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Neutrophils and oral squamous cell carcinoma: lessons learned and future directions

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Summary sentence:
This review focuses on how neutrophils modulate the behavior of oral squamous cell carcinoma and the possible use of neutrophils as biomarkers of OSCC
Abbreviations

OSCC - Oral Squamous Cell Carcinoma
INFb - Interferon Beta
TGFb - Transforming Growth Factor Beta
MMP9 - Matrix metallopeptidase 9
AKT - Protein kinase B
NET - Neutrophil extracellular traps
PMN - Polymorphonuclear neutrophils
NLR - Neutrophil to Lymphocyte Ratio
HNSCC - Head and neck squamous cell carcinoma
HNP - Human Defensins
NGAL - Neutrophil Gelatinase Associated Lipocalin
SCC - Squamous Cell Carcinoma
HNSCC – Head and neck squamous cell carcinoma
TNF - Tumor Necrosis Factor
HSPG - Heparin sulfate side chains of proteoglycans
TACI - Calcium modulator and cyclophilin ligand interactor
TNFa - Tumor Necrosis Factor Alpha
ROS - Reactive Oxygen Species
TRAIL - TNF-related apoptosis-inducing ligand
MAPK - Mitogen-activated protein kinases
iNOS - Calcium insensitive Nitric Oxide Synthase
NO - Nitric Oxide
TAN - Tumor Associated Neutrophils
MMP8 - Matrix Metalloproteinas 8
MT1MMP - Membrane type 1-matrix metalloproteinase 1
VEGF - Vascular endothelial growth factor
TIMP-1 - TIMP metallopeptidase inhibitor 1
TIMP - Tissue inhibitors of metalloproteinases
SN - Supernatant
CREB - cAMP response element-binding protein
MIF - Macrophage migration inhibitory factor
Abstract

The role of cells of the innate immune system in the pathogenesis of squamous cell carcinoma has been the subject of intense research in recent years. In particular, neutrophils have recently been shown to have either a pro-tumor or anti-tumor phenotype in different cancers. Here we review the role of neutrophils as tumor microenvironment and signaling modulators of oral squamous cell carcinoma (OSCC) and their possible role as biomarkers of OSCC prognosis. Current evidence supports a pro-tumorigenic role for neutrophils in OSCC but more research is needed to clarify the precise mechanisms involved.
Introduction

Oral squamous cell carcinoma is the 5th leading cause of death in Canada [1] and the overall high mortality rate (average 57% survival in 5 years) has changed minimally over the previous 10 year period. A total of 42,440 new cases and 8,390 deaths are estimated in the United States in 2014 [2]. The high mortality rate of patients with oral cancer is associated with its late detection and subsequent increase in the occurrence metastatic disease. The 5-year survival rate for oral cancer drops from approximately 60% to 30% for patients with metastatic disease [1]. Recent advances have been made in understanding the genetic changes and molecular mechanisms regulating OSCC progression, including the role of neutrophils in this process.

Neutrophils are key mediators of the innate immune system. Neutrophil activation is essential to protect the host system against infections and promote normal healing [3]. For many decades, leukocytosis has been associated with a poor prognosis in different types of malignancies [4]. Despite this, the specific role of neutrophils and macrophages in the pathogenesis of cancer has only recently become the subject of intense research [5, 6] with special focus on the association between inflammation and cancer progression. This idea was initiated by Pekarek et al. who showed that neutrophils can induce tumor angiogenesis [7], prompting a significant number of research groups to investigate possible neutrophil pro-tumor or anti-tumor roles as well as their potential uses as diagnostic and prognostic markers. Recent work has shown that neutrophils are important players in cancer biology [8, 9] and different neutrophil sub-populations may have opposing roles in
cancer progression where an N1 population was demonstrated to have anti-tumor properties and an N2 population of neutrophils was shown to promote tumor progression. The N1 phenotype is induced by INFβ and is characterized by reduced MMP9 expression, increased reactive oxygen species (ROS) formation, and apoptosis [10]. The N2 neutrophils develop a pro-tumor phenotype after TGFβ stimulation, leading to increased Arginase, MMP9 and collagenase expression, AKT activation, and promotion of leukocyte recruitment [9]. This N2 phenotype is particularly relevant for OSCC patients, since OSCC show increased expression of IL-1β, IL-6 and TGFβ [11]. These proposed opposing roles for neutrophils suggest that they may be important biomarkers for OSCC and perhaps targets to control cancer progression [12].

During the past 4 years, an increasing number of publications have shown that neutrophils are present in a variety of tumors, including renal cell carcinomas and head and neck carcinomas [13] and that they may contribute to tumor progression [14, 15], metastasis [16], and extracellular traps (NET)-dependent tumor metastasis [17]. The general roles of neutrophils in cancer pathogenesis and prognosis have been reviewed elsewhere [6, 12], but the particular roles of neutrophils in the diagnosis, prognosis and progression of oral squamous cell carcinomas have not yet been reviewed. Considering the constant presence of neutrophils in the oral tissues due to the oral biofilms, there is an increased interest in analyzing how the presence of neutrophils modulates OSCC behavior. The literature on OSCC and neutrophils is limited compared to other cancers and we
added publications on head and neck squamous cell carcinomas (HNSCC) to this discussion.

In the next sections, we will analyze our current knowledge about the roles of neutrophils in the progression of oral squamous cell carcinoma in three main categories: biomarkers, microenvironment changes, and intercellular signaling (Figure 1).

**Neutrophils as biomarkers of OSCC**

Significant efforts are being made to identify new diagnostic and prognostic markers to better manage OSCC. Neutrophils are highly proteolytic and motile cells, allowing them to have direct contact with various cells of the tumor microenvironment. It is possible that the proteins they release can either directly or indirectly be used for early detection, staging and prognosis of OSCC lesions. Due to their accessibility in peripheral blood and saliva, neutrophils are excellent candidates to develop new biomarkers for oral cancer. Here we describe the role of neutrophils and neutrophil derived proteins as biomarkers of OSCC.

*Neutrophil infiltration*

Similar to the literature on other tumors, recent reports have shown that high neutrophil infiltration in OSCC is associated with poor clinical outcomes. Trellakis et al. used a retrospective histological analysis of head and neck squamous cell carcinomas to show an association between PMN infiltration and squamous cell
carcinomas prognosis. Increased neutrophil infiltration as detected by CD66b immunostaining correlated with poor patient survival [13]. These findings were consistent with a recent report by Wang et al. who showed that tongue squamous cell carcinomas with high neutrophil infiltration displayed increased lymph node metastasis, higher clinical stage and increased chances of tumor recurrence [18].

Neutrophil-to-lymphocyte ratio (NLR)

The neutrophil-to-lymphocyte ratio (NLR) is a well established marker of poor prognosis in various conditions including cardiovascular diseases [19] and cancers [20, 21] including head and neck carcinomas [22]. An increase in NLR is indicative of an ongoing inflammatory state with decrease in regulatory pathways. Millrud and coworkers have correlated the activation pattern of leukocytes and survival of HNSCC patients [23] where the prognostic markers (CD71, CD98, CD4/8 ratio, CD16/14) and a high NLR correlated with poor prognosis. Perisanidis et al. evaluated 97 patients with biopsy proven OSCC who received neoadjuvant chemotherapy and showed that a high NLR (>1.9) is an independent marker for poor prognosis in OSCC patients [24]. Although this finding suggests that the NLR may be an important OSCC prognostic biomarker, larger prospective studies are needed to further clarify the use of NLR, the mechanisms and relevance behind the changes in lymphocyte and neutrophil counts in OSCC.

Neutrophil-secreted proteins
Numerous studies have used neutrophil-secreted products as diagnostic and prognostic markers of cancer. Among these, human defensins (HNP), TNF family proteins and neutrophil gelatinase associated lipocalin (NGAL) have been studied in the OSCC patients.

Human defensins (HNP1, HNP2 and HNP3) are known to induce cytotoxic effects in numerous target cells, including SCC cells [25]. In a search for a possible role for HNPs in cancer progression, Lundy et al. investigated the presence of HNPs in OSSC and reported a 2-12 fold increase in their presence in localized tumor areas. This finding also correlated with an increase in neutrophil infiltrates [26]. HNPs were also elevated in the saliva of OSCC patients but the clinical significance of this finding has yet to be clarified [27-29].

Members of the TNF superfamily of secreted proteins, including APRIL [30], TRAIL and DR5 [31] were also investigated as possible oral cancer biomarkers. The proliferation-induced ligand APRIL has been shown to regulate tumor cell survival and proliferation through binding to heparin sulfate side chains of proteoglycans (HSPG) or to the calcium modulator and cyclophilin ligand interactor (TACI)[32]. Jablonska et al. analyzed the expression of APRIL in peripheral blood neutrophils of patients with OSCC and found a correlation between high expression and poor prognosis. TRAIL is a soluble TNFa family ligand produced by numerous cells and may be a promising candidate to promote cancer suppression. TRAIL induces apoptosis by activation of DR receptors, including DR1 and DR5, and its expression and release are decreased in late stage squamous cell carcinoma [31]. Since many cells in the tumor microenvironment may secrete TRAIL, further studies are needed
to better understand the role and the prognostic importance of neutrophil-derived TRAIL in OSCC progression.

Neutrophil gelatinase associated lipocalin (NGAL) is a regulator of iron and hydrophobic-compounds transport, and has an important role in protecting MMP9 from degradation. Recent studies have described a pro-tumor role for NGAL in different tumors, including breast and esophageal cancers [33]. NGAL has also been shown to be up-regulated in well differentiated OSCC while poorly differentiated tumors showed a weak expression, suggesting a possible role for this protein in tumor staging [34].

*Reactive oxygen species*

Reactive oxygen species have an increasing number of roles in different cellular processes, including phagocyte killing, chemotaxis, apoptosis, and intracellular signaling [35]. The formation of ROS by neutrophils is decreased in most cancers [9]. Peripheral blood neutrophils from patients with head and neck squamous cell carcinoma had lower ROS production [36] and reduced apoptosis [13]. This mechanism is dependent on the p38 MAPK pathway. Similarly, Jablonska and others showed that iNOS expression and NO production are significantly reduced in peripheral blood neutrophils of OSCC patients [37]. Although ROS are not considered biomarkers of cancer progression, future studies will clarify the signaling changes in TANs and the reduction of ROS in these cells. This may contribute to the understanding of the crosstalk between cancer cells and TANs.
Neutrophils remodeling the cancer microenvironment

One of the most interesting recent findings suggests that neutrophils are recruited to the niches of distant cancer metastasis, and may be a key player in the establishment of metastatic spread [16]. Similarly, Huh et al. found that neutrophils were recruited and increased the metastatic spread of melanoma through an IL-8 dependent mechanism [38].

Matrix Metaloproteases

The cancer microenvironment is a complex niche that is constantly being remodelled by fibroblasts, inflammatory and cancer cells. Neutrophils secrete MMP8 (collagenase), MMP9 (gelatinase), elastase, cathepsin G, proteinase 3 and other matrix proteases that contribute to the remodelling of the tumor microenvironment. These proteases can degrade the extracellular matrix directly and facilitate the invasion of cancer cells [39], or alternatively activate MT1MMP in the tumor cells and indirectly facilitate cancer progression [40]. Moilanen et al. showed that MMP8 is also secreted by head and neck squamous cell carcinomas, including oral cancers [41]. MMP9 is also known to regulate tumor angiogenesis, a key factor in tumour progression (see below). In tumors where macrophages are the main source of MMP9, inhibition of macrophage recruitment induces a compensatory response, increasing the recruitment of MMP9+ neutrophils which
have a pro tumor phenotype [42]. Further studies are needed to verify the significance of this mechanism in OSCC.

Angiogenesis

Neutrophils are known to promote tumor angiogenesis through upregulation of MMP9 and VEGF [43]. Bausch and others have shown that MMP9 is a VEGF-independent angiogenic factor with an additive effect to VEGF-induced angionenesis in hepatocellular carcinoma. Complete inhibition of angiogenesis requires both MMP9 and VEGF inhibition [44].

Unlike other cells, neutrophils secrete proMMP9 without the inhibitor TIMP1, providing a readily active MMP9, which is critical for tumor angiogenesis and intravasation. Inhibition of IL-8 decreases neutrophil recruitment to the tumor, angiogenesis and metastasis (Bekes et al 2011). The combination of TIMP (MMP9 inhibitor) and anti-IL-8 antibody induces a substantial decrease in local angiogenesis and intravasation of tumor cells in a given area [45]. This is a fundamental link between inflammation and cancer progression, highlighting the importance of neutrophils in cancer spread. The role of MMP9 in the progression and spread of oral squamous cell carcinoma is not yet completely understood.

Neutrophil extracellular traps (NET) and adhesion molecules

Recent evidence shows that neutrophils may also contribute to the metastatic spread of cancers by facilitating the seeding of circulating cancer cells [46]. Several mechanisms have been described to explain the neutrophil-mediated
metastatic spread, including NETs. NETs are composed of extruded neutrophilic DNA that can be seen under normal systemic inflammatory/infectious responses. Cools-Lartigue et al. have showed that NETs sequester circulating cancer cells and increase the formation of liver micrometastasis [17]. There are no publications describing the role of NETs in OSCC metastasis. Further studies are needed to understand the clinical relevance of this mechanism in OSCC.

Modulation of cell function and signaling

Both neutrophils and cancer cells influence the behaviour of each other in the cancer microenvironment [39, 47]. Recent key studies have identified potential pathways involved in the crosstalk between cancer cells and neutrophils. We will focus this discussion on the regulation of cell function and signaling between OSCC and neutrophils.

Dumitru and co-workers recently showed that tumor associated neutrophils increased cortactin phosphorylation in oropharyngeal squamous cell carcinomas, promoting cancer migration, leading to a poor prognosis [48]. This is a promising result that links the presence of TAN with cytoskeletal changes in the cancer cells. More importantly, cortactin is an essential actin binding protein, regulating leading edge formation and invadopodia formation in cancer cells [49].

Trellakis and coworkers performed a functional analysis of peripheral blood neutrophils to show that the peripheral blood of HNSCC patients had an increase in
PMN, CXCL8, CCL4 and CCL5 [13]. In vitro analysis showed an increased migration and increased survival of PMN exposed to FaDu cells or FaDu cells supernatant (SN). This effect was significantly reduced by CXCL8 inhibition. Stimulation of PMN with FaDu SN also increased the secretion of CCL4, MMP9 and lactoferrin. These results show that head and neck squamous cell carcinomas establish a feedback loop with neutrophils leading to increased inflammation.

Cancer cells can also change the activation state of neutrophils. Using a protein phosphorylation array, Dumitru et al. showed that neutrophils challenged with FaDu cancer cells showed a strong activation of p38/MAPK, CREB, and p27. The activation of p38/MAPK was associated with an increase in chemotaxis, cell survival and secretion of CCL4 and CXCL8. P27 and CREB also regulated the release of MMP9 by neutrophils. The authors also demonstrated an increase in expression of MMP9 and CCL4 by CD66b positive cells (neutrophils) in HNSCC [50].

Neutrophils show increased survival when exposed to supernatants from different tumors, including squamous cell carcinoma, as shown by different groups [51]. Several mechanisms are implicated in the prolonged survival of neutrophils, including the release of cytokines and hyaluronan by tumor cells and other cells in the microenvironment [52, 53]. The macrophage migration inhibitory factor (MIF) was recently shown to modulate the activation and increase survival of neutrophils [53]. MIF significantly increased neutrophil chemotaxis, reduced apoptosis, and increased the expression of CCL4 and MMP9 through a CXCR2-dependent mechanism. Samples from cancer patients were also used to show that MIF expression in tumors correlate with neutrophil recruitment and poor survival. The
observation that neutrophils survive longer in the tumor microenvironment challenges the initial understanding that neutrophils, as extremely short-lived cells, could not participate effectively in tumor progression that occurs over an extended period of time. Also, increased neutrophil survival translates into sustained inflammation and secretion of neutrophil inflammatory mediators that may contribute to tumor progression.

Concluding remarks

*Increasing roles of innate immune cells in cancer biology*

Considering the increased interest in the development of targeted therapies, immune cells have become a primary target for prognosis and possible treatment of cancers and this is valid for almost all malignancies, including OSCC. The dogma of the short-lived, “non-specific” neutrophil in cancer is disappearing and is being replaced by the concept of the neutrophil as a protagonist in cancer progression.

There are many reasons that support this hypothesis. First, the neutrophil has extremely efficient motility machinery, allowing it to be recruited quickly to areas of early cancer development and interact with tumor cells in the very early steps of malignant transformation. Neutrophils can move in and out of the tumor microenvironment, conveying important signaling cues and can also be detected in the peripheral circulation. Second, contrary to initial beliefs, neutrophils in contact with cancer cells have a prolonged life cycle and can be a resident of the tumor microenvironment for extended periods of time. Third, neutrophils are equipped
with a variety of matrix remodeling proteases that can help shape the tumor microenvironment.

**Neutrophils, cancer and the oral microenvironment**

Considering the oral microenvironment, the constant presence of neutrophils in the saliva may be an important factor in early malignant transformation, signaling and, most importantly, a marker for disease progression that is easily accessible with a mouth rinse, without the need of blood collection [54, 55]. Also, there are numerous conditions that are characterized by changes in the neutrophil population in the mouth, including periodontal disease [55, 56]. These changes in neutrophils may be involved in the observed increase in the rate of oral epithelial malignant transformation in chronic inflammatory states [57, 58]. Certainly, more research is needed to address these topics. Another important question is whether the modulation of neutrophil activity can influence the development or prognosis of oral cancers. There is evidence to suggest that maintaining good oral hygiene and therefore low neutrophil counts in the saliva correlate with small prevalence of oral cancer [59, 60]. Finally, the accessible oral microenvironment could also be a potential candidate for local treatments for early OSCC.

**Future directions**

There are still many unanswered questions regarding the role of neutrophils in cancer pathogenesis in general. The literature on oral squamous cell carcinoma and neutrophils is limited compared to other models. New exciting results show tat
neutrophils inhibit seeding in the premetastastic lung in a breast cancer model [61] and recruit T regulatory cells that may contribute to a decreased antitumor response [62]. Further studies analyzing the role of these mechanisms in OSCC are needed.

The most challenging topic to be addressed in the next few years is how to modulate neutrophil activity to increase tumor surveillance and early suppression. Identifying the signaling switches is essential to developing treatments to precisely target this interaction. Also, considering the particularities of OSCC, how can we develop localized treatment strategies? Since oral neutrophils are readily available, research is needed to evaluate the genetic signature changes of neutrophils exposed to OSCC.

The most promising area of research is the development of a sensitive and specific diagnostic/prognostic marker for OSCC, which may be associated with saliva tests. Analyzing neutrophils from OSCC patients’ saliva might prove to be a good prognostic marker.

Authorship

MAOM designed and prepared the manuscript. JG contributed to manuscript preparation. MG contributed to the design and the revision of the manuscript.

Acknowledgements
Thanks to Dr. Grace Bradley for the helpful discussion and support of this work. This supported is supported by a Dental Research Institute grant. MG is supported by CIHR.

Conflict of interest disclosure

The authors have no conflict of interest to disclose.

References


Figure Legends

Figure 1. The role of neutrophils in the progression of OSCC. Neutrophils have important roles in the progression of OSCC, including the remodeling of cancer microenvironment, modulating cell function and signaling and a potential biomarker. This figure summarizes the available information on each of these categories.
**Biomarkers**

- Neutrophil to lymphocyte ratio
- Neutrophil infiltration
- Neutrophil secreted proteins
  - Defensins
  - TNF superfamily
  - NGAL

**Modulation of cell function and signalling**

- Neutrophils
  - Increased survival
  - Increased CCL4, CXCL8, MMP9 and lactoferrin
- Cancer cells
  - Increased migration
  - Cortactin phosphorylation

**Tumor microenvironment**

- Angiogenesis
- Matrix remodelling
- Modulation of inflammatory response