

Ayşe Basak Engin\*, Rıza Onder Günaydin, Sacit Altug Kesikli, Dietmar Fuchs and Ali Sefik Hosal

# Serum neopterin concentrations and tryptophan degradation pattern in patients with late stage larynx carcinoma

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**Keywords:** indoleamine 2,3-dioxygenase; larynx cancer; neopterin; tryptophan.

**Abstract:** As the disease-free 5-year-survival of late stage laryngeal carcinoma patients is extremely low, indoleamine-2,3-dioxygenase-1 (IDO)-induced tryptophan degradation may represent an immune escape mechanism which plays an important role in cancer spreading in advanced stage laryngeal cancers. We examined whether the late stage laryngeal cancer enhances tumor immune evasion by the expression of systemic IDO activities and chronic cellular immune activation. Twenty-two of 42 male laryngeal cancer patients were classified as late stage cancer according to American Joint Committee on Cancer (AJCC) criteria. Their serum neopterin, tryptophan and kynurenine concentrations were compared with 30 cancer-free individuals. IDO activity was approved by correlation between serum neopterin and kynurenine/tryptophan. Late stage cancer patients preoperatively showed a significantly higher IDO activity compared to controls and early stage cancer cases. Six months after tumor removal, late stage cancer patients although having higher serum neopterin concentration compared to early stage patients or controls, they showed a significant decrease in IDO activity and tryptophan consumption. Increased systemic IDO activity may provoke the escape of tumor cells from the immune surveillance of the host. High IDO activity is due to the presence of tumor mass. Persistence of high serum neopterin levels despite tumor removal may indicate poor prognosis.

## Introduction

Head and neck squamous cell carcinoma is the sixth most common neoplasm in the world. Despite advances in treatments involving surgery, radiation, and chemotherapy, the 5-year survival is <50%, because of local recurrence of this aggressive malignancy [1]. Although surgery is a favorable prognostic factor for late stage laryngeal cancer, it does not impact on overall survival [2]. In this respect, head and neck squamous cell carcinomas are highly immunogenic tumors in which tumor-infiltrating cells consist largely of CD4+ lymphocytes [3]. Using an antibody against CD4 is a potent strategy for eliminating immunosuppressive natural CD4+ Treg cells and inducing lasting immunity to cancer [4]. Head and neck cancers often induce profound immunosuppression which contributes to disease progression and interferes with immune-based therapies. Body fluids of head and neck cancer patients are enriched in exosomes potentially engaged in negative regulation of anti-tumor immune responses [5]. In this context, by considering the data obtained from clinical trials, immunological escape mechanisms and key immunotherapies are discussed in head and neck squamous cell carcinomas [6].

Tryptophan degradation rate by tumor cells and high serum IDO activity may be critical for the balance between host immune response and the capacity of the tumor to evade normal host immune defense [7]. In this respect, the proinflammatory cytokine, interferon-gamma (IFN- $\gamma$ ) is thought to be associated with anti-tumoral cellular immunity [8]. In fact, indoleamine 2,3-dioxygenase (IDO) is overexpressed in response to IFN- $\gamma$  in a variety of malignancies. This enzyme causes immunosuppression through breakdown of tryptophan (Trp) in the tumor and tumor-draining lymph nodes [9]. Additionally, another substance synthesized by activated monocytes/macrophages in response to IFN- $\gamma$  is neopterin. Measurement of neopterin concentrations in body fluids provides

\*Corresponding author: **Ayşe Basak Engin**, Department of Toxicology, Faculty of Pharmacy, Gazi University, 06330 Hipodrom, Ankara, Turkey, E-mail: abengin@gmail.com

**Rıza Onder Günaydin and Ali Sefik Hosal:** Department of Otorhinolaryngology Head and Neck Surgery, Faculty of Medicine, Hacettepe University, Ankara, Turkey

**Sacit Altug Kesikli:** Department of Basic Immunology, Cancer Institute, Hacettepe University, Altindag, Ankara, Turkey

**Dietmar Fuchs:** Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Innsbruck, Austria

information about T helper cell 1 (Th1)-derived cellular immune activation [10, 11]. Recently, although a number of clinical studies examined the role of IDO in the patients with cancer, we could not find any data considering larynx cancers [7, 12]. The disease-free 5-year-survival rate for patients suffering from late stage laryngeal carcinoma is between 32.4% and 6.7%. The tumor size and free margins proved to be absolute significant prognostic factors [13]. Therefore, the immunosuppressive environment is of great importance for advanced stage laryngeal cancers. In this study, it was examined whether the late stage of laryngeal cancer enhances IDO activity and in accordance with this, alters the increased frequency of serum neopterin concentration.

## Materials and methods

### Patients and methods

In total, 42 male laryngeal squamous cell carcinoma patients with a median age of 58.5 years (range: 41–73 years) were included in this study. Thirty male cancer-free voluntary individuals with similar characteristics served as control. All participants' rights were protected and informed consent was obtained according to the Helsinki Declaration. Ethical approval was obtained from Hacettepe University, Medical Faculty Local Research Ethics Committee. Peripheral venous blood samples were collected from voluntary individuals and patients with histologically confirmed to have primary laryngeal squamous cell carcinoma, into anticoagulant free blood collection tube and used for the serum separation. All samples were obtained in the early morning, protected from direct light and kept in  $-20^{\circ}\text{C}$  until assayed. Patients had no other known malignancies, infections, or chronic diseases and had not received preoperative chemotherapy and/or radiotherapy. Age, tumor site, tumor stage, histological grade, total radiation dose and surgery margins of the patients were recorded. The patients were classified according to American Joint Committee on Cancer (AJCC), tumor-node-metastasis (TNM) classification; seven patients were stage 1, 13 were stage 2, 10 were stage 3 and 12 were stage 4. Afterwards 42 patients were allocated into two groups; early stage (stage 1 and 2,  $n=20$ ) and late stage (stage 3 and 4,  $n=22$ ).

Serum neopterin concentrations were determined by a commercially available enzyme immunoassay kit (ELISA, Demeditec Diagnostics, Kiel, Germany), according to the manufacturer's instructions. The optical density was measured at 450 nm. Trp and kynurenine (Kyn) concentrations in serum were determined by reversed-phase high-pressure liquid chromatography following precipitation of protein with trichloroacetic acid. Trp was measured via fluorescence detection at 285 nm excitation wavelengths and 365 nm emission wavelengths, while Kyn was detected by UV absorption at the 360 nm wavelengths, simultaneously [14]. In order to determine the IDO activity, Kyn to Trp ratio (Kyn/Trp) was calculated by dividing Kyn concentrations (nmol/L) by Trp ( $\mu\text{mol/L}$ ), subsequently the correlation between the serum neopterin and Kyn/Trp ratio was examined [15]. The presence of IDO activity was approved in the case of

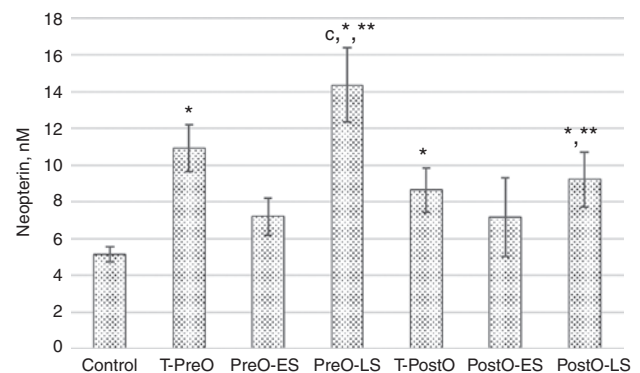
significantly positive correlation between the serum neopterin and Kyn/Trp ratio.

### Statistical analysis

Data were analyzed by using the statistical package SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA). All results were expressed as the mean  $\pm$  standard error of mean (SEM). After checking the data by Kolmogorov-Smirnov test, non-parametric data of the two independent groups were compared with Mann-Whitney U-test and  $p < 0.05$  was considered statistically significant. Correlations were assessed using Spearman's rank test.

## Results

Preoperative neopterin concentrations in total cancer patients were significantly higher than that of cancer-free controls ( $p=0.0001$ ). Although the neopterin levels of early stage cancer patients and healthy controls were similar, late stage group had significantly higher neopterin compare to both control and early stage cancer individuals (Figure 1). While the increased frequency of neopterin concentrations was 15% in early cancer cases, at the late stage it was 59%. Preoperative Trp degradation rate was significantly higher in late stage cancer patients. However, first detectable degradation product of Trp, Kyn was only slightly higher but not significantly different in late stage laryngeal cancer ( $p=0.01$ , late stage vs. control). The increases of neopterin and Kyn/Trp concentrations in the various subgroups of patients seemed to



**Figure 1:** Alterations in the neopterin levels of the control and larynx cancer patient groups.

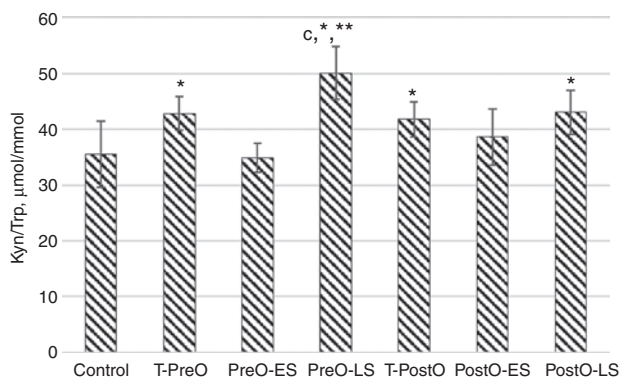
T, Total larynx cancer patients group; PreO, pre-operative; PostO, post-operative; ES, early stage larynx cancer; LS, late stage larynx cancer;  $c, p < 0.01$ , Spearman correlation, PreO-LS neopterin – PreO-LS Kyn/Trp;  $*p < 0.01$ , control vs. patient group;  $**p < 0.01$ , ES vs. LS patient groups.

develop in a very parallel manner. On the other hand, late stage cancer patients showed significantly higher Kyn/Trp ratio versus both control and early stage cancer individuals ( $p=0.0004$  and  $p=0.0146$ , respectively) (Figure 2). A highly significant correlation was observed between the preoperative values of neopterin and Kyn/Trp ratio in the late stage cancer group ( $p=0.0073$ ,  $r=+0.555$ ) and it was recognized as enhanced IDO activity.

However, postoperative evaluation of laryngeal cancer patients revealed that the late stage group had still higher serum neopterin concentration when compared to early stage patients or controls. After the operation, despite the overall increased mean values of the groups, the frequencies of increased neopterin concentrations decreased to 5% and 14%, respectively, for the early stage and late stage cancer patients. On the other hand, neither Trp nor Kyn levels showed a significant rise. Although the postoperative Kyn/Trp ratio of the late stage carcinoma group showed a significant increase in comparison to controls, we did not observe a significant correlation with the neopterin levels. Surprisingly the lack of increase in postoperative IDO activity was attributed to the reduction of tumor tissue, even if not completely removed.

## Discussion

Altered immune, inflammatory, and angiogenic responses have been noticed in head and neck cancer, and many of these responses have been associated with a poor clinical outcome [16]. Unfortunately, patients with head and



**Figure 2:** Alterations in the kynurenine/tryptophan levels of the control and larynx cancer patient groups. T, Total larynx cancer patients group; PreO, pre-operative; PostO, post-operative; ES, early stage larynx cancer; LS, late stage larynx cancer;  $c, p < 0.01$ , Spearman correlation, PreO-LS neopterin – PreO-LS Kyn/Trp; \* $p < 0.01$ , control vs. patient group; \*\* $p < 0.01$ , ES vs. LS patient groups.

neck squamous cell carcinoma have profound immune defects that are associated with increased recurrence [1]. Upon induction by Th1-type cytokine, IFN- $\gamma$  [17, 18], tumors expressing IDO at a high level may effectively escape from the immune surveillance of the host by degrading local Trp [19]. Indeed, in cancer patients, degradation of Trp is significantly accelerated. In this case, serum Trp concentrations decrease; Kyn and Kyn to Trp ratio increases [20]. Increase in IDO activity and the accumulation of Trp metabolites results in a strong inhibitory effect on the development of immune responses by blocking T cell activation, inducing T cell apoptosis or promoting the regulatory T cell (Tregs) differentiation [21]. Kyn binding to human aryl hydrocarbon receptor (AHR) is essential to generate Tregs that suppress adaptive immunity [22]. Thereby, IDO-expressing cells have been considered to create a state of immunological unresponsiveness towards tumor-derived antigens [23]. In particular, the enzyme IDO has recently attracted special attention [24] and may be expressed constitutively by tumor cells as part of the genetic changes involved in malignant transformation [19, 25]. Although IDO expression by tumor cells has been shown to be correlated with a poor clinical prognosis [15], there is no convincing data considering the degradation of Trp by IDO in laryngeal carcinoma cases. In our study, patients with late stage laryngeal cancer preoperatively showed significantly higher IDO activity ( $p=0.0146$ ) and Trp degradation pattern ( $p=0.0015$ ) in contrast to the early stage patients. The presence of IDO activity was approved in the case of positively significant correlation between the serum neopterin and Kyn/Trp ratio. Thus, the highly significant correlation between neopterin concentrations with increased Kyn/Trp clearly indicated that the formation of Kyn is related to IDO activity by IFN- $\gamma$  stimulation in late stage laryngeal cancer cases. Surprisingly, late stage laryngeal carcinoma cases could not demonstrate higher IDO activity and excess Trp consumption following removal of tumor mass within 6-months period. Eventually, the correlation between neopterin and Kyn/Trp ratio was disrupted. Thereby, it is claimed that tumor-originated IDO limits immune cell proliferation by depleting locally available Trp and/or producing its cytotoxic metabolites [26]. Fallarino et al. showed that IDO has been implicated in immune-tolerance because it can inhibit the immune response, either by exhausting L-Trp in the medium or producing toxic metabolites that can cause apoptosis in T cells [27].

On the other hand, an increase in neopterin concentrations during cancer growth indicates a chronic cellular immune response, but it is not specific for malignant cell proliferation [28]. If the presence of the tumor

is previously confirmed by the tissue diagnosis, higher neopterin levels may be considered as cancer-related chronic immune activation. Nevertheless, increased frequency of neopterin levels among the advanced cancer cases was an evidence of enhanced macrophage response to tumor antigen. However, the neopterin levels of the patients with late stage laryngeal cancer were still higher postoperatively than the other groups, but it was found to be below the cut-off value. These results suggested that without considering the tumor margin, surgical removal of tumor mass reduces the Trp degradation and IDO activity but cannot further affect the cell-mediated immunity. Postoperative evaluation of serum neopterin levels in laryngeal cancer patients revealed that late stage group had still higher serum neopterin concentration when compared to early stage patients. High IDO activity results in the progression of tumor mass, persistence of high serum neopterin levels despite the tumor removal may suggest poor prognosis. Actually, the mean serum neopterin level in the non-tumor group is also below the standard cut-off value, 10 nmol/L [29]. Higher neopterin measurements not only provide an insight into the present state of cell-mediated immune response but also allow monitoring and prognosis of disease progression [30]. Our results indicated that, patients with late stage cancer were still immunosuppressed, when compared to patients with non-metastatic disease, despite surgical removal of the tumor [31]. Furthermore, IDO inhibition may significantly enhance the antitumor activity of various chemotherapeutic and immunotherapeutic agents. These data are consistent with the results and showed that increased IDO expression is an independent prognostic variable for reduced overall survival in cancer patients [21]. Although the overall increase in postoperative neopterin levels of cancer cases is marked, in advanced cancer group increase in neopterin concentration is highly significant [32]. Increased neopterin concentrations associate with the endogenously formed IFN- $\gamma$ , which is released as a result of the host-tumor interaction [33, 34]. While the increase of neopterin reflects the activation of monocyte-derived cells like macrophages or dendritic cells [35], the changes of IDO activity may indicate the possible contribution of tumor cells to Trp metabolism.

## Conclusion

Increase in serum IDO activities of late stage laryngeal squamous cell carcinoma patients causes serious metabolic alterations which may facilitate the tumor progression

irrespective of histopathological parameters. This study showed that besides the clinical manifestations, identification of systemic IDO activity may provide a preliminary information at diagnosis, regarding tumor stage in patients with laryngeal cancer. While, tumor removal in advanced cases results in diminished IDO activity, persistent serum neopterin concentration indicates poor prognosis.

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