Analysis of Serum Cytokine Levels in Larynx Squamous Cell Carcinoma and Dysplasia Patients

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ABSTRACT

Background: Although the imbalance of cytokines in Head and Neck Squamous Cell Carcinoma (HNSCC) is well known, there is scarce data regarding its occurrence during dysplasia, before the malignant transformation. **Objective**: To determine whether laryngeal dysplasia patients show a different cytokine profile than patients with cancer and healthy controls. **Methods:** Seventeen newly diagnosed, untreated larynx squamous cell carcinoma (SCC) and six laryngeal dysplasia patients as well as 22 healthy controls were analyzed for circulating cytokines. A flowcytometry Th1/Th2 cytokine array kit was used to quantitatively measure Interleukin-2 (IL-2), IL-4, IL-6, IL-10, Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ) levels. Additionally, IL-8 levels were determined through ELISA. **Results:** IL-6, IL-8 and IL-10 were determined to be statistically increased in SCC patients ($p<0.05$). IL-8 and IL-10 levels were also higher in SCC patients than dysplasia patients ($p<0.05$). Additionally, IL-6 and IL-10 were all found to be markedly increased in dysplasia patients compared with controls ($p<0.05$). **Conclusion:** Our results demonstrate an imbalance of IL-6 and IL-10 not only in HNSCC but also in laryngeal dysplasia.

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Keywords: Bead Array, Cytokine, Dysplasia, HNSCC, Larynxm Carcinoma

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INTRODUCTION

Cytokines are a group of soluble, low-molecular-weight proteins that mediate immune and inflammatory responses. Currently 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 (1). The Th1 cytokines are interferon-gamma (IFN-γ), interleukin-12 (IL-12) and IL-18, Th2 cytokines comprise IL-4, IL-5, IL-10 and IL-13 and the Th17 cytokine is IL-17 (1). Th1 cytokines stimulate cellular immune responses, while Th2 cytokines predominantly regulate humoral responses. In addition, IL-17 is currently known to regulate inflammatory responses and plays several roles in autoimmunity (1).
Cytokines are also classified as pro-inflammatory (e.g. IL-1, IL-6, IL-8, tumor necrosis factor alpha (TNF-α), IFN-γ and anti-inflammatory cytokines (e.g. IL-4, IL-10, tumor growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF)(2). 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 (HNSCC)(3,4). Involvement of cytokines in the pathogenesis of HNSCC has been investigated by a number of recent studies (4-8). 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 (9). The main source of cytokines are immune cells, however, various tumor cells are shown to make autocrine mediators to support their own growth and evade immune responses (6,10). Among these tumors are HNSCC, which 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)(4). It is 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 (5,11).This could be of great value since there are currently no reliable markers to predict either tumor development or relapse in treated patients.
A number of recent studies demonstrated that HNSCC is associated with a decrease in Th1 cytokine levels and an increase in Th2 cytokine levels, which is thought to be used as a mechanism to evade anti-tumor immune response (2,4,12). This shift towards the Th2 cytokine response is in fact a common event seen in HNSCC and many other solid tumors, such as colorectal cancer, renal cell carcinoma, prostate cancer, and melanoma (13,14).Therefore, current studies in search of immunotherapeutic approaches to cancer aim to shift the balance in favor of Th1 responses (4,9).
Although the studies mentioned above and many others have investigated the involvement of cytokines in HNSCC, to our knowledge, have not yet included laryngeal dysplasia patients in their analysis. As it is commonly known, dysplasia is the pre-malignant state, of which 14% end up with malignant transformation(15). Therefore, we first aimed to determine whether laryngeal dysplasia patients showed a different cytokine profile the patients with cancer and healthy individuals. Second, we also made a detailed comparative analysis of cytokine levels in various pathological and clinical stages of larynx squamous cell carcinoma (SCC).

MATERIALS AND METHODS

Patients. Upon histopathological analysis, a total of 23 patients composed of 17 primary larynx SCC and six laryngeal dysplasia cases, were recruited for this study. The SCC patients had not previously received chemotherapy and/or radiotherapy and had no other known malignancies. As for controls, 22 healthy volunteers, who had not been diagnosed with any immunological disorders and who had not received any immunomodulatory treatments within the last six months and who had no co-existing infectious diseases, were included. Cancer patients were staged according to the 2002/3 American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) classification. Demographics of the study subjects are shown in Table 1.

Table 1. Demographical properties of the study groups.

Group	No. of Patients	Median Age yrs	Age Range yrs	Sex	
				Female	Male
Larynx SCC	17 (73.8%)	57	45-77	1	16
Dysplasia	6 (13.3%)	69	51-78	0	6
Healthy	22 (48.9%)	34	17-53	3	19
Total	45 (100.0%)	50	17-78	4	41

This study was approved by the local ethics committee, Antalya Education and Research Hospital Ethics Committee and an informed consent was obtained from each patient prior to sample acquisition.

Assays for IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ Measurement. Peripheral venous blood samples were collected in sterile test tubes, centrifuged at 1,000 ×g for 15 minutes and serum samples were stored at -20°C until used. The BD™ CBA Human Th1/Th2 Cytokine Kit II (Cat. No. 551809) was used to quantitatively measure IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ protein levels in a single sample using the beadarray technology as described by the manufacturer. Briefly, the assay uses beadarray technology to simultaneously detect the cytokines, mentioned above, in very small samples. Six bead populations with different fluorescence intensities that have been coated with capture antibodies specific for IL-2,IL-4, IL-6, IL-10, TNF-α, and IFN-γ proteins were mixed together to form the bead array. The results were then visualized using the red channel of a FACSCanto II flowcytometer. Capture beads were then mixed with recombinant standards or unknown serum samples and were incubated with the PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex was measured to determine the concentration of that cytokine. After acquiring samples on the flowcytometer, we used FCAP Array™ software to generate final results. For calculations, data obtained from a set of diluted standards was used to generate a standard curve for each cytokine and the

final calculations of cytokine concentrations were made accordingly. Additionally, since IL-8 was not included in our array, we separately measured IL-8 levels in the samples using an ELISA kit (BD Biosciences, Cat. No: 555244).

Statistical Analysis. Serum levels of individual cytokines between groups were compared by Student *t*-test with Welch correction. Statistical analyses were performed using the Graph-Pad-In-Stat software. P values less than 0.05 was considered to be statistically significant.

RESULTS

Cytokine Levels in Patients and Controls. Cytokine levels measured in the sera of patients and controls are displayed in Table 2.

Table 2. Cytokine levels of study groups.

Cytokine	Mean ± SE (pg/ml)	Range (pg/ml)
IL-6		
SCC	3.82 ± 1.74	0.50-31.00
Dysplasia	3.98 ± 1.74	0.69-11.94
Controls	0.61 ± 0.08	0.00-1.33
IL-8		
SCC	227.15 ± 81.19	2.50-855.12
Dysplasia	24.02 ± 11.57	5.02-81.07
Controls	34.33 ± 12.60	0.00-237.23
IL-10		
SCC	26.67 ± 4.59	0.00-59.01
Dysplasia	3.31 ± 0.63	1.82-6.12
Controls	1.99 ± 0.70	0.00-14.13

Analysis of serum cytokine levels of larynx SCC, dysplasia patients and healthy controls revealed changes in the cytokine profiles. Because we were unable to recruit age-matched healthy individuals into the study, we had to use healthy controls of younger ages. However, the ages of dysplasia and the cancer groups were well matched. IL-6, IL-8 and IL-10 levels were determined to be statistically increased in SCC patients compared with the healthy controls (p=0.000, p=0.016, p=0.009, respectively; Figures 1, 2 and 3).

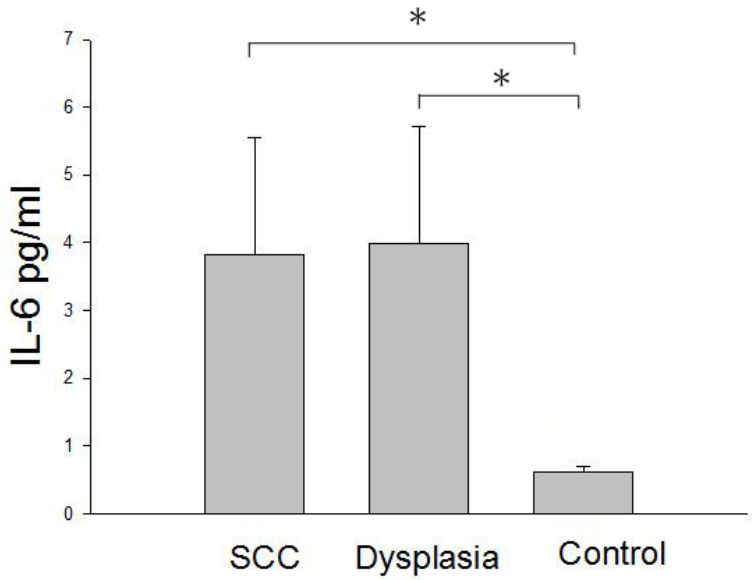

Figure 1. Serum IL-6 levels in patients with SCC and dysplasia as well as healthy controls. IL-6 was significantly increased in both SCC and dysplasia compared with controls. There was no statistical difference between patients with dysplasia and cancer; *p<0.05.

IL-8 and IL-10 were also determined to be markedly increased in patients with SCC compared with patients with dysplasia (p=0.012, p=0.016, respectively; Figures 2 and 3).

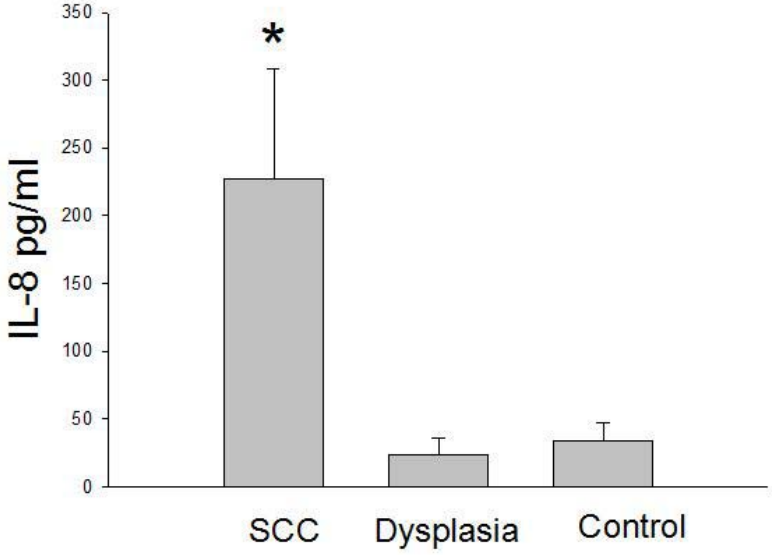

Figure 2. Serum IL-8 levels in patients with SCC and dysplasia as well as healthy controls. IL-8 was significantly increased in patients with SCC patients compared with patients with dysplasia and controls. There was no statistically significant difference between the patients with dysplasia and healthy controls; *p<0.05.

Similarly, IL-6 and IL-10 were found to be higher in patients with dysplasia than in the healthy controls (p=0.040, 0.008, respectively; Figures 1 and 3).

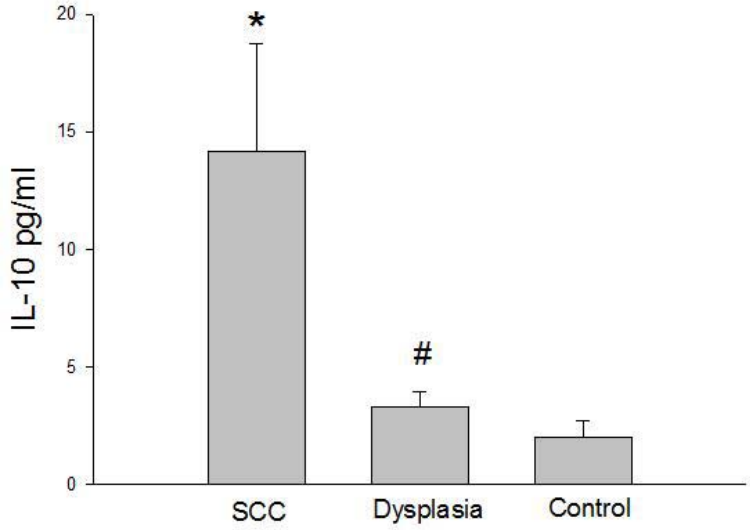

Figure 3. Serum IL-10 levels in patients with SCC and dysplasia as well as controls. IL-10 was significantly increased in SCC patients compared with patients with dysplasia and healthy controls. IL-10 was also significantly elevated in patients with dysplasia compared with controls;*p<0.05, # p<0.05

In addition, mean IL-2 and IL-4 levels were found to be 2.9 ± 1.49 pg/ml and 3.41 ± 2.89 pg/ml in SCC group, respectively, while they were not detectable in the healthy controls and dysplasia patients. Therefore, no statistical analysis was performed. Similarly, TNF-α and IFN-γ were not detected in any of the tested groups.
Statistical analysis of the relationship between cytokine levels and clinicopathological properties of the 17 larynx SCC patients revealed that there was no significant difference between the size of tumors, lymph node metastases, stage, differentiation and cytokine levels (Table 3).

DISCUSSION

HNSCC is one of the most common cancers of humans with high morbidity and mortality rates (2). It is commonly believed that various cellular and biochemical changes lead to the malignant transformation of normal epithelial cells. During this transformation, the cells go through various steps including, hyperplasia, dysplasia, carcinoma in situ, and invasive cancer. Among the risk factors are tobacco use, alcohol consumption, betel nut chewing, and human papillomavirus infection. However, the exact mechanisms for malignant transformation are not currently known (2).

Table 3. Cytokine levels in subgroups of patients with Larynx SCC.

T Status	n	Cytokine Levels (pg/ml)			P Values		
		IL-6	IL-8	IL-10	IL-6	IL-8	IL-10
T1-T2	11	2.37 ± 0.57	218.71 ± 105.19	29.91 ± 24.21	p=0.960	p=0.688	p=0.191
T3-T4	6	6.46 ± 4.91	242.62 ± 138.27	20.73 ± 9.12			
N status							
N0	10	2.33 ± 0.63	239.57 ± 113.99	32.62 ± 26.60	p=0.696	p=0.922	p=0.380
N1-N3	7	5.93 ± 4.18	209.40 ± 121.49	18.17 ± 8.12			
M Status							
M0	17	3.82 ± 1.74	227.15 ± 81.19	26.67 ± 4.59			
Differentiation							
Good	6	2.76 ± 0.93	158.71 ± 139.37	5.43 ± 1.87	p=0.481	p=0.688	p=0.269
Moderately	11	4.39 ± 2.67	264.48 ± 102.99	38.26 ± 23.94			
Stage							
I-II	10	2.33 ± 0.63	239.57 ± 113.99	32.62 ± 26.60	p=0.696	p=0.922	p=0.380
III-IV	7	5.93 ± 4.18	209.40 ± 121.49	18.17 ± 8.12			

Previous studies provided evidence suggesting that cytokines, such as IL-6, IL-8 and IL-10, are involved in the pathogenesis of the HNSCC, even though it is not clear whether they play roles in the transformation or they are merely over-produced as a result of the immune response against the malignancy. In addition, IL-6 and IL-10 are not only produced by the immune cells, but also by tumor cells. It is speculated that these cytokines are used by tumor cells to evade and/or suppress the immune response. Although the involvement of the cytokines in HNSCC has been studied in detail, no data exists in the research regarding whether or not the cytokine imbalance takes place before the malignant transformation. We, therefore, comparatively analyzed dysplasia patients with newly diagnosed untreated larynx SCC patients. Herein, we report that there is an increase in circulating IL-6 and IL-10 levels in dysplasia and laryngeal SCC patients.

Our finding of increased IL-6 in SCC patients is in agreement with previous studies (Riedel *et al*. (11) and Duffy *et al*. (16), Druzgal *et al*. (6), Hathaway *et al*. (8), Hoffmann *et al*. (10). These studies also determined that although tumor size and histological differentiation were not associated with the increase in IL-6 (11), higher stage (11,16) and lymph node metastases (11) were positively correlated with IL-6 levels. In contrast, we were unable to observe such an association. This discrepancy might have resulted from the divergence of study groups. We only studied larynx SCC patients while these studies analyzed HNSCC including larynx, oral cavity, oropharynx, hypo pharynx patients. Although we used the most sensitive method currently available, the techniques used by the studies mentioned above vary. It is well established that IL-6 is an important growth factor for a variety of different cells including lymphocytes, keratocytes, neurons, osteoclasts and endothelial cells (11,16). Furthermore, IL-6 is not only produced by the immune cells but also by the HNSCC tumor cells to be used as an autocrine growth factor to support their own survival (3,10).

Larynx SCC patients had concentrations of IL-8 which were markedly increased compared to the controls, in line with two previous studies by Linkov *et al*. (5) and Hoffman *et al*. (10). Similarly, Druzgal *et al*. (6) and Hathaway *et al*. (8) also demonstrated some elevation in IL-8, which, did not have statistical significance. Contrary to our findings, Gokhale *et al*. (17) reported that IL-8 is not elevated in newly diagnosed patients but in patients with recurrence and metastasis. Likewise, Hathaway *et al*. (8) also report that, although IL-8 is not markedly increased in patients, it is associated with the tumor size. Since our sample group did not include recurrent patients, we were unable to perform such analysis. Furthermore, our patient group did not reveal any significant association between IL-8 and tumor size, lymph node metastasis, stage and tumor differentiation. Metastasis is a complicated and poorly understood phenomenon. 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 (4). Various factors, produced either by normal or malignant cells, including IL-8, VEGF and FGF, are shown to stimulate this process (4).

Our results demonstrated a substantial increase in circulating IL-10 levels in larynx SCC patients. Similarly, Jebreel *et al*. (13) and Alhamarneh *et al*. (18) reported that IL-10 is more detectable in HNSCC patients than in controls. However, Jebreel *et al*. (13) did not compare the mean IL-10 levels because their data did not fit a continuous variable pattern, while Alhamarneh *et al*. (18) found that the mean IL-10 levels were not significantly different. In another study, Hathaway *et al*. (8) determined that IL-10 along with other Type 2 cytokines are increased in active HNSCC patients, although this

elevation was also not statistically significant. Levels of IL-10 have been shown to be associated with tumor size (13,18) and the overall stage of the disease (18). Although our sample groups are similar, we were unable to find any association with either the tumor size or the stage or any other clinicopathological parameters. In contrast, Linkov *et al*. (5), Druzgal *et al*. (6), Mojtahedi *et al*. (7) and Hoffmann *et al*. (10) all reported that IL-10 levels are not different between HNSCC patients and healthy controls. All in all, we were unable to find a study in academic literature reporting a statistically significant difference in mean serum IL-10 level similar to ours. IL-10 is one of the most important immune mediators that tumors use to evade host immune responses. It is a Th2 cytokine, known to suppress cellular immune responses by down regulating both proinflammatory cytokines and HLA class I expression. It is also known to support the growth of various tumors and increase their metastatic potential the tumors (1).

The interesting aspect of our study was the analysis of dysplasia patients in comparison with the cancer patients and controls. We determined that circulating IL-6 and IL-10 levels of dysplasia patients were higher than healthy controls. Interestingly, IL-6 levels of three dysplasia patients were substantially (7 to 20 fold) higher than healthy controls, while they are overall similar with those of the cancer patients. This may suggest that the imbalance of IL-6 occurs before the malignant transformation. It is known that approximately %14 of the dysplasia patients develop cancer (15). However, it is currently not possible to predict which of these patients will be more susceptible to developing cancer. Prospective studies with larger numbers of dysplasia patients are needed to test this hypothesis. Similarly, two recent studies demonstrated that the levels of IL-6, IL-8 and IL-10 were involved in bronchopulmonary dysplasia (BDP) in newborns (19,20). First, Rocha *et al*. have reported that IL-6, IL-8 and IL-10 in cord blood are associated with BDP (19). Second, Koksal *et al*., have demonstrated that IL-6 is positively and IL-10 is negatively associated with BDP (20).

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REFERENCES

1. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, Klunker S, Meyer N, O'Mahony L, Palomares O, Rhyner C, Ouaked N, Schaffartzik A, Van D, V, Zeller S, Zimmermann M, Akdis CA: Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2011; 127:701-21.
2. Wang F, Arun P, Friedman J, Chen Z, Van WC. Current and potential inflammation targeted therapies in head and neck cancer. Curr Opin Pharmacol. 2009; 9:389-95.
3. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008; 454:436-44.
4. Pries R, Wollenberg B. Cytokines in head and neck cancer. Cytokine Growth Factor Rev 2006; 17:141-6.
5. Linkov F, Lisovich A, Yurkovetsky Z, Marrangoni A, Velikokhatnaya L, Nolen B, et al. Early detection of head and neck cancer: development of a novel screening tool using multiplexed immunobead-based biomarker profiling. Cancer Epidemiol Biomarkers Prev. 2007; 16:102-7.

6. Druzgal CH, Chen Z, Yeh NT, Thomas GR, Ondrey FG, Duffey DC, et al. A pilot study of longitudinal serum cytokine and angiogenesis factor levels as markers of therapeutic response and survival in patients with head and neck squamous cell carcinoma. Head Neck. 2005; 27:771-84.
7. Mojtahedi Z, Khademi B, Yehya A, Talebi A, Fattahi MJ, Ghaderi A. Serum levels of interleukins 4 and 10 in head and neck squamous cell carcinoma. J Laryngol Otol. 2012; 126:175-9.
8. Hathaway B, Landsittel DP, Gooding W, Whiteside TL, Grandis JR, Siegfried JM, et al. Multiplexed analysis of serum cytokines as biomarkers in squamous cell carcinoma of the head and neck patients. Laryngoscope. 2005; 115:522-7.
9. Topping KP, Fletcher LM, Agada FO, Alhamarneh O, Stafford ND, Greenman J. Head and neck tumour immunology: basic concepts and new clinical implications. J Laryngol Otol. 2009; 123:9-18.
10. Hoffmann TK, Sonkoly E, Homey B, Scheckenbach K, Gwosdz C, Bas M, et al. Aberrant cytokine expression in serum of patients with adenoid cystic carcinoma and squamous cell carcinoma of the head and neck. Head Neck. 2007; 29:472-8.
11. Riedel F, Zaiss I, Herzog D, Gotte K, Naim R, Hormann K. Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. Anticancer Res, 2005; 25:2761-5.
12. Salazar-Onfray F, Lopez MN, Mendoza-Naranjo A. Paradoxical effects of cytokines in tumor immune surveillance and tumor immune escape. Cytokine Growth Factor Rev. 2007; 18:171-82.
13. Jebreel A, Mistry D, Loke D, Dunn G, Hough V, Oliver K, et al. Investigation of interleukin 10, 12 and 18 levels in patients with head and neck cancer. J Laryngol Otol. 2007; 121:246-52.
14. Li Z, Chen L, Qin Z. Paradoxical roles of IL-4 in tumor immunity. Cell Mol Immunol. 2009; 6:415-22.
15. Weller MD, Nankivell PC, McConkey C, Paleri V, Mehanna HM. The risk and interval to malignancy of patients with laryngeal dysplasia; a systematic review of case series and meta-analysis. Clin Otolaryngol. 2010; 35:364-72.
16. Duffy SA, Taylor JM, Terrell JE, Islam M, Li Y, Fowler KE, et al. Interleukin-6 predicts recurrence and survival among head and neck cancer patients. Cancer. 2008; 113:750-7.
17. Gokhale AS, Haddad RI, Cavacini LA, Wirth L, Weeks L, Hallar M, et al. Serum concentrations of interleukin-8, vascular endothelial growth factor, and epidermal growth factor receptor in patients with squamous cell cancer of the head and neck. Oral Oncol. 2005; 41:70-6.
18. Alhamarneh O, Agada F, Madden L, Stafford N, Greenman J. Serum IL10 and circulating CD4(+) CD25(high) regulatory T cell numbers as predictors of clinical outcome and survival in patients with head and neck squamous cell carcinoma. Head Neck. 2011; 33:415-23.
19. Rocha G, Proenca E, Guedes A, Carvalho C, Areias A, Ramos JP, et al. Cord blood levels of IL-6, IL-8 and IL-10 may be early predictors of bronchopulmonary dysplasia in preterm newborns small for gestational age. Dis Markers. 2012; 33:51-60.
20. Koksal N, Kayik B, Cetinkaya M, Ozkan H, Budak F, Kilic S, et al. Value of serum and bronchoalveolar fluid lavage pro- and anti-inflammatory cytokine levels for predicting bronchopulmonary dysplasia in premature infants. Eur Cytokine Netw. 2012; 23:29-35.