



ORIGINAL ARTICLE / ORIGINAЛНИ РАД

Smoking and inflammation in laryngeal squamous cell carcinoma

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SUMMARY

Introduction/Objective Epidemiological studies have established cigarette smoking as one of the most significant risk factors in pathogenesis of laryngeal squamous cell carcinoma (LSCC). One of the possible underlying mechanism is chronic inflammation, but published data regarding the effect of tobacco on systemic immune response is inconsistent.

The goal of this study was to evaluate concentrations of serum proinflammatory cytokines [interleukin (IL)-6, IL-1 β , and tumor necrosis factor (TNF)- α] in patients with LSCC and in healthy subjects according to cigarette smoking.

Methods Fifty-nine LSCC patients and 44 healthy controls were enrolled in the study. Samples of peripheral blood and details of tobacco use were gathered from the examinees. Flow cytometry was performed to analyze serum concentrations of IL-6, IL-1 β , and TNF- α . The results were compared according to active smoking status.

Results Statistical analysis revealed no significant difference between smoking LSCC patients and smoking healthy subjects. Additionally, investigated cytokines were not significantly different in healthy subjects according to smoking status. In non-smoking participants with LSCC, concentrations of serum IL-1 β and TNF- α were higher ($p < 0.05$) in comparison with smoking LSCC patients.

Conclusion Findings of our study may indicate that smoking leads to the suppression of proinflammatory response in LSCC patients, whilst proinflammatory response is unaffected by cigarettes in healthy subjects.

Keywords: smoking; IL-6; IL-1 β ; TNF- α ; laryngeal squamous cell carcinoma

INTRODUCTION

Carcinogenesis is a multifactorial and multistage process in which gene-environment interactions play crucial role. Smoking is well-established as a significant risk factor in laryngeal squamous cell carcinoma (LSCC). One of the most accepted hypotheses in carcinogenesis is chronic inflammation. Inflammation and immune modulation induced by tobacco and asbestos are broadly associated with lung cancer, alcohol consumption, and inflammation of the pancreas with pancreatic cancer, hepatitis B infection with liver cancer, inflammatory bowel disease (Crohn's disease and ulcerative colitis) with colorectal cancer. Despite the established evidence of the causal relationships between smoking and elevated cancer risk, the underlying mechanism has not been completely understood. The fact that not all smokers develop cancer suggests individual susceptibility for developing malignant disease. Recently published literature reports genetic and epigenetic changes induced by tobacco carcinogens in head and neck carcinoma [1]. Additionally, in previously published literature, nicotine was found to exert both pro-inflammatory and anti-inflammatory effects [2].

Virchow noticed over a 100 years ago that histologic appearance of tumor tissue resembles

the histologic change seen in unhealed wound [3]. Conversely, Coley [4] reported regression of the malignant tumor following bacterial infection. Today, inducing strong infection and inflammation by *Mycobacterium bovis*, bacillus Calmette-Guerin (BCG) in bladder carcinoma is standard antitumor therapy.

Association between cancer and inflammation is reflected by the presence of numerous proinflammatory cytokines in cancer. It is believed that mediators released by host inflammatory cells or cancer cells are involved in tumor initiation, promotion, and progression. Given that inflammation can have both protumorigenic and anti-tumorigenic effect, it seems that the role of inflammation in tumorigenesis depends on the interaction between tumor cells, immune cells, and inflammatory cells. Since deregulated inflammation is a significant factor in carcinogenesis of numerous malignant tumors, identifying the mechanisms by which inflammation is deregulated in cancer may improve antitumor therapeutic strategies.

The goal of this research was to reveal the relations between smoking and concentrations of serum proinflammatory cytokines TNF- α , IL-6, and IL-1 β in patients with LSCC and in healthy subjects.

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METHODS

The research was performed as a cross-sectional study of 59 patients with LSCC (40 smokers, 19 non-smokers). All the patients were diagnosed at a tertiary referral center. The diagnosis of LSCC was confirmed clinically, histopathologically, and radiologically. The control group included 44 subjects (14 smokers, 30 non-smokers), healthy volunteers with normal fiberoptic laryngeal findings.

Informed consent was collected from both patients and controls following the hospital's ethics committee-approved protocol. Exclusion criteria were as follows: any other previous or present malignant or autoimmune disease, history of allergies, co-existing infectious disease, systemic corticosteroid or any immunomodulating therapy.

We defined active smoking as consuming more than 20 cigarettes per day during the period of the last five years.

Samples of peripheral venous blood (5 ml) were taken from all LSCC patients and healthy individuals included in the study, then allowed to clot for 30 minutes. Blood samples were centrifuged at 1,000 g for 15 minutes. Serum was separated, aliquoted and stored at -80°C until cytokine detection. Flow cytometric kit (FlowCytomix™ Multiple Analyte Detection System, Human FlowCytomix™ Inflammation Panel, eBioscience, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to measure the serum levels of TNF- α , IL-6, and IL-1 β on the flow cytometer (Beckman Coulter XL-MCL, USA), which was connected with BMS FlowCytomix Pro 2.2 Software in accordance with the manufacturer's instructions. By the manufacturer's instructions, the standard range was 27–20,000 for TNF- α , IL-6, and IL-1 β .

Statistical tests were performed using GraphPad Prism 5 (Graph Pad Software, San Diego, CA, USA). Mann-Whitney U (nonparametric) test was used for comparison between the groups. The results were rendered as mean \pm SD (standard deviation). If p was < 0.05 , we considered the difference statistically significant.

RESULTS

Cytokine levels in smoking LSCC patients and smoking control groups

Concentrations of serum cytokines in smoking LSCC patients and smoking control individuals are presented in Table 1. No statistically significant difference was observed between these two groups of patients.

Table 1. Distribution of cytokine levels in smokers

Cytokine	Cytokine level, mean \pm SD (pg/mL)		p
	LSCC smokers	Control smokers	
IL-6	39.40 \pm 69.54	53.93 \pm 91.18	0.7894
IL-1 β	191.3 \pm 351.9	239.3 \pm 408.4	0.6711
TNF- α	143.2 \pm 231.3	178.8 \pm 312.7	0.7002

LSCC – laryngeal squamous cell carcinoma; SD – standard deviation

Cytokine levels in LSCC patients according to smoking

Statistical analysis revealed significantly higher concentrations ($p < 0.05$) of IL-1 β and TNF- α in non-smoking LSCC patients compared to smoking patients. The results are shown in Table 2, Figure 1, and Figure 2.

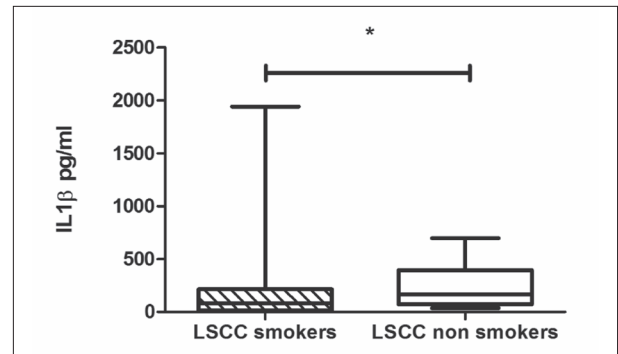


Figure 1. Comparison of interleukin (IL)-1 β serum levels in smoking and non-smoking laryngeal squamous cell carcinoma (LSCC) patients

* $p < 0.05$

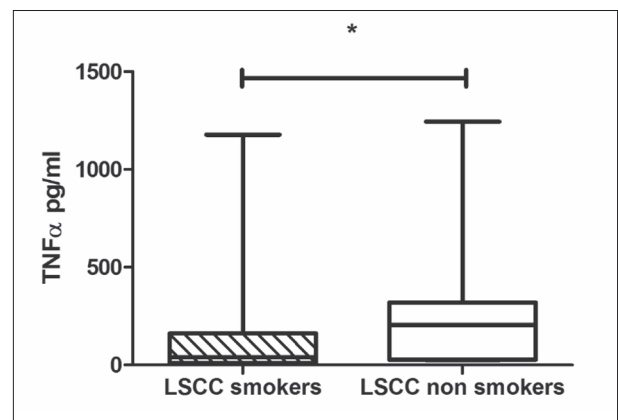


Figure 2. Comparison of interleukin TNF- α serum levels in smoking and non-smoking laryngeal squamous cell carcinoma (LSCC) patients

* $p < 0.05$

Cytokine levels in the Control Group according to smoking

Proinflammatory cytokines were not significantly different between controls who smoke and controls who do not smoke (Table 2).

DISCUSSION

As chronic infection and inflammation may lead to malignant cell transformation, a malignant tumor may also induce chronic inflammation. The intrinsic inflammatory pathway activated by genetic changes that cause neoplasia leads to an excessive production of inflammatory cytokines. This mechanism is observed in the activation of oncogenes such as MYC, RAS, RET, or inactivation of tumor suppressors. On the other hand, both extrinsic (alcohol)

Table 2. Distribution of cytokine levels in laryngeal squamous cell carcinoma patients and control subjects according to smoking

Cytokine	Cytokine level mean \pm SD (pg/mL)		p	Cytokine level mean \pm SD (pg/mL)		p
	LSCC patients			Controls		
	Smokers	Non-smokers		Smokers	Non-smokers	
IL-6	39.4 \pm 69.54	45.56 \pm 48.16	0.4336	53.93 \pm 91.18	55.42 \pm 67.81	0.1895
IL-1 β	191.3 \pm 351.9	247.7 \pm 199.3	0.0450	239.3 \pm 408.4	161.6 \pm 158.4	0.5812
TNF- α	143.2 \pm 231.3	282.2 \pm 343.7	0.0304	178.8 \pm 312.7	120 \pm 142.1	0.7314

LSCC – laryngeal squamous cell carcinoma; SD – standard deviation

and intrinsic (K-RAS) pathways of inflammation play a role in pathogenesis of pancreatic cancer [5].

TNF- α is prototypical proinflammatory cytokine and has significant role in host defense to bacterial, viral, and parasitic infections [6]. Although originally found to be toxic for cancer cells in high doses, TNF- α increases the colonization of the peritoneum and neovascularization of developing tumor deposits in ovarian cancer [7]. *In vitro* studies revealed therapeutic effect of TNF- α antibodies in liver, colorectal, and pancreatic cancer, although the exact mechanism remains unclear [8]. Infliximab, a specific TNF- α inhibitor, displays potential as an antitumor drug [9].

IL-6 is regarded as a key growth factor for both malignant and inflammatory cells. It is associated with cell cycle progression and suppression of apoptosis. Previous studies demonstrated the role of IL-6 in pathogenesis of multiple myeloma [10]. It has been reported that elevated serum concentrations of IL-6 can be found in HNSCC; they can also represent an independent factor of long-term prognosis of LSCC patients [11, 12]. Several authors also showed elevated serum concentrations of IL-6 in LSCC compared to healthy subjects [13, 14].

IL-1 β is strongly connected to inflammatory diseases and cancer. This cytokine has a significant role in the host defense from bacteria, viruses, and fungi [15]. Some recent studies show that epigenetic changes of IL-1 β may represent an important factor in the carcinogenesis [16].

Inflammation due to smoking is one of the proposed mechanisms in cancer. It is interesting to note that the incidence of HNSCC in the Basque region is one of the highest in Europe, while tobacco and alcohol consumption is one of the lowest compared to other regions in Europe [17]. It is clear that carcinogenesis depends on the interaction between environmental factors, such as smoking, and genetic and immune host factors.

When we compared serum cytokine profile of LSCC patients who are active smokers and smoking healthy subjects, we did not observe any difference (Table 2). Surprisingly, according to our results, inflammation is not greater in cancer patients who smoke compared to inflammation in healthy smoking individuals.

Correspondingly, after comparing serum proinflammatory cytokines in control subjects, statistical analysis showed no difference between smokers and non-smokers (Table 2). In contrast to our results, Zeidel et al. [18] found increased production of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in asymptomatic smokers.

More than 60 carcinogens are identified in the cigarette smoke, although the underlying mechanism of smoking

in carcinogenesis is still unclear. [19]. Chronic exposure to tobacco carcinogens leads to mutations of the K-RAS oncogene and the p53 tumor suppressor gene, oxidative damage, deregulated apoptosis, and cell cycle [19]. Epigenetic changes are also considered vital in the metabolism of tobacco carcinogens, thus enlarging the effect of smoking in carcinogenesis.

Statistical analysis on subgroups of LSCC patients who actively smoke and of those who do not, revealed that non-smokers had statistically significant ($p < 0.05$) elevated serum concentrations of TNF- α and IL-1 β compared to smokers (Table 2, Figures 1 and 2). According to our results, cigarette smoking leads to a reduced proinflammatory response in LSCC patients. These observations may suggest that patients with LSCC are more susceptible to bacterial, viral, parasitic, and fungal infections, considering the role of IL-1 β and TNF- α in host defensive mechanisms [6, 18].

Data regarding the effect of tobacco on systemic immune response is inconsistent. Suppression of inflammatory response is in accordance with Shiels et al. [20], who concluded that smoking leads to the suppression of systemic immune marker levels. *In vitro* studies also showed decreased production of IL-1 β , IL-2, IFN- γ , and TNF- α by nicotine [21, 22]. Conversely, other authors showed increment of serum proinflammatory cytokine due to smoking [23, 24].

It is questionable whether inflammation is a sufficient factor to promote carcinogenesis. Chronic inflammation is observed in many other diseases apart from cancer. Chronic inflammation is present in Chronic obstructive pulmonary disease (COPD), while macrophage innate response, pro Th-1 and Th-17 response to bacteria is suppressed [25, 26]. Although the most prevalent, tobacco and inflammation, cannot be considered the only etiological factors in laryngeal carcinogenesis. Several studies have suggested an association between laryngeal cancer and heavy metal exposure, industrial heat, mustard gas, hair dye, nickel, wood dust, rubber, diesel and gasoline fumes, formaldehyde, asbestos, organic solvents, mineral oil, coal dust.

Studies which include subjects' self-reported data on tobacco and alcohol consumption have certain limitations. This is primarily related to the fact that the measures of smoking exposure rely on self-reported data. Khariwala et al. [27] revealed that the carcinogen exposure in HNSCC patients does not correlate with self-reported tobacco use. The possible explanations for such inaccuracies could be that the patient is facing physicians' expectations, fear, or guilt as he is treated for malignancy. Likewise, in healthy subjects, potential shame or embarrassment because of unhealthy habit can occur. The need for a more objective analysis of

tobacco exposure and carcinogen dose is also reflected in the fact that the number of cigarettes per day may not be the accurate measure of exposure. Variability in puffs per cigarette, depth of inhalation, type of cigarette or cigar can significantly influence the actual carcinogen exposure.

Like in our study, most of the published data refer to the serum cytokine levels. It is possible that cytokines serum levels may not represent the adequate tumor-host interaction since it may differ from the concentrations of immune mediators in tumor microcirculation.

CONCLUSION

The results of our study demonstrate the complex relationship between carcinogenesis, inflammation, environmental factors, and host factors. Our results show that smoking leads to significantly decreased ($p < 0.05$) serum levels of TNF- α and IL-1 β in smoking LSCC patients compared to non-smoking patients. This data may suggest that these patients are more susceptible to bacterial, viral, parasitic, and

fungal infections, reflecting an immunosuppressive effect of cigarette smoking in LSCC patients, while we did not perceive this effect of smoking in healthy subjects. Further investigations are needed to elucidate perplexed network of smoking, carcinogenesis, and host immunity.

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All authors conceived and planned the work that led to the paper and interpreted the evidence it presents. All authors participated in data collection and analysis, in writing of the paper, and they all approved the final version.

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Пушење и инфламација код планоцелуларног карцинома ларинкса

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САЖЕТАК

Увод/Циљ Епидемиолошке студије јасно показују да је пушење цигарета један од најзначајнијих етиолошких фактора у патогенези ларингеалног планоцелуларног карцинома (LSCC). Једно од могућих објашњења дејства дувана на карциногенезу је хронична инфламација. Међутим, подаци из литературе су често опречни у погледу утицаја пушења на системски имунски одговор.

Ова студија је имала за циљ одређивање концентрација проинфламаторних цитокина у серуму [фактор некрозе тумора (TNF)- α , интерлеукин (IL)-6, IL-1 β] код болесника са LSCC и здравих испитаника у односу на пушење цигарета.

Метод У испитивању је учествовало 59 болесника са LSCC и 44 здрава испитаника. Од свих учесника у студији узети су подаци о пушењу цигарета, као и 5 ml периферне венске крви. Проточном цитометријом урађено је израчунавање концентрација цитокина у крви. Статистичком анализом

поређене су концентрације цитокина испитаника у односу на пушење.

Резултати У групи испитаника са LSCC, серумске концентрације цитокина IL-1 β и TNF- α биле су статистички значајно веће ($p < 0,05$) у групи непушача поредећи их са пушачима. Примењени статистички тестови нису показали постојање значајне разлике концентрација испитиваних цитокина у контролној групи у односу на то да ли испитаници пуше. Такође, концентрације испитиваних проинфламаторних цитокина у групи пушача са LSCC нису се разликовале у односу на здраве пушаче.

Закључак Пушење има имunosупресивни ефекат на проинфламаторни одговор код болесника са LSCC. Код здравих пушача нема имunosупресивни ефекат. Такође, нема разлике у системском проинфламаторном одговору између пушача са LSCC и здравих пушача.

Кључне речи: пушење; IL-6; IL-1 β ; TNF- α ; планоцелуларни карцином ларинкса