoepidermoid cancer of the salivary glands. No significant heterogeneity was observed in all studies. Overexpression of VEGF indicates an unfavorable prognosis for patients with squamous cell carcinoma of the oral cavity, adenocystic and mucoepidermoid cancer of the salivary glands [25; 26]. In the era of personalized medicine and the treatment of malignant neoplasms based on the identification of biomarkers, it is important to find those therapeutic targets that need to be influenced to achieve maximum response to therapy, especially for squamous cell carcinoma of the oral cavity, since this pathology is very heterogeneous and poorly studied.

CONCLUSION

Based on the immunohistochemical study of tumor tissue biopsies, it can be concluded that in the presence of resistance to cetuximab, the number of vessels stained with the VEGF marker in the microcircular bed increases. Statistically significant results were obtained demonstrating the relationship between the degree of VEGF expression and the response to therapy with certain drugs. The data obtained are of particular clinical interest and can be used to predict the results of treatment in patients with squamous cell carcinoma of the oral cavity.

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