

TNF- α evaluation in tonsil cancer

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Abstract

Squamous cell tonsil carcinoma is the most frequent form of oropharyngeal cancer, representing 70–80% of the total of head and neck malignant tumors. Poor clinical symptoms make that 60–80% of patients with squamous cell tonsil carcinoma have a late diagnosis, in the third and fourth stages, when the tumor exceeds the organ limits, invading the pharyngeal wall or the tongue base, being associated with metastases in the laterocervical lymphatic ganglions. The tumor necrosis factor alpha (TNF- α) represents an important inflammation mediator associated to carcinogenesis and even to tumor progression. We evaluated the seric values of TNF- α in a group of patients with tonsil cancer in comparison to a group of patients with chronic tonsillitis, as well as the reaction of mastocytes and macrophages in the two types of tonsil lesions. Seric levels of TNF- α in squamous cell tonsil carcinoma were quite high, varying from 1000 to 2000 pg/mL, and in four patients, with poorly differentiated tonsil carcinoma in the fourth stage, the TNF- α values varied from 2000 to 4000 pg/mL. In the patients undergoing radiotherapy, the TNF- α seric levels were within normal limits. In chronic tonsillitis, the TNF- α seric level varied from 10 to 200 pg/mL. There were not observed any significant differences between the two types of tonsil lesions, regarding the macrophages and mast cells density on the surface unit.

Keywords: TNF- α , tonsil cancer, tumorigenesis, macrophages, mast cells.

Introduction

Oropharyngeal cancer represents 1% of all newly diagnosed cancer cases every year, and approximately 15% of malignant localizations in the head and neck area. Every year, all over the world there are recorded approximately 150 000 new cases of head and neck cancer [1]. Global incidence places oropharyngeal cancer on the 6th place [2], but the incidence varies from one country to another. In Europe, the disease incidence is approximately 1–2% of the total malignant tumors in developed countries such as England, Switzerland, Germany, Denmark, while in Asian countries – in the Southeast of Asia – it is found in about 45% [3–5]. In Romania, the disease incidence is approximately of 4.2% [1].

The oropharyngeal cancer etiology remains unknown, although there have been incriminated certain risk factors such as: smoking, alcohol, poor oral hygiene, wearing of inadequate dental prosthesis, chewing hazels or betel nuts, the presence of some infectious agents such as papilloma viruses [6–8], together with other factors considered as favoring neoplasia (family antecedents, preneoplastic state, genetic predisposition).

Squamous cell tonsil carcinoma is the most frequent form of oropharyngeal cancer, representing 70–80% of the total malignant tumors in this area [9–11]. World-widely, the incidence of squamous cell tonsil cancer has significantly increased in the last two decades, from 0.6 to 1.45 cases in 100 000 persons, especially in men and adults aged between 40 and 59-year-old [12]. Clinical data show that squamous cell tonsil carcinoma is aggressive,

with local or regional recurrences and early dissemination, a reason for which 60–80% of the patients diagnosed with tonsil squamous cell tonsil carcinoma present metastases in the laterocervical lymphatic ganglions at the moment of diagnosis [13].

The carcinogenesis process is frequently associated with a chronic inflammatory process. Chronic inflammation is involved in various stages of tumorigenesis, starting from cellular transformation to the formation of tumoral microenvironment and metastasizing [14, 15]. Even though there have not been yet understood the molecular and cellular mechanisms of the connection between chronic inflammation and tumorigenesis, numerous studies have shown that TNF- α , an important inflammation mediator, plays a crucial part in the inflammatory process associated to carcinogenesis [16].

TNF- α is one of the most important pro-inflammatory cytokines involved in the growth, differentiation and survival of various cells. This is produced by various types of cells, such as macrophages, neutrophils, fibroblasts, keratinocytes, NK-cells, T- and B-cells and tumoral cells [17].

In our study, we proposed to determine the seric values of TNF- α in patients with tonsil cancer, in comparison to other inflammatory conditions (chronic tonsillitis), and also to establish some correlations between the TNF- α seric values and the histological aspect of tonsil tumors.

Materials and Methods

The study was performed on a number of 28 patients with tonsil cancer and 19 adult patients with chronic

tonsillitis, hospitalized within the ENT Clinic of the Emergency County Hospital of Craiova, Romania, between 2012 and 2013, who benefited from a surgical intervention with a therapeutic purpose. The patients were subjected to a general clinical examination, ENT examination, lung X-ray, laterocervical echography and cervical tomography for the evaluation of tumor extension and presence of ganglionic metastases. Also, there were performed the usual biological examinations: hemogram (complete blood count), VSH, samples of dysproteinemia, urine examination, coagulation and blood type tests. The determination of TNF- α seric values was performed by a "sandwich"-like immunoenzymatic method (ELISA), PeliKine Compact-Sanquin kit (The Netherlands).

After the clinical and paraclinical examination, there was established the diagnosis of the disease and its stage, and there was performed a surgical procedure involving the tonsil tumor removal, with an intention of radicality, or there was performed a simple tonsillectomy in the case of chronic tonsillitis. From the surgical exeresis pieces there were sampled fragments of tonsil tissue for the histopathological study. The harvested biological material was fixed in 10% neutral formalin solution for 48 hours and included in paraffin, according to the histopathological protocol. After inclusion, the biological material was cut into seriated sections of 4 μ m thickness with a microtome (Microm HM350) equipped with a section transfer system on water bath (Section Transfer System, STS). For the classical histological study, the histological cuts were displayed on ordinary histological blades, and for the immunohistochemical study, the sections were placed on special poly-L-Lysine covered blades (Sigma-Aldrich). After a quick dry period of 5–10 minutes on a hot plate, the sections were transferred into an incubator, at 60°C, and kept over night, a time during which the biological material perfectly adhered to the histological slide blade. For highlighting the microscopic structures, there were used the stainings with Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) green light trichromic.

For the immunohistochemistry study, after antigen retrieval, the sections were allowed to cool down at room temperature and incubated for 30 minutes in a 1% hydrogen peroxide solution. Subsequently, the sections were washed in phosphate-buffered saline (PBS), followed by a blocking step of 30 minutes in 1% skim milk. Next, the slides were incubated with the primary antibodies overnight at 4°C, and the next day, the signal was amplified for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako). Finally, the signal was detected with 3,3'-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin staining.

In our study, we used the following antibodies (Table 1):

Table 1 – Antibodies used in the immunohistochemical study

Antibody	Code	Clone	Antigen retrieval	Specificity	Dilution	Source
CD68	M0814	KP1	Sodium citrate buffer, pH 6	Macrophages	1:200	Dako

Antibody	Code	Clone	Antigen retrieval	Specificity	Dilution	Source
Tryptase	M7052	AA1	Sodium citrate buffer, pH 6	Mast cells	1:1000	Abcam

Results

The age of patients with tonsil cancer varied from 45 to 73-year-old, and the age of patients with chronic tonsillitis from 32 to 61-year-old. Of the 28 patients with tonsil cancer, 21 were men and seven were women, the ratio between men and women being 3/1. Of the patients with chronic tonsillitis, 10 were women and nine men, the gender ratio being of cca. 1/1. Regarding the tumor stage, by using the TNM classification criteria and according to *AJCC* (American Joint Committee on Cancer) 2002, of the total of 28 patients with tonsil cancer, 22 patients were diagnosed in the 3rd stage of cancer, and six in the 4th stage. Regarding the tumor differentiation degree, there was observed that 15 (53.57%) cases presented well-differentiated squamous cell carcinomas, nine (32.14%) cases had moderately-differentiated carcinomas, and four (14.29%) cases had poorly differentiated carcinomas.

The TNF- α seric levels in all patients with chronic tonsillitis were moderately high (with normal values <10 pg/mL), but none of the 19 studied cases had values higher than 200 pg/mL. Of the patients with tonsil cancer, six presented normal seric values of TNF- α ; the rest of 22 patients presented quite high TNF- α values, over 1000 pg/mL, in some cases being recorded values up to 4000 pg/mL.

By correlating the TNF- α seric values with the stage of tumoral lesion, we observed that six patients with squamous cell tonsil carcinoma in the 3rd stage, previously treated by radiotherapy, presented normal values of TNF- α ; the rest of 16 patients, who did not receive any radiotherapy had higher seric values of TNF- α , between 1000 and 2000 pg/mL.

In the patients with fourth stage tonsil cancer, the TNF- α seric values were over 1000 pg/mL, four of them even presenting values between 2000 and 4000 pg/mL.

By correlating the TNF- α seric values with the tumor differentiation degree, there was observed that in the patients with well-differentiated squamous cell carcinoma, the TNF- α seric values were high, being situated between 1000 and 1500 pg/mL, except for six patients treated with radiotherapy, in whom the TNF- α seric values were within normal limits. The TNF- α seric values were high in the patients with moderately differentiated squamous cell carcinoma, between 1000 and 1500 pg/mL, and in the patients with poorly differentiated carcinoma, the TNF- α values were over 2000 pg/mL.

The histopathological and immunohistochemical studies were intended to highlight and provide a quantitative and qualitative evaluation of two cell types involved in the TNF- α synthesis, namely macrophages and mast cells, both in chronic tonsillitis and tonsil carcinomas.

In chronic tonsillitis, macrophages, electively highlighted with the CD68 antibody, appeared more numerous, intensely reactive, large sized, with a heterogeneous cytoplasm, in the germinal centers of the lymphoid follicles, while their number was extremely low in the lymphoid crown (Figure 1). The interfollicular lymphoid

tissue contained a variable number of macrophages, of much smaller sizes, heterogeneously disseminated and poorly reactive to the CD68 antibody (Figure 2). The average number of macrophages on the section surface varied between 442 and 520 cells/mm².

Mastocytes were absent at lymphoid follicle level, but they were present in the interfollicular lymphoid tissue, being more frequently identified perivascularly. The

immunohistochemical tryptase reaction appeared more extended and more diffuse than in macrophages, an aspect most probably caused by mastocyte degranulation and antigen dissemination (tryptase) on large areas around mast cells (Figures 3 and 4). The average number of mast cells varied from one patient to another and even from one area to another on the same histopathological piece, between 196 and 258 cells/mm².

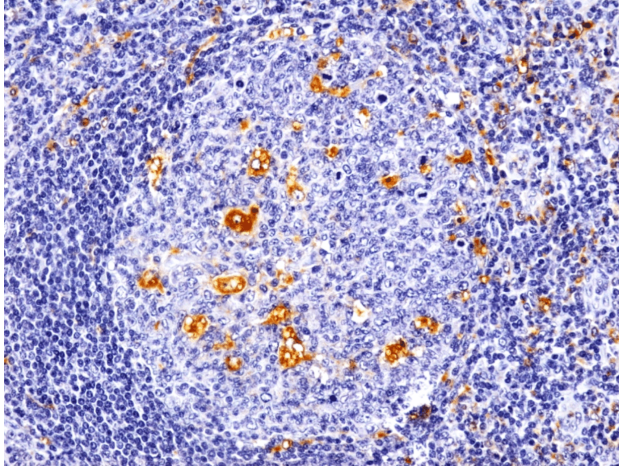


Figure 1 – Microscopic image of a lymphoid follicle in chronic tonsillitis, where we may observe its hypertrophy, with the increase of the germinal center and reduction of lymphoid crown. At germinal center level, there highlighted numerous macrophages (brown color), large sized, intensely reactive. Immunomarking with anti-CD68 antibody, $\times 200$.

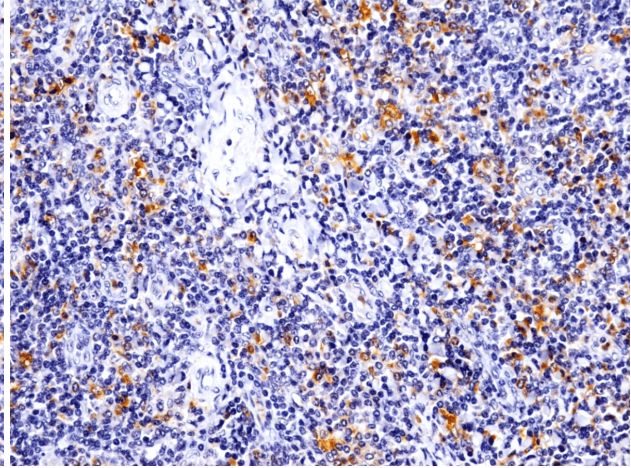


Figure 2 – Interfollicular lymphoid tissues, with small sized macrophages, heterogeneously disseminated. Immunomarking with anti-CD68 antibody, $\times 200$.

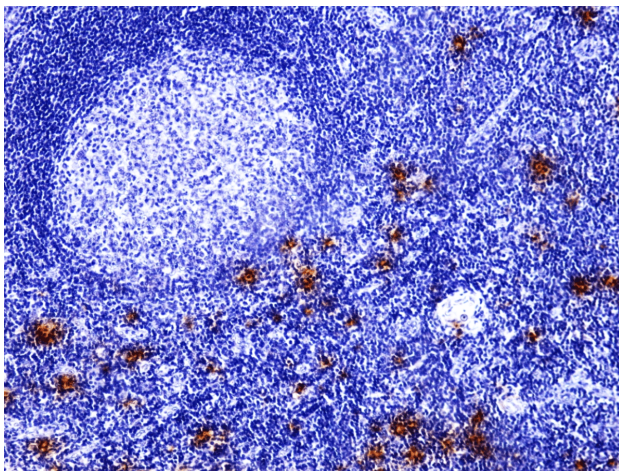


Figure 3 – Microscopic aspect at tonsil lymphoid tissue where there may be observed the absence of mastocytes at lymphoid follicle level and their heterogeneous distribution in the rest of the lymphoid tissue. Immunomarking with anti-tryptase antibody, $\times 100$.

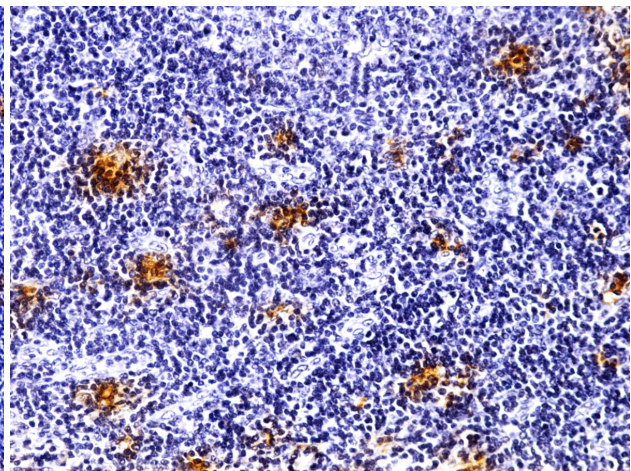


Figure 4 – Immunohistochemical image of mast cells present in the interfollicular lymphoid tissue. There may be observed the relatively high number of these cells, the intense and diffuse reaction to tryptase caused by the mast cell degranulation phenomenon. Immunomarking with anti-tryptase antibody, $\times 200$.

In the squamous cell tonsil carcinoma, the density of macrophages and mast cells was correlated with the quantity of tumoral stroma and with the development degree of the peritumoral inflammatory process. In some cases, we found a relatively poor stroma, especially in well-differentiated tonsil carcinomas (Figure 5); in other cases, especially in moderately or poorly differentiated carcinomas, the tumoral stroma appeared more abundant and strongly infiltrated with inflammatory type cells (Figure 6). Macrophages were identified both in the tumoral tissue and in the peritumoral

stroma. The number of macrophages was a variable one, from one tumor lesional degree to another (Figures 7 and 8). Thus, in the well-differentiated squamous carcinomas, the number of intratumoral macrophages varied between 180 and 322 cells/mm² and between 34 and 73 cells/mm² in poorly differentiated carcinomas, respectively; instead, in the peritumoral stroma, the number of macrophages varied between 86 and 220 cells/mm² in the well-differentiated carcinoma and between 262 and 480 cells/mm² in the poorly differentiated carcinoma, respectively.

Regarding mast cells, they were identified by using the anti-tryptase antibody only in the tumoral and peritumoral stroma (Figures 9 and 10). Their number varied according to the intensity of the inflammatory process,

between 244 and 360 cells/mm². There could not be established any correlation between the number of mast cells and the differentiation degree of the squamous cell tonsil carcinoma.

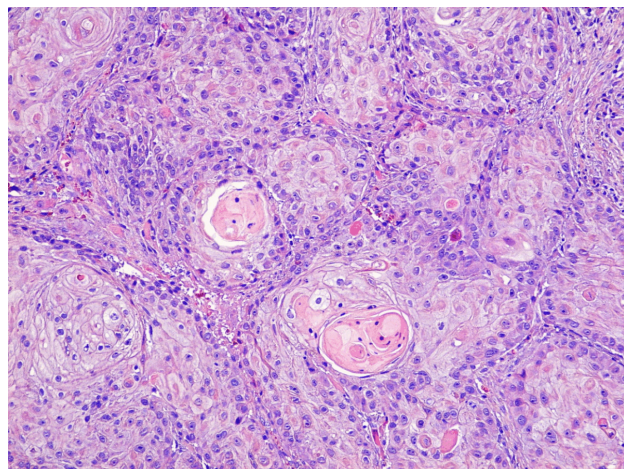


Figure 5 – Area of well-differentiated squamous cell tonsil carcinoma, with poor tumoral stroma. HE staining, ×200.

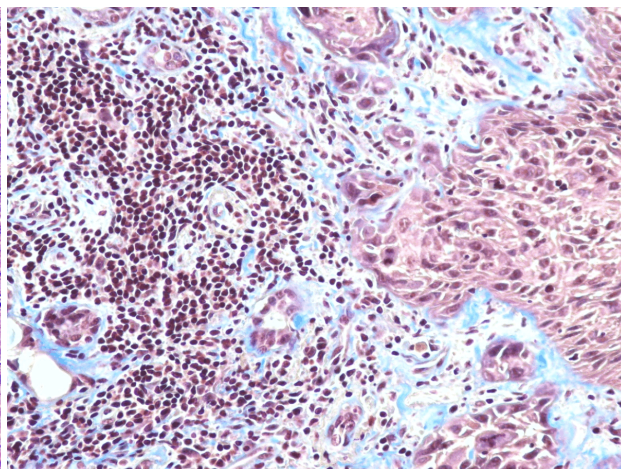


Figure 6 – Moderately differentiated squamous cell tonsil carcinoma, associated with a rich tumoral stroma, strongly infiltrated by inflammatory type cells. GS trichromic staining, ×200.

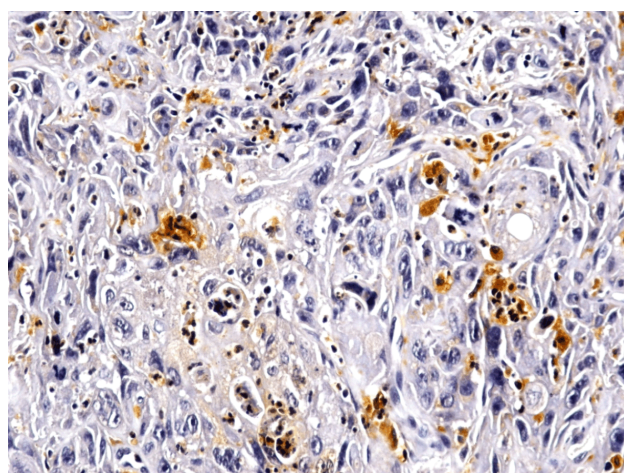


Figure 7 – Moderately differentiated squamous cell carcinoma, associated with an intratumoral relatively small number of macrophages. Immunomarking with anti-CD68 antibody, ×200.

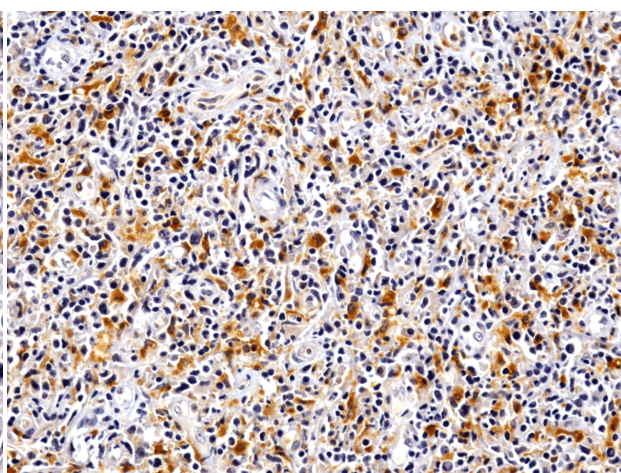


Figure 8 – Poorly differentiated squamous tonsil carcinoma with a very large number of macrophages present in the tumoral stroma. Immunomarking with anti-CD68 antibody, ×200.

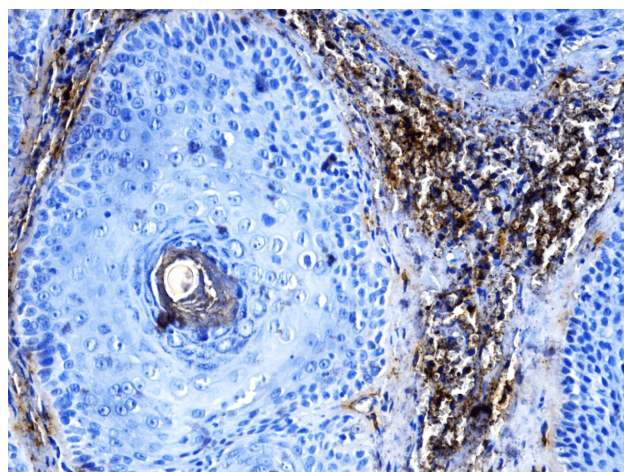


Figure 9 – Intense mast cell reaction in the tumoral stroma of a well-differentiated tonsil carcinoma. Immunomarking with anti-tryptase antibody, ×200.

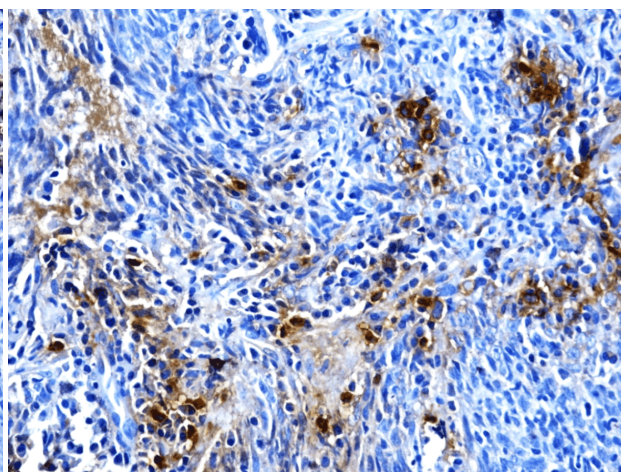


Figure 10 – Moderate reaction of mast cells in a poorly differentiated tonsil carcinoma. Immunomarking with anti-tryptase antibody, ×200.

Discussion

The study of the relation between inflammation and cancer has gained a special momentum in the last 20 years, after numerous clinical and epidemiological studies brought evidence that chronic inflammation may favor the onset and progress of the carcinogenesis process [18–20]. Moreover, there was proven that some bacteria or viruses are directly involved in carcinogenesis [21–23].

The existence of a relation between chronic inflammation and cancer was suggested even since 1863, when Virchow showed that some cancer types were observed in chronic inflammation areas [24].

It is well known the fact that chronic inflammation may produce sustained tissue impairment, cellular proliferation (stimulated by cellular and tissular lesions), as well as tissue repair. Cellular proliferation in the context of a chronic inflammatory process is usually correlated with morphological changes of the “metaplastic” type, a reversible change of the cellular phenotype [25]. Time persistence of the inflammatory process leads to a “dysplasia” onset, a disorder of the cellular proliferation, characterized by the appearance of atypic cells, a process considered a premalignant lesion [26]. The microenvironment in chronic inflammatory processes is characterized by the presence of a large number of lymphocytes and macrophages [27, 28]. Macrophages, together with other leukocytes, produce high quantities of oxygen and nitrogen-reactive species, which may cause DNA changes underlying the onset of the carcinogenesis process [29, 30]. Macrophages and T-lymphocytes may release TNF- α and the macrophage migration inhibitory factor, which may accelerate the DNA impairment [31].

Numerous studies support the idea that TNF- α plays an essential part in the inflammatory process. It stimulates macrophages in the synthesis of other proinflammatory cytokines (IL-1, IL-6, IL-12, IL-15), increases vascular permeability, causes a massive leukocyte infiltration of inflammatory foci and stimulates the production of the vascular endothelium growth factor, which constitutes the promoter of angiogenesis process. TNF- α is directly involved in carcinogenesis, having a mutagenic effect in comparison to the one of ionic radiations, or produces DNA lesions through oxygen-reactive species [32]; moreover, it inhibits precancerous cell death and stimulates inflammation associated with tumorigenesis, by activating the κ B nuclear signaling factor κ B (NF- κ B) [33].

In our study, we determined the TNF- α seric values in chronic inflammatory tonsil processes and in tonsil cancer, comparing its values with macrophage and mast cell density in this type of lesions. In chronic tonsillitis, the TNF- α value was 10–20 times higher than the normal seric value; instead, in tonsil cancer, the TNF- α values increased over 100 times, reaching values over 400 pg/mL. The exception was observed in tonsil cancer treated by radiotherapy, where there were normal TNF- α values. The data we obtained recommend the use of TNF- α seric level as a marker in the differential diagnosis between inflammatory and neoplastic lesions of tonsils, since some tonsil cancers may evolve without any symptoms, in the tonsil depth, and during the clinical examination, they may present a normal or an inflammatory aspect

[34, 35]. Also, TNF- α could be used as an observation marker for the neoplastic lesion evolution after radiotherapy.

Another aspect observed in our study was the one showing that TNF- α seric values were much higher in poorly differentiated carcinomas, in comparison to the well-differentiated squamous cell carcinomas, which suggests that TNF- α seric values could correlate with the tumoral grading and, thus, with the neoplasia severity, having the possibility of being used as a tumor prognosis factor. The results of our study, even though performed on a relatively small number of patients with tonsil cancer, come to underline the TNF- α importance in tonsil tumor diagnosis, in the context where various studies of the same type presented controversial or inconclusive results [36–38].

Regarding the macrophage and mast cell reaction, we did not observe any significant differences between the two types of tonsil lesions, namely between chronic tonsillitis and tonsil cancer, although it is well-known the fact that both cellular types intervene in the development of both the inflammatory process and the carcinogenesis one. According to some authors [28, 39, 40], macrophages, lymphocytes, neutrophils, dendritic cells and mastocytes are essential cells in the carcinogenesis of epithelial tumors, as they synthesize a multitude of cytokines and chemokines that facilitate tumorigenesis.

TNF- α is mainly produced by activated macrophages, and also by CD4-lymphocytes, NK-cells, neutrophils, mastocytes and eosinophils. The fact that, in our study, very high values of TNF- α in tonsil carcinoma did not correlate with the increase of macrophage and mast cell number, shows that this cytokine is synthesized by quite many cells present in the tumoral microenvironment, including by tumoral cells.

Conclusions

In our study, the TNF- α seric values in squamous cell tonsil carcinoma were quite high, varying from 1000 to 2000 pg/mL. In four patients with poorly differentiated fourth stage squamous cell tonsil carcinoma, the TNF- α values varied between 2000 and 4000 pg/mL. In the patients undergoing radiotherapy, the TNF- α seric values were within normal limits. In chronic tonsillitis, TNF- α seric values varied between 10 and 200 pg/mL. In comparison to chronic tonsillitis, the TNF- α values were 5 to 20 times higher. Regarding the macrophage and mast cell reaction, there could not be observed any significant differences between the two types of tonsil lesions.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

All authors equally contributed in the present study.

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