



INTERLEUKIN-8 AND LARYNGEAL SQUAMOUS CELL CARCINOMA

Michele Grasso¹, Massimo Ralli^{1*}, Griselda Agolli¹, Marco Fiore², Mauro Ceccanti³, Paola Tirassa², Marco de Vincentiis⁴, and Antonio Greco¹

¹Department of Sense Organs, Sapienza University of Rome, Italy

²Institute of Cell Biology and Neurobiology, IBCN-CNR, Rome, Italy

³Centro Alcologico Regione Lazio, Rome, Italy

⁴Department of Oral and Maxillofacial Sciences, Sapienza University of Rome, Italy

*Laryngeal squamous cell carcinoma (LSCC) is the second most common neoplasm of the upper aerodigestive tract after cancer of the oral cavity. Over the past two decades, even though patients have benefited greatly from the latest advances in surgical techniques, chemotherapy and radiation therapy, the survival rate of LSCC has not improved significantly. It is reported that changes in the expression of cytokines and growth factors have implications in the malignant transformation of many cancers including head and neck squamous cell carcinoma and, more recently, LSCC. It has been hypothesized that some of these cytokines may be used as additional diagnostic markers in the sera of patients because of their excessive production by the tumor cells. This could be of great value since there are currently no reliable markers to predict either tumor development or relapse. Interleukin-8 (IL-8), a chemokine (C-X-C motif) ligand 8 (CXCL8), is now reported to play an important role in cancer invasion, angiogenesis and metastasis. Recent studies have shown an increased concentration of IL-8 in patients with LSCC and a positive association with lymph node metastasis and T classification. Interleukin-8 levels were not significantly associated with shorter overall survival and cancer progression-free survival. The investigation of the mechanisms of origin, invasion, and metastasis of the cancer is one of the emergent and most promising scientific fields in head and neck cancer, especially in LSCC. Biomarkers such as IL-8 could have a role as a screening test and as a support of the clinical decisions for appropriate therapy and postoperative care in individual patients. **Biomed Rev 2018; 29: 47-55***

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* Corresponding author: Dr Massimo Ralli, Department of Sense Organs, Sapienza University of Rome, Viale del Policlinico 155, Rome 00186, Italy

Tel: +39 06 4997 6808, Email: massimo.ralli@uniroma1.it

INTRODUCTION

Laryngeal squamous cell cancer (LSCC) is the second most common neoplasm of the upper aerodigestive tract after cancer of the oral cavity (1). Over the past two decades, even though patients have benefited greatly from the latest advances in surgical techniques, chemotherapy and radiation therapy, the survival rate of LSCC has not improved significantly (2, 3). In 2016, an estimated 13,430 new cases of laryngeal cancer have been diagnosed, with approximately 3620 patients dying from the disease. Laryngeal squamous cell cancer occurs more commonly in men than in women (5.8 cases per 100,000 vs 1.2 per 100,000, respectively). In addition, there are racial disparities observed in laryngeal cancer, with African Americans presenting at a younger age and having a higher incidence and mortality compared with Caucasians (2).

Several risk factors have been implicated in the pathogenesis of laryngeal cancer; the most significant are tobacco and alcohol consumption (4). Tobacco use has been shown to have a linear association with the development of laryngeal cancer, with a risk for smokers that is 10 to 15 times higher than the risk for nonsmokers, and the heaviest smokers have as much as a 30 times greater risk. Research has also demonstrated a linear relationship between the amount of alcohol consumed and the risk of laryngeal cancer. In a study that examined the role of alcohol and tobacco in laryngeal cancer, the multivariate odds ratio was 2.46 for nonsmoking heavy drinkers (defined as > 8 drinks per day) and 9.38 for nondrinking smokers (5-11). It has also been shown that alcohol and tobacco have a multiplicative effect on the risk of laryngeal cancer.

Exposure to other environmental factors is thought to potentially increase the risk of LSCC, such as asbestos, polycyclic aromatic hydrocarbons, and textile dust (12). Dietary factors have also been noted, with red meat increasing the risk of laryngeal cancer, while a diet varied in fruit and vegetables potentially has a protective effect. The role that both gastroesophageal and laryngopharyngeal reflux play in the disease process is still controversial and under investigation.

Although the human papillomavirus (HPV) is a proven driver of the majority cancers of the oropharynx, it was initially thought that the virus did not play a role in laryngeal cancer (13-18). However, new research demonstrates the presence of HPV and/or the surrogate marker p16 (cyclin-dependent kinase inhibitor 2A/multiple tumor suppressor 1) in a minority of laryngeal tumors, although the biologic and prognostic relevance of this finding is unclear. It is estimated that the prevalence of HPV ranges from 20% to 30% in

laryngeal cancer; however, this percentage varies widely between studies and depends on the detection method used. More work is needed to determine the clinical relevance of HPV/p16-positive status in laryngeal cancer, as this remains controversial (13-21).

Approximately 60% of patients present with advanced (stage III or IV) disease at diagnosis. Unfortunately, laryngeal cancer is one of the few oncologic diseases in which the 5-year survival rate has decreased over the past 40 years, from 66% to 63%, although the overall incidence is declining. This highlights the need for further research and innovation in the field (5, 22-24). Among the causes of the low survival rate are the lack of screening methods (clinical examination remains the single solution to early detection) and the late presentation of patients to the specialist as a result of little worrying symptoms (hoarseness). The lack of suspicion of cancer at the first consultation is associated with delay in the second consultation among patients who were not diagnosed with cancer initially (1, 25).

CYTOKINES IN HEAD AND NECK CANCER

It has been reported that changes in the expression of cytokines and growth factors may have implications in the malignant transformation of many cancers including head and neck squamous cell carcinoma (HNSCC) and, more recently, LSCC (2, 26). Cytokines are a group of soluble, low-molecular-weight proteins that mediate immune and inflammatory responses. Cytokines are classified into three groups: T-helper 1 (Th1), T-helper 2 (Th2) and T-helper 17 (Th17), as determined by their biological properties (27). Th1 cytokines stimulate cellular immune responses, while Th2 cytokines predominantly regulate humoral responses. Th17 is currently known to regulate inflammatory responses and plays several roles in autoimmunity (27). Cytokines are also classified as pro-inflammatory (IL-1, IL-6, IL-8), tumor necrosis factor-alpha (TNF- α), interferon (IFN)- γ and anti-inflammatory cytokines (IL-4, IL-10), tumor growth factor-beta (TGF- β), and vascular endothelial growth factor (VEGF) (28). As for nerve growth factor (NGF), several studies led to divergent hypotheses about the role of NGF, its specific distribution pattern within the tissues and its implication in induction as well as progression of carcinogenesis. However, other recent studies have shown that NGF may have direct clinical relevance in certain tumor cell prevention (29-32).

Involvement of cytokines in the pathogenesis of cancer, and especially HNSCC, has been investigated in a number

of recent studies (33-36). These studies have categorized cytokines as (a) factors that affect tumor growth, (b) factors that can be used as prognostic markers and (c) those that are possible immunotherapeutic targets (37). Although the main source of cytokines are immune cells, many tumor cells have been shown to make autocrine mediators to support their own growth thus evading the immune response (38); they include HNSCC that produce IL-4, IL-6, IL-8, IL-10, granulocyte macrophage-colony-stimulating factor (GM-CSF), VEGF, prostaglandin E2 (PGE2) as well as basic fibroblast growth factor (bFGF).

It has been hypothesized that some of these cytokines may be used as additional diagnostic markers in the sera of patients because of their excessive production by the tumor cells (39). This could be of great value since there are currently no reliable markers to predict either tumor development or relapse in treated HNSCC patients. Recent studies have demonstrated that HNSCC is associated to a decrease in Th1 and an increase in Th2 cytokine levels, which is thought to be used as a mechanism to evade anti-tumor immune response. In fact, this shift towards the Th2 cytokine response is a common event in HNSCC and many other solid tumors, such as colorectal cancer, renal cell carcinoma, prostate cancer, and melanoma. Therefore, current studies in search of immunotherapeutic approaches to cancer aim to shift the balance in favor of Th1 response.

NEUTROPHIL-TO-LYMPHOCYTE RATIO

An increased neutrophil-to-lymphocyte ratio (NLR) is associated with poorer prognostic outcomes in numerous types of cancer, including HNSCC (40-43). However, a small number of studies have demonstrated the prognostic role of NLR in patients with laryngeal cancer (44). The association between NLR and survival outcomes in 654 patients with LSCC was evaluated (45) and the authors reported that blood and biochemical parameters, including NLR, platelet-to-lymphocyte ratio and albumin-to-globulin ratio were associated with clinical characteristics of the patients, with the exception of histologic grade. Survival analysis demonstrated that NLR at cutoff values subdivided patients into different survival outcomes; subsequent to adjustments for age and other clinical features, NLR was identified to be an independent prognostic factor for overall survival and progression-free survival. Increased levels of cytokines, including IL-6 and IL-8 in tumor tissues were associated with NLR values. In summary, pre-treatment NLR was associated with the

prognostic outcomes for patients with laryngeal cancer and may assist to establish prognostic factors for these patients.

INTERLEUKIN-8

Interleukin-8, a pro-inflammatory factor of the CXC chemokine family that was originally classified as neutrophil chemoattractant, is now reported to play an important role in cancer invasion, angiogenesis and metastasis (46-51). The cancer cells themselves can also secrete IL-8 in an autocrine or paracrine manner, such as in breast cancer, gastric cancer, colon cancer, cervical cancer, pancreatic cancer and leukemia (52). Moreover, in human colon cancer cell lines, constitutive expression of IL-8 has been linked to metastatic potential and has been suggested to play a role in the development of distant metastases. *In vivo* analysis also showed that IL-8 would be a sensitive marker in predicting prognosis and monitoring disease progression of the pancreatic cancer patients (52).

INTERLEUKIN-8 IN HEAD AND NECK CANCER

One of the factors that is known to positively drive metastasis is angiogenesis, which has been reported to be associated with decreased survival of HNSCC patients (2, 53-55). IL-8, along with other factors produced either by normal or malignant cells such as VEGF and bFGF has been shown to contribute to angiogenesis, tumorigenesis, and metastasis in patients with HNSCC (2, 33, 56).

Serum levels of IL-8 are consistently elevated in patients with recurrent or metastatic SCCHN and elevated levels may correlate with advanced or aggressive disease (34, 36, 57); in general, IL-8 production is linked with tumor vascularization, metastatic phenotype, tumor growth, and overall poor prognosis. A clear switch from cytokine Th1 to cytokine Th2 in HNSCC patients, low levels of IL-2 and IFN- γ in advanced stages, as well as a positive correlation of increased levels of both IL-2 and IL-12 with the early stages of laryngo-pharyngeal cancer were reported (58). Loco-regional metastases were correlated with increased levels of IL-8 and IL-10 and drastic decrease of IFN- γ . In advanced cancer stages, it has been found that the most affected were IL-2 and IFN- γ correlated with increased levels of Th2 cytokines, supporting the hypothesis that the ratio between different Th1 and Th2 cytokines could represent a useful marker for clinical and pathological evaluation of cancer patients.

Interleukin-8 can be significantly triggered by SDF-1/CXCR4 interaction in HNSCC and its secretion is regulated by Akt phosphorylation after SDF-1 stimulation (59). These

results point out the importance of SDF-1/CXCR4 interaction in HNSCC angiogenesis and provides a new targeting therapy utility, disrupting SDF-1/CXCR4 interaction combined with downstream-induced angiogenic factors in HNSCC would be beneficial to improve clinical outcome.

According to Swenson and colleagues IL-8 and VEGF expression is based on interactions between NF- κ B, AP-1, and NF-IL6 (60). They identified at least 1.5-fold dose-dependent induction of AP-1, VEGF, and IL-8 promoter/reporter gene activity after 24-hour exposure to cigarette smoke condensate (CSC) reporting that tobacco carcinogens up-regulate AP-1 activity and AP-1 dependent IL-8 and VEGF gene expression in head and neck cancer (60). This up-regulation may promote an angiogenic phenotype that favors invasion in both premalignant and squamous cancer cells of the head and neck. CSC could therefore significantly stimulate AP-1 activation of both genes, resulting in increased IL-8 and VEGF secretion, and these processes could be down-regulated with introduction of a dominant negative *A-Fos* gene. These data demonstrate a role for tobacco carcinogen stimulation of pro-angiogenic cytokines, thus promoting an environment suitable for development and metastatic spread of head and neck cancer cells.

INTERLEUKIN-8 IN LARYNGEAL CANCER

The role of IL-8 in the development and progression of LSCC has been recently investigated (61). In the last decade, four studies focused on the role of IL-8 in LSCC, enrolling a total of 214 patients with laryngeal cancer, and 6 patients with laryngeal dysplasia (52, 58, 62, 63). IL-8 sample was taken by peripheral venous blood, while the types of assays for IL-8 measurement were ELISA kits. The main results of these studies are summarized in Table 1.

Eyigor *et al* investigated the relationship between the size of tumors, lymph node metastases, stage, differentiation and cytokine levels and reported no significant differences between these variables. IL-8 level in patients affected by laryngeal cancer ranged from 24.9 pg/ml (52) to 227.15 pg/ml (62). Patients with laryngeal dysplasia presented 24.02 pg/ml. IL-8 cytokine level in controls reached the maximum of 34.33 (62). These results are in line with two previous studies (33, 38), although they focused on HNSCC and did not specify how many patients had laryngeal cancer compared with other sites in head and neck (38). Similarly, Druzgal *et al* (34) and Hathaway *et al* (36) also demonstrated some elevation in IL-8, although without statistical significance. Contrary to the

findings of Eyigor *et al*, Gokhale (57) reported that IL-8 was not elevated in newly diagnosed patients but in patients with recurrence and metastasis of HNSCC. Likewise, Hathaway *et al* (36) also reported that, although IL-8 is not markedly increased in cancer patients, it is associated with the tumor size. Serum IL-8 level of patients with LSCC was associated with lymph node metastasis and T classification (52). High levels of IL-8 were not significantly associated with shorter overall survival and progression-free survival. Univariate and multivariate analyses showed that high IL-6 serum level, contrarily to IL-8, was an independent adverse prognostic variable for progression free and overall survival (52).

Patients with T2 pharyngeal cancer showed that the average value of IL-8 was much higher than in patients with T2 stage laryngeal cancer (58). In exchange, in T3 group, nearly 25% of patients exhibiting high or detectable levels of IL-8 had laryngeal cancer. Concerning the Th1/Th2 levels according to loco-regional metastases, the authors identified a specific pattern consisting in a low level of IFN- γ and high levels of IL-8 and IL-10. Serum level of IL-8 was high in patients with loco-regional metastases and was low, although noticeable, in patients with reactive nodes. Loco-regional metastases were correlated with a specific cytokine pattern consisting of increased levels of IL-8 and IL-10 and a drastic decrease in IFN- γ , demonstrating that the alteration of the immunological balance in these patients with the activation of an immune response may be inefficient to exclude metastatic processes. The switch towards a Th2-type response in advanced cancer patients and the high levels of IL-8 and IL-10 could be responsible of promoting the tumor growth through contiguity. The higher level of IL-8 in patients with loco-regional metastases demonstrates the importance of this cytokine as an indicator of the presence of local metastases, potentially contributing to the correct evaluation of patients and an adequate therapy selection.

The presence of a significant positive relationships between TNF-receptor-associated-factor (TRAF)-6 mRNA expression and IL-8 secretion when stimulated with phytohaemagglutinin (PHA) in patients with laryngeal carcinomas (63). These authors found that patients with carcinomas characterized by the highest TRAF6 mRNA expression had higher IL-8 concentration. They also noted the positive relationship between toll-like receptor-4 (TLR-4) mRNA status and IL-8 concentration, but this association had borderline statistical significance. This data is in agreement with other studies reporting a higher of TLR4 activity on inflammatory cells

Table 1. Recent studies that investigated the role of interleukin-8 (IL-8) in laryngeal squamous cell carcinoma (LSCC). The studies included 214 patients with laryngeal cancer and 6 patients with laryngeal dysplasia.

Reference	Nº of patients	Sample taken; types of assays for IL-8 measurement	Cytokine levels in patients; Mean \pm SE (pg/ml)	Cytokine levels in controls; Mean \pm SE (pg/ml)	Comments
Eyigor et al (2014) (62)	17 patients with LSCC; 6 patients with laryngeal dysplasia; 22 healthy controls	Peripheral venous blood samples; ELISA	Levels of IL-8: 227.15 \pm 81.19 in patients with L SCC ($p < 0.05$); 24.02 \pm 11.57 in patients with laryngeal dysplasia;	Levels of IL-8: 34.33 \pm 12.60	No significant difference between tumor size, lymph node metastases, stage, differentiation and cytokine levels
Hao et al (2013) (52)	92 patients with primary diagnosis of LSCC; 50 age and gender matched healthy blood donors with no previous cancer history served as healthy controls.	Peripheral venous blood samples; ELISA	24.9 [3.6–56.0], ($p < 0.0001$)	14.5 [2.3–42.6], ($p < 0.0001$)	Serum levels of IL-8 were only associated with lymph node metastasis ($p = 0.01$) and T classification ($p = 0.011$)
Bleotu et al (2013) (58)	50 patients with laryngeal ($n = 41$) or pharyngeal ($n = 9$) cancer	Peripheral venous blood samples; ELISA	123.47 \pm 282.66	0.0 \pm 0.0	Loco-regional metastases were correlated with increased levels of IL-8 and IL-10 and drastic decrease of IFN γ .
Starska et al (2012) (63)	55 patients with LSCC. The control group of healthy donors consisted of 51 individuals.	Peripheral blood mononuclear cells; ELISA	119.69 \pm 29.95 ng/mL; proinflammatory cytokine production following mitogenic stimulation was 231.56 \pm 29.95 ng/mL. The secretion pattern of carcinomas with the invasion of cartilage was 97.4 \pm 18.81 ng/mL, as well as 243.5 \pm 81.77 ng/mL with mitogenic stimulation. Proinflammatory cytokine production of tumors characterized by mode of invasion with no distinct borderlines was 139.5 \pm 53.33 ng/mL, as well as 266.9 \pm 84.45 ng/mL following mitogenic stimulation, respectively. In carcinomas with 14-17 points TFG was 122.5 \pm 38.24 ng/mL, as well as 270.5 \pm 56.17 ng/mL in experiments with mitogenic stimulation.		In all cases of LSCC the evaluation of proinflammatory cytokine expression, disclosed the presence of significant positive relationships between TRAF6 mRNA expression and IL-8. secretion when stimulated with PHA ($r = 0.83$, $p = 0.009$). Patients with carcinomas characterized by the highest TRAF6 mRNA expression, were found to demonstrate higher IL-8 concentration. It was also noted a positive relationship between TLR4 mRNA status and IL-8 concentration, but this association had borderline statistical significance ($r = 0.67$, $p = 0.06$).

from patients with laryngeal carcinomas (64). Specifically, more invasive laryngeal carcinomas characterized by a higher disseminated tumor invasion (with no distinct borderlines of the tumor front or with diffuse growth), demonstrated a lower mRNA expression of each TLR pathway molecule in immunocompetent cells. Moreover, it was observed that the more advanced histological laryngeal neoplasm (with higher scores according to TFG total points), the lower TLR4 and TRAF6 mRNA expression in peripheral blood mononuclear cells. This data is in agreement with the findings of studies reporting that the TLR pathway molecules are the key mediators of immune mechanisms, which result in activation of antigen-specific immunity, leading to production of the proinflammatory mediators (63). Higher whole blood levels of IL-6, IL-8 and TNF- α were related to a higher activity of TLR signaling molecules, connected with a less disseminated invasion of laryngeal carcinomas. These findings demonstrate evidence of unsuppressed anti-tumor immunological response in these types of neoplasms. Moreover, these results suggest the presence of alterations in immunological activity and changes in the secretion of proinflammatory cytokines by circulating immunocompetent cell, which lead to inhibition of tumor progression in advanced laryngeal cancer. Based on these preliminary findings, it could be hypothesized that the TLR signaling molecules might be good potential biomarkers of tumor behavior in LSCC as they could potentially determine the aggressiveness of laryngeal cancer acting through regulatory mechanisms of interleukin production.

CONCLUSIONS

The mechanisms of cancer origin, invasion, and metastasis is one of the emergent and most promising scientific fields in head and neck cancer, especially in LSCC. Tumor, lymph node and metastasis (TNM) classification is not sufficient for the estimation of tumor aggressiveness; moreover, different conclusions may result from a heterogeneous group of investigated HNSCC cancers in different locations such as oral cancers, laryngopharyngeal carcinomas, nasal cavities and paranasal sinuses tumors (1, 4, 22-24, 65-68).. Biomarkers such as IL-8 deserve further investigation as they could have a role as a screening test for head and neck cancer. To date, there is little and still contrasting evidence on the role of IL-8 in LSCC; however, preliminary data available in the literature are encouraging and could suggest a role for IL-8 as a support of the clinical decisions for appropriate therapy and postoperative care in individual patients affected by laryngeal cancer.

CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement in any organization with any financial interest in the subject matter discussed in this review article.

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