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# Unobvious Neutrophil Extracellular Traps Signification in the Course of Oral Squamous Cell Carcinoma: Current Understanding and Future Perspectives

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## Abstract

**Background:** The current standards of treatment for oral squamous cell carcinoma (OSCC) include surgery, radiotherapy, and chemotherapy. In recent years, research on the effectiveness of immunotherapy in the treatment of OSCC has also been conducted.

**Purpose:** Studies indicate that nonspecific immune mechanisms involved in the course of the anticancer response also need to be taken into account.

**Research Design:** This review summarizes the results of our research on the active participation of neutrophils, which are previously underestimated, in the antitumor response in the course of OSCC, taking into account the ability of these cells to generate neutrophil extracellular traps (NETs).

**Results:** We proved that the formation of NETs accompanies not only inflammatory changes but also the neoplastic process and that lipopolysaccharide (LPS) or interleukin 17 (IL-17) plays a critical role in inducing the formation of NETs during the OSCC. The greatest achievement of our published findings was the demonstration of the formation and release of NETs from neutrophils cocultured with tumor cells, as well as after stimulation with supernatant from the SCC culture with a PI3K-independent Akt kinase activation mechanism. Moreover, the pioneering achievement of our studies was the localization of NET structures in the tumor tissue, as well as the observation of high concentrations of NET markers in the serum of OSCC patients with low concentrations in the saliva, indicating the differences in the course of immune response between the periphery and the local reactions.

**Conclusions:** The data presented here provide surprising but important information on the role of NETs in the course of OSCC, thus pointing to a promising new direction in the development of management strategies for early noninvasive diagnosis and monitoring of the disease course, and perhaps immunotherapy. Furthermore, this review raises further questions and elaborates on the process of NETosis in cancer.

## Keywords

neutrophil extracellular traps, oral squamous cell carcinoma, neutrophils, polymorphonuclear neutrophils, cancer, tumor

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## Introduction

The immune system identifies and destroys cancer cells, and this process is called immune surveillance. However, it can also promote cancer development.[1] In 2004, Dunn et al. presented their research on "immunoediting" of the immune system in the course of cancer.[2] The immunoediting process assumes

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plasticity of the relationship between the tumor and the immune system in three stages.[3,4] In the first stage, which is referred to as immune surveillance, an immune response directed against the tumor develops. Clinical symptoms are absent in this stage, and the immune system can effectively eradicate the genetically damaged cells responsible for tumorigenesis.[5,6] The prolonged battle between the immune system and tumor cells is followed by the second stage, in which cancer develops in a favorable tissue microenvironment. Tumor cells continue to divide, but the immune system keeps them under control. Immune balance is maintained without any clinical symptoms, and at this stage, the detection of disease may be purely incidental.[3,6,7] In the final stage of the "immunoediting" process, the cancer cells reach a state where they effectively escape, block, and disrupt the functions of the immune system. The tumor escapes from immune surveillance and causes progressive damage to the surrounding tissues and clinical picture.[6,8-11] It is proposed that understanding the mechanism behind the escape of tumor cells from immune surveillance is important for understanding the pathophysiology of tumor and the development of effective immunotherapeutic strategies.

Cell-type reactions are the primary elements of the body's immune response to the tumor process.[12] In addition, neutrophils, such as polymorphonuclear neutrophils (PMNs), play a key role in the innate immune response. Neutrophils are rapidly recruited, and they readily recognize tumor-transformed cells and eliminate them through nonspecific mechanisms, thus constituting the first line of defense.

PMNs primarily employ three defense strategies: phagocytosis, degranulation of antimicrobial peptides, and generation of reactive oxygen species, along with the formation of neutrophil extracellular traps (NETs).[13-15] Besides, neutrophils play an important role in the integration of nonspecific and specific immune responses, as well as in tissue repair processes.[16] In the case of dysregulation of these specialized defensive mechanisms, inflammatory tissue damage can occur. Several features of neutrophil hyperreactivity and its role in the development of various pathological conditions, including cancer, remain unknown.

Our previous studies have shown significant changes in the neutrophil biological activity in patients diagnosed with oral squamous cell carcinoma (OSCC).[17-24]

### *Neutrophil Extracellular Traps in Cancer*

In 2004, a breakthrough discovery of the mechanism of neutrophil action involving the formation of neutrophil extracellular traps was published, rekindling the scientific interest in neutrophils.[15] NETs structures that are made of DNA strands and neutrophil granule proteins are part of the nonspecific immune response, which bind pathogens and prevent their spreading while maintaining high local concentrations of toxic agents.[25] Experiments have shown that NETs act as effective traps for a wide range of pathogens, especially bacteria. The first reports on NETs indicated their key role against in vivo infections. The newly discovered antibacterial mechanism of neutrophils confirmed the crucial role of these cells in recognizing and killing microbes. On the contrary, reports on the unfavorable role of NETs in the human body are also published. Most of the research studies now focus on the possibility of the induction of autoimmunization processes as a result of prolonged inflammation accompanying NETs. Earlier reports suggested the unfavorable role of neutrophils in the pathogenesis of many diseases involving increased inflammation, which was initially thought to be due to the effect of these cells and is currently attributed to NETs.[26-28]

Only a few reports have discussed the role of NETs in the cancer process, with contrasting results, thus pointing to the functional duality of NETs. Detailed studies have shown that numerous components of NETs exhibit cytotoxic effects on cancer cells, eg, myeloperoxidase (MPO), which kills melanoma cells and inhibits their growth after implantation. Individuals with MPO deficiency have been found to have a higher risk of cancer. Histones—another important component of NETs—can destroy endothelial cells and thus blood vessels supplying the tumor. Neutrophil extracellular traps also include defensins which can lysate tumor cells.[29-32] However, NETs, together with proteases, can degrade the extracellular matrix and promote the metastasis of tumor cells. In addition, the presence of matrix metalloproteinase 9 (MMP-9) in NETs, which blocks tumor cell apoptosis, can promote tumor growth. MMP-9 can also promote angiogenesis and neovascularization within the tumor.[33-35] NETs seem to physically bind tumor cells and prevent their proliferation. However, studies of some authors indicate that NETs favor the development of metastasis through this pathway.[36-38] Furthermore, certain components of NETs have been shown to induce platelet activation, platelet aggregation, red blood cell accumulation, and the release of von Willebrand factor, which are the principle components of thrombi.[30,39] These findings suggest the role of NETs in the pathomechanisms of metastasis and/or thrombosis in cancer patients.

## Oral Squamous Cell Carcinoma

Since the oral cavity has a complex anatomical and histological structure, malignant tumors occurring in this location are characterized by great diversity, due to both their origin and location. OSCC constitutes more than 90% of these cancers.[40] Squamous cell carcinoma can develop due to numerous external factors such as exogenous carcinogens (tobacco smoke), cocarcinogens (alcohol), mechanical trauma, poor oral hygiene, viral and fungal infections, intense sunlight, and a diet low in fruits and vegetables, as well as internal (genetic factors and immune defects) and social factors. Oral mucosal cancers differ in their clinical course and prognosis but share some common features. These tumors are characterized by a high growth rate. They spread by infiltration, quickly destroy tissue barriers, and also metastasize, mainly through the lymphatic vessel route. The first clinical symptoms are nonspecific and often ignored, with pain appearing late.[40-42] Worldwide, approximately 300,000 new cases of OSCC have

been diagnosed annually, which is 3% of all cancers.[43,44] In recent years, the incidence of OSCC has increased rapidly, with about 20% of patients developing either concurrent or subsequent development of another cancer.[45] In an increasingly younger population such as those in developing countries, the incidence of malignant neoplasms, including oral cancers, is expected to increase. Despite advances in diagnostics and treatments, the 5-year survival rate of patients with OSCC is low.[46,47] This is primarily attributable to the increase in exposure to carcinogens, the low detection rate of these cancers in their early stages, the significant severity of the disease when patients present for treatment, and the inadequate prevention strategies (diagnosis and treatment of precancerous conditions). Due to dysfunctions in basic vital functions such as breathing, food intake, and/or speech, oral cancers rapidly impair the health of the entire body.[48]

## *Neutrophil Extracellular Traps in Oral Squamous Cell Carcinoma—Summary of Own Research*

Our study aimed to elucidate the importance of neutrophil extracellular traps in OSCC, which may significantly contribute to the understanding of the innate mechanisms of the immune response against tumor cells in situ. The results of the study may contribute to the development of new therapeutic strategies for the treatment of cancer and reduce mortality in patients with OSCC.

Confocal microscopy and flow cytometry were used in our experiment. The cellular studies were complemented by the analysis of the signaling pathways involved in NETs using the Western blot technique and the evaluation of soluble NET biomarkers using immunoenzymatic methods. The hypothesis of the formation of NETs in response to direct contact between neutrophils and tumor cells was validated. These processes were complemented by the histopathological evaluation of the tumor tissue to determine the presence of neutrophil trap structures.

Previous studies have proven that a long-lasting neutrophil trap can not only mechanically impair the circulation in microvessels, but also enzymatically damage tissues, recruit other cells, and strongly promote the inflammatory process.[49-51] Given the previously established association between inflammation and the tumor process[52,53] and considering the scant data on the role of NETs in the tumor process, the formation of NETs in patients with inflammation and patients with cancer of the same location, ie, within the oral cavity, was compared. The results proved that the formation of NETs accompanies not only inflammatory changes but also the neoplastic process of the oral mucosa.[54] Similar results were obtained by Li et al, showing elevated plasma NET levels in patients with OSCC.[55] An increase in the formation and release of NETs can have a twofold effect on host defense mechanisms.[56,57] Not only do NETs contribute to the neutralization of harmful pathogens or cancer cells, but the potent toxic effects of the proteins released into NETs can damage the surrounding tissues. A high number of NETs near blood vessel walls can facilitate the binding of tumor cells to the endothelium, promote their extravasation, participate in metastasis, and facilitate immune escape by binding circulating tumor cells (CTCs) to platelets.[58-60]

The high number of NETs released in patients with inflammation was accompanied by an increase in the number of myeloperoxidase-(MPO) positive neutrophils, indicating an active process of NET formation. However, the situation was different in patients with OSCC. In the early stage of OSCC, the number of MPO-positive neutrophils was low, probably due to the neutrophil trap release. As an additional element in the analysis of the formation of neutrophil extracellular traps, circulating cell-free DNA (cfDNA) was introduced, the concentration of which has been shown to be higher in the serum of cancer patients in numerous scientific reports. Along with the increase in the formation of NETs during inflammation and in the early stages of cancer, an increase in the amount of cfDNA in neutrophil supernatants and serum was observed, which indicates PMNs as a source of cfDNA.[54]

The results of studies by other authors confirm the increase in the production of thrombin and fibrin associated with NETs in patients with OSCC. Moreover, HUVECs stimulated with NETs biomarkers displayed a procoagulant phenotype.[55] Not only thrombosis, but pneumonia, peritonitis, and sepsis are also significantly associated with high mortality rates from metastatic disease, as shown in many types of cancer.[61,62] These results suggest that lipopolysaccharide (LPS) plays a critical role in inducing the formation of NETs during the early stages of cancer. However, the fact that the number of MPO-positive neutrophils decreased after LPS stimulation in both inflammatory and cancer patients needs further exploration. This is likely due to the loss of MPO-positive cells in favor of the formation of NETs. This is supported by the inverse relationship observed between the amount of cfDNA in the supernatants of PMNs and MPO-positive neutrophils after LPS stimulation.[54]

In continuation of the previous studies on the role of cytokines in the formation of NETs,[63] the effect of IL-17 on the cells of the patient groups studied was evaluated, and the ability of IL-17 to stimulate the formation of NETs in the neutrophils of inflammatory and cancer patients was demonstrated, which was further confirmed by an increase in the amount of cfDNA in the supernatants of the stimulated cells. The surprisingly stronger effect of IL-17 in the formation of NETs than that of LPS in advanced cancer patients indicates the important role of this cytokine in late-stage cancer.[54]

Similar changes in the release of NETs in both inflammatory and cancer patients seem to confirm the close association between inflammation and cancer and the potential role of neutrophil traps in tumorigenesis. In addition, the data pointing to neutrophils as a source of cfDNA are relevant. The role of IL-17 in advanced cancer seems to confirm the protumor activity of IL-17.[54] Other authors have also demonstrated that the predisposition of OSCC patients' neutrophils to release NETs depends on cytokines, including IL-8, IL-6 and TNF-α.[55]

The results of the abovementioned studies became a natural starting point for subsequent studies to evaluate the process of NET formation by neutrophils of OSCC patients in response to direct or indirect contact with the cells of the squamous cell carcinoma (SCC) line compared with the results obtained in the cells from healthy subjects. The overall study plan included the analysis of the parameters of NETs after coculture of neutrophils with SCC or after stimulation with SCC culture supernatants. An increase in the parameters associated with the spontaneous release of NETs in unstimulated neutrophils from cancer patients was accompanied by a high expression of p-Akt (T308), p-Akt (S473), and p-PI 3k proteins, indicating the involvement of the PI3K/Akt pathway in NETosis in cancer patients. The increase in the formation of NETs in response to the direct contact with tumor cells of the SCC line not only confirms the involvement of neutrophil extracellular traps in the course of the tumor process but also indicates the interactions between the cells.[64] As of now, it is proposed that peripheral blood neutrophils do not provide information on the course of the immune response to the tumor in situ and are not capable of direct response. According to previous reports, such an ability is shown only by tumor associated neutrophils (TANs).[65,66] As shown in our study, the increase in the formation of NETs was accompanied by changes in p-Akt expression after coculture with SCC cells, indicating the activation of the Akt/PKB pathway in neutrophils in response to the appearance of tumor cells. Surprisingly, a concomitant low expression of p-PI 3k was observed, which may be due to the presence of PI3K inhibitory factors with an ability to activate Akt. This also gives rise to the hypothesis of Akt kinase activation excluding PI3K kinase.[64]

Studies suggest that tumors induce changes in distant organs by preparing their tissues for colonization by CTCs through the release of proinflammatory cytokines.[67] As shown in previous reports, neutrophils mobilize and accumulate at future sites of metastasis, release NETs, and bind CTCs, taking part in the formation of a "premetastatic niche" and promoting the formation of a tumor with an aggressive phenotype.[68-70] These results confirm that the formation of NETs increases in response to factors released by tumor cells. In addition, the formation of a higher number of neutrophil traps after stimulation with the supernatant obtained from the SCC culture than that observed after direct contact with SCC indicates a strong influence of factors from the primary tumor on the formation of NETs that favor the formation of the metastasis microenvironment. The increase in cfDNA and MPO levels after stimulation of neutrophils from cancer patients with the supernatant from the SCC culture confirms the microscopic findings and indicates a rapid cellular response to products released by tumor cells resulting in the release of NETs.[64]

The greatest achievement of this work was the demonstration of the formation and release of NETs from neutrophils cocultured with tumor cells, as well as after stimulation with supernatant from the SCC culture. Another valuable contribution is the demonstration of a PI3K-independent Akt kinase activation mechanism that leads to the formation of NETs in SCC patients.[64]

The subsequent step in the study of the mechanism of neutrophil extracellular trap formation in the course of cancer was to evaluate the expression of selected NET biomarkers in various biological materials, namely tumor tissue, neutrophils, saliva, and serum. The first stage of the analysis involved examining the presence of the major NET elements in the tumor tissue of OSCC patients, which allowed us to verify the direct contact of neutrophils with tumor cells in vivo. Given the high levels of the NET markers observed in cancer, the relationship between the NET markers and the activity of NADPH oxidase was considered,[71] which initiated a cascade of reactions leading to the NET formation.[72] The activity of the enzyme based on the expression of individual subunits and isoforms of the oxidase was also assessed. Assuming that the efficient degradation of NETs in a sufficiently short time is crucial to the effects of NETosis, the evaluation of DNase 1—the enzyme conditioning the degradation of NETs in vivo[73]—was included in the panel of studies. At the same time, the possibility of using saliva as a diagnostic material for the evaluation of NET markers in this group of patients was considered due to the direct contact of saliva with the tumor and the noninvasive method of sampling.[71]

In our pioneering studies, the presence of neutrophil extracellular traps in tumor tissues of patients with OSCC was demonstrated. Neutrophil infiltration and extracellular localization of MPO and histones intertwined with the DNA strands of the trap within the tumor tissue indicate the local formation of NETs and thus their involvement in the course of OSCC. The significant size of the tumor observed accompanied by the extracellular presence of neutrophil trap markers in tumor tissues in all patients diagnosed with lymph node metastasis confirms that NETs may be actively involved in metastasis in these patients.[71] Millurd et al showed the presence of neutrophil elastase, one of the NET-building proteins, in the biopsies of several head and neck squamous cell carcinoma (HNSCC) patients.[74] A low level of expression of p67-phox and pan Rac subunits with the normal expression of p47-phox in the neutrophils of cancer patients may indicate a delayed response, as shown in previous studies. The reduced expression of oxidase subunits may also be attributable to the depletion of the cellular action potential after the enzyme is released outside the cell, as indicated by the increase in serum NOX1 (NADPH oxidase (1) and NCF2 (neutrophil cytosolic factor (2) levels.[71] Histone citrullination is a vital parameter that determines the normal course of the formation of NETs,[75] which depends on Rac1 and Rac2 proteins, among others.[76] Very low expression of citrullinated histone H3 in the neutrophils of OSCC patients may be related to the low expression of p67-phox and pan Rac. This finding is supported by the positive correlation between p67-phox protein and citrullinated histone H3, which indicates a strong relationship between oxidase activation and the

formation of NETs. The low concentrations of NOX1 in the saliva of OSCC patients indicate that the activation and involvement of NADPH oxidase in the formation of NETs are deficient and may be the cause of the impaired antimicrobial response leading to inflammation and ultimately a risk factor for cancer progression. The surprising differences in proteins associated with neutrophil trap generation between saliva and serum in OSCC patients confirm the possibility of higher differences in neutrophil activity in the tumor microenvironment than in the periphery. High levels of DNase 1 in serum were accompanied by low levels in saliva in cancer patients, which, together with the negative correlation of NOX1 and DNase, may be associated with disease progression involving NETs.[71]

The pioneering achievement of this work was the localization of NET structures in the tumor tissue, as well as the observation of high concentrations of NET markers in the serum of OSCC patients but low concentrations in the saliva, indicating the differences in the course of immune response between the periphery and the local reactions within the oral cavity. The findings demonstrate the need to include saliva analysis in the diagnostic management of oral cancer differentiation.[71]

Despite emerging research on the role of NETs in carcinogenesis, the exact mechanisms regulating the formation of neutrophil traps in cancer are still unknown. Given the changes in the levels of NET biomarkers in OSCC patients observed in this study, research aimed at evaluating the expression of genes that are crucial in the NETosis process in this group of patients was conducted. This research focused on the mRNA expression of MPO, the primary biocidal protein involved in the formation of NETs; PADI4 (peptidylarginine deiminase (4), an enzyme that promotes the formation of neutrophil traps by histone citrullination; and NCF1, a subunit of phox47complex of NADPH oxidase involved in the formation of NETs. Preliminary results showed changes in the mRNA expression of key enzymes for NETs in the neutrophils from OSCC patients compared with the expression obtained in the cells from healthy individuals. Based on these findings, it can be concluded that the abnormal formation of NETs in patients with SCC is due to the overactivity of neutrophils in response to microenvironmental factors rather than due to genetic causes. The observed changes in mRNA expression for the proteins studied are consistent with the previously obtained results at the protein level, which seems to constitute an unfavorable prognostic factor in these patients [data not published]. Chen et al. proposed a novel prognostic model of HNSCC patients based on the evaluation of 6 NET-related genes: LTF, CYBB, SELPLG, GADPH, ANHA3, and CSF2.[77] Li et al. identified seven prognostic-related NET genes: ITGA5, LINC00460, LINC02454, NIFK, NUTF2, PDGFa, TNFRSF12a named NET-score, which was highly associated with the clinicopathological and immune traits of the patients with HNSCC.[78] Other researchers have proposed a novel AGR-NLR score (albumin/globulin ratio - neutrophil/lymphocyte ratio) for assessing the overall survival of OSCC patients.[79]

Studies by other authors indicate that changes in NET formation are also associated with potentially malignant oral disorders (OPMD) and may be associated with the high risk of transformation to OSCC. Jabłońska et al showed increased amounts of NETs induced by TGF- β in patients with oral lichen planus (OLP) – precancerous lesion. The presence of NETs, as an effect of TGF-β activity, may be a potential switching factor for pro-tumor N2 phenotype neutrophils. The NET generation phenomenon seems to connect inflammation, potentially malignant diseases, and neoplasms located in the oral mucosa.[80]

## Conclusions

The results presented here demonstrate that neutrophils play an important role in cancer biology. The dogma of short-lived, functionally nonspecific neutrophils has been replaced by the concept of a protagonist in cancer development. Since neutrophils are recruited rapidly, they are in direct contact with tumor cells at the very early stages of tumor development. "Crosstalk" between neutrophils and tumor cells contributes to extending the lifespan of these leukocytes, thus favoring their hyperactivity. It is highly likely that proteins produced by neutrophils can directly or indirectly influence the "remodeling" of the tumor microenvironment. Furthermore, recent findings indicate that neutrophils are actively involved in "premetastatic niche" preparation. The results of our study unambiguously indicate the involvement of neutrophil extracellular traps in the course of OSCC. The findings presented here point to a promising direction for the development of management strategies for early noninvasive diagnosis and monitoring of the course of OSCC and perhaps also immunotherapy, particularly focusing on the different courses of immune responses to the developing tumor locally and systemically.

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