Romanian Biotechnological Letters *Vol. 22, No. 2, 2017*

ORIGINAL PAPER

Expression of some inflammatory and angiogenic factors in oropharyngeal and laryngeal squamous cell carcinomas

Received for publication, August, 03, 2015
Accepted, October, 20, 2015

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Abstract

Oropharyngeal and laryngeal cancers are among the most aggressive malignancies. Cooperation between neoplastic cells, intratumoral angiogenic process and immune system cells plays an important role in initiating and developing of a malignant tumor. The present study was conducted on 5 patients with oropharyngeal and laryngeal squamous cell carcinoma, all of them being chronic tobacco smokers and alcohol consumers. Using relative quantification by qPCR, the mRNA levels corresponding to 18 genes encoding inflammation and angiogenesis factors, in tumor versus peritumoral tissue (the latter, used as „control") have been investigated. The results showed that a persistant inflammatory process of the upper aero-digestive tract - due to chronic diseases (e.g. chronic sinusitis, chronic tonsillitis, gastroesophageal reflux), associated with high risk factors like alcohol and tobacco consumption, is a major factor in inducing and sustaining the development of oropharyngeal and laryngeal neoplasia; one important mechanism activated by inflamation at the growing tumor site is angiogenesis which is a very intense process in this type of malignancies.

Keywords: gene expression patterns, inflammation, angiogenesis

1. Introduction

Oropharyngeal and laryngeal neoplasias are some of the most aggressive cancers among head and neck squamous cell carcinomas (HNSCCs), rapidly invasive in surrounding organs, especially in the neighbouring lymph nodes [1]. This is why they are usually diagnosed in advanced stages (III, IV), with low rate of survival, even with combined surgery, chemo and radiotherapy. HNSCCs are the sixth more common cancer type in the world, with the 5-year survival rate below 50% in the past 30 years [2]. This type of neoplasia is mainly occurring in men and is related to a series of behavioural factors like tobacco smoking and alcohol intake, to which can also be added air pollution and infections with human papilloma viruses [2]. A malignant tumor is a tissular entity whose survival and growth depends on its capacity to suppress the immune response and to induce angiogenesis and lymphangiogenesis. Recent studies provide strong arguments that tumor formation is the result of the interplay between cancerous cells, extracellular matrix, the extent of tumor neovascularization and immune cells [2-4]. Understanding the molecular mechanisms of tumor development may indicate potential

therapeutic targets. Both, the innate and adaptive components of immune system are considered to be implicated in promoting and preventing tumor growth. Inflammation appears and persists in a premalignant tissue through accumulation of immune cells and their products. Initially, subsets of T and NK (Natural Killer) lymphocytes act to eliminate the tumoral cells but mechanisms of immune suppression induced by the tumor cells can inhibit the cytotoxic effect of lymphocytes. The inflammation accelerates the malignant transformation in patients predisposed to cancer development [5]. ***The tumoral cells*** inhibit the immune response by producing soluble mediators (prostaglandin E2 (PGE2), tumoral growth factor beta (TGF-β), interleukin 6 (IL-6), IL-10 and adenosine; they also induce apoptosis of tumor infiltrating lymphocytes [2]. By producing proinflammatory cytokines (the colony stimulating factor-1 (CSF-1), monocyte chemoattractant protein-1 (MCP-1), chemokine ligand 2, 3, 4, 5, and 8 (CCL2, 3, 4, 5, and 8), vascular endothelial growth factors (VEGFs), interleukin-8 (IL-8), and TGF-β), the neoplastic cells recruit macrophages (activated monocytes), neutrophils, myeloid-derived suppressor cells (MDSCs) and lymphocytes at the growing tumor site [2,4,5]. ***The tumor-associated macrophages (TAMs)*** – macrophages recruited in the tumor microenvironment – release multiple angiogenic factors, including interleukin-1 (IL1), IL6, IL8, transforming growth factor alpha (TGF-α), fibroblast growth factor (FGF-2), VEGF, epithelial growth factor (EGF), platelet-derived growth factor (PDGF), and tumoral growth factor beta (TGF-β) [2, 6, 7]. ***Tumor associated neutrophils (TANs)*** – can have both pro- and antitumor activity [4]. They sustain tumor angiogenesis by releasing proangiogenic factors as VEGF, IL-8, MMPs and elastases [5]. Recruitment of a ***myeloid-derived suppressor cell population (MDSC)*** at the tumor site (by VEGF, GM-CSF, TGF-β, IL-6, PGE2, and Cyclooxygenase 2 (COX-2)) [2] suppresses adaptive immunity and fosters angiogenesis through the secretion of VEGFA, basic fibroblast growth factor (bFGF) and TGF-β. MDSCs also inhibit NK cell functions, effector T-cell expansion, activation and migration, and expand the immunosuppressive regulatory T-cell population [3]. ***T lymphocytes*** represent up to 10% of all cells in a tumor; they are present in the tumor mass, in the peritumoral tissue, and in the draining lymphoid organs. There are pro- and antitumor phenotypes, varying with disease type and stage [2,4]. ***The NK (Natural Killer) cells*** have cytolytic activity on tumoral cells. They are stimulated to become active killer cells by cytokines as IFN-γ, released by T cells as well as by activated NKs. NK depletion promotes metastasis in experimental models, suggesting that NK cells may have an important role in tumor cell surveillance [8]. ***B cells*** have been shown to suppress and support T-cell function, resulting in differential effects on tumorigenesis. Independent of T-cell function, B cells promote tumor progression by enhancing pro-tumoral inflammation [3, 4]. In this study we have analyzed the gene expression profiles of 18 pro-inflammatory and angiogenic factors present in the tumor microenvironment, produced by malignant- and immune cells, in five patients with oropharyngeal and laryngeal neoplasias.

2. Material and methods

Biological material

Five patients with pharyngeal- (two of them), and laryngeal (the other three) squamous cell carcinoma (primary tumors, non radio-/chemotherapy subjected before surgical remove), from the Oto-Rhyno-Laryngology Clinic of the Ilfov County Emergency Hospital, were investigated. All patients signed an informed written consent for their biological samples (blood and tissue fragments) to be used for cytological and molecular investigations, and to report and publish individual patient data, considering the requested confidentiality on their identity. The research protocol was approved by the Ethics Committees of the participant institutions. The study was

performed in accordance with the Declaration of Helsinki. The biological material was represented by tissue fragments from pharyngeal and laryngeal malignant primary tumors surgically removed from the patients. Two different types of tissue were analysed: tumoral tissue (from inside of the tumor), and as control – apparently/macroscopically normal tissue from the tumor vicinity. The tissue samples have been conserved in RNA *later*® RNA Stabilization Solution (cat.no.AM7021, AMBION, Inc., Austin, Texas, USA) at -20°C. For total RNA isolation, 100 mg of tissue per sample have been used.

Methods

For each patient, the total RNA isolation from tumoral and non-tumoral (control) tissue was performed using the *NucleoSpin RNA II-Total RNA Isolation Kit* (ref.740955.50, MACHEREY-NAGEL GmbH & Co. KG, 52355 Düren, Germany). The reverse transcription of total RNA was performed with the *High-Capacity cDNA Reverse Transcription Kit* (cat.no.4368814, APPLIED BIOSYSTEMS, Foster City, CA 94404, USA); 900 ng of total RNA were reverse transcribed, and the reaction efficiency was considered to be 100%, as stated by the manufacturer. For the quantitative PCR (qPCR) reaction, *TaqMan® Array 96-Well Plates Gene Signature for Human Angiogenesis* (cat.no.4414071, APPLIED BIOSYSTEMS, Foster City, CA 94404, USA) were used; these plates are prefilled with primers and a specific fluorochrome- labeled hydrolisis probe (the *TaqMan*® probe), for 92 genes involved in angiogenesis, and 4 housekeeping genes: *18S* (for RNA 18S), *GAPDH* (for glyceraldehyde-3-phosphate dehydrogenase), *HPRT1* (for hypoxanthine phosphoribosyltransferase 1) and *GUSB* (for β-glucuronidase). The *TaqMan® Gene Expression Master Mix (2x)* (cat.no.4369016, APPLIED BIOSYSTEMS, Foster City, CA 94404, USA) was used; the final reaction volume was of 20 µl, from which 10 µl of master mix, and 10 µl of sample containing 25 ng of cDNA template. The amplification reaction was performed on a Real-Time PCR 7500 (Applied Biosystems) device, using the following program: 95°C-10 min; 40 cycles of 95°C-15 sec, 60°C-1 min, run in standard mode. The data were analysed using the *DATAASSIST™ Software* designed for sample comparison based on the comparative Ct (ΔΔCt) method [9] for calculating the relative quantification (RQ) parameter, regarding gene expression levels in two different samples. The housekeeping genes considered as endogenous control were *18S* and *GAPDH*.

3. Results

Considering the TNM clasification, all the five investigated patients presented advanced stages of the neoplastic transformation (**Table 1)**.

A set of 18 types of mRNAs corresponding to genes whose products are involved in the inflammatory and/or angiogenic processes, have been analysed (**Table 2**).

CSF-3 – the Colony Stimulating Factor 3, named also G-CSF (Granulocytes Colony Stimulating Factor) – a protein secreted by the blood platelets – is involved in proliferation, maturation and surviving of granulocytes. CSF-3 is overexpressed also in malignant cells, sustaining their growth, invasiveness capacity and recurence after surgical removing of the tumor [10]. ***CSF3*** gene was found to be overexpressed in tumor in four of the five patients; it appeared underexpressed in tumoral tissue, only in patient 1.

CXCL-2 – the CXC motif chemokine ligand 2, known also as Growth-Related Gene Product β (GRO-β) – is secreted by neutrophils and some tumoral cells; it is involved in neutrophils recruitment, migration and proliferation of endothelial cells, sustaining, this way, angiogenesis [11, 12]. ***CXCL2*** gene was overexpressed in tumor, in patients 2, 4, 5, and underexpressed in patients 1 and 3.

Table 1. Clinical data and anatomo-pathological diagnosis of the investigated patients.

Patient code	Age	Sex	Clinical diagnosis / TNM	Anatomo-pathological diagnosis	Associated diagnoses	Current state
P1	55	M	Oropharyngeal neoplasm / $T_3N_{2b}M_0$	Well differentiated, keratinized squamous cell carcinoma; latero-cervical lymph node with metastasis.	Obesity, chronic tonsillitis, weight loss	Deceased
P2	71	M	Laryngeal neoplasm / $T_3N_{2b}M_0$	Keratinized squamous cell carcinoma; latero-cervical lymph node with metastasis.	Chronic tonsillitis, weight loss	Stable
P3	62	M	Oropharyngeal neoplasm / $T_3N_{2b}M_0$	Moderately differentiated squamous cell carcinoma, invasive in the tonsil tissue; lymph node metastasis absent.	Type 2 diabetes, obesity, chronic tonsillitis, chronic bronchitis	Relapse
P4	53	M	Laryngeal neoplasm / $T_3N_3M_0$	Moderately to poorly differentiated, nonkeratinized squamous cell carcinoma, invasive, with necrotic areas; latero-cervical lymph node with metastasis.	Obesity, chronic tonsillitis, chronic sinusitis, gastroesophagial reflux	Deceased
P5	73	M	Laryngeal neoplasm / $T_3N_{2c}M_0$	Moderately differentiated, invasive squamous cell carcinoma; lymph node metastasis absent.	Chronic tonsillitis, weight loss	Deceased

Table 2. The gene expression level, as CT values, in tumoral (T) versus peritumoral (Pt) tissue.

	CT values									
Gene	**1 Pt**	**1 T**	**2 Pt**	**2 T**	**3 Pt**	**3 T**	**4 Pt**	**4 T**	**5 Pt**	**5 T**
CSF-3	*30.4*	*31.9*	**35.8**	**32**	**NE**	**35**	**37.7**	**32.3**	**36**	**29**
CXCL-2	*25*	*28.7*	**28.8**	**25.4**	*27.2*	*30*	**29**	**24**	**28.4**	**25.3**
CXCL-12	*21.8*	*25.7*	**26**	**23.6**	*26.1*	*27*	*25.5*	*28.2*	*26.4*	*27.4*
IL-8	**26.4**	**24.3**	**32.3**	**26.2**	**29.3**	**29**	**31.5**	**22.2**	**30.6**	**18.6**
TGF-A	**29.6**	**26.1**	**34.7**	**30.1**	*28.7*	*35*	**34**	**25**	**36.5**	**25.3**
TGF-B1	**26**	**25.3**	**28.7**	**23.8**	*27.6*	*31*	**28.8**	**25**	**31.1**	**24.7**
MMP-2	*23.2*	*24.0*	**26.7**	**23.6**	*27.4*	*29.0*	**26.6**	**25.5**	**28.3**	**21.4**
PDGF-B	28.0	28.3	**28.5**	**26.6**	*29.2*	*31.0*	**28.4**	**27.7**	**30.1**	**25.3**
PDGFR-A	*25.0*	*26.5*	**27.3**	**23.8**	*27.5*	*30.0*	*27.1*	*27.7*	**29.1**	**24.8**
PDGFR-B	**27.6**	**26.0**	**26.0**	**24.4**	*27.6*	*29.0*	26.2	26.0	**27.3**	**23.0**
VEGF-A	**27.8**	**25.4**	27.1	27	*27.5*	*29*	**26.4**	**24.3**	**28.3**	**25.3**
VEGF-B	*25.7*	*25.6*	*24.7*	*25.7*	*25*	*28*	*25.6*	*26.5*	**26.7**	**26**
VEGF-C	*30.9*	*31.7*	**32.1**	**29.6**	*29.8*	*33*	**31**	**29.3**	**32.8**	**27.9**
CXCL-10	**31**	**26.6**	**32.6**	**22.1**	*29.6*	*NE*	**33.5**	**24.8**	**36.8**	**27.2**
IFN-G	*31.8*	*32.8*	**39.1**	**27.7**	*35.2*	*38*	**37.5**	**30.1**	**NE**	**39**
IL-12A	**35.6**	**32.3**	**36.4**	**31.4**	*35.5*	*36*	**36**	**31.1**	**37.1**	**34**
TNF	**31.5**	**30.5**	**33.6**	**28.3**	*31.7*	*34*	**32.5**	**26**	**36.4**	**28.4**
TNFSF-15	**30.8**	**29.6**	**36**	**28**	*28.1*	*35*	**35**	**28.2**	**33.2**	**26.8**

NE – no expression (CT value was over 40). **Bold** – overexpression in tumoral tissue; *Italics* – underexpression in tumor; normal writing – similar expression in both tissues.

CXCL-12 – the CXC motif chemokine ligand 12, also known as SDF-1 (stromal cell-derived factor-1) – is produced by many cell types, among which are monocytes and B lymphocytes.

This protein sustains proliferation, migration, invasiveness of tumoral cells and metastasis, by induction of metaloproteinase 9 (*MMP9*) gene expression; it is also an angiogenic factor [12,13]. ***CXCL12*** gene was found to be overexpressed in tumoral tissue compared to the peritumoral one, only in patient 2.

IL-8 – Interleukin 8 (known also as CXCL8) – is the most studied inflammatory cytokine promoting angiogenesis [6]. Increased expression of IL-8 was observed in cancer cells, endothelial cells, infiltrating neutrophils, lymphocytes and tumor associated macrophages (TAMs); it promotes the migration of tumor-derived endothelial cells more than of normal endothelial ones, by stimulating the activity of the metalloproteinase 9 (MMP-9); it stimulates proliferation, surviving, migration and invasiveness capacity of malignant cells [6, 14-16]. In all investigated patients, *IL8* appeared overexpressed in tumoral tissue, compared to the peritumoral one, with significant increased values in patients 4 and 5.

TGFα – Transforming growth factor alpha - is a member of the epidermal growth factor ligands (EGF) family. It is produced by keratinocytes, brain cells, and also by macrophages. TGFα binds to the epithelial growth factor receptors (EGFRs), inducing cell proliferation [17,18]. TGFα has shown proangiogenic and proneurogenic effects, is involved in wound healing, but also in tumorigenesis both by its angiogenic and mitogenic activities. In the present study, the gene ***TGFA*** had a tumoral significant overexpression in patients 4 and 5; it was found to be underexpressed in tumor, only in patient 3.

TGFβ – Transforming growth factor beta comprise a family of three cytokines – TGFβ1, 2 and 3 – encoded by distinct genes. TGFβ1 is expressed in endothelial, hematopoietic and connective-tissue cells. TGFβ has an ambivalent potential: it can act also as a tumorigenesis suppressor or as a tumor promoting factor: in normal epithelial cells and in early stages of oncogenesis, TGFβ stops the cell cycle in G1 stage, inducing differentiation or promoting apoptosis [19]; in a tumor microenvironment, mutations in the TGFβ pathway allow uncontrolled cell proliferation. The cancer cells and the surrounding stromal cells increase their TGFβ production which stimulates angiogenesis, cell motility, immunosuppression and invasiveness and metastatic capacity of tumoral cells [20]. In our study, the mRNA levels corresponding to *TGFβ1* encoding gene have been found significantly increased in tumoral tissue, in patients 2, 4 and 5; this gene was underexpressed in patients 1 and 3.

MMP2 – Matrix metalloproteinase 2 (gelatinase A); a member of the matrix metallo-proteinase (MMP) family, which degrades type IV collagen, the major structural component of basement membranes [21]. MMP2 is involved in vascularization regulation and in the inflamatory response. In tumoral tissues, MMP proteins are produced by TAMs and TANs, dendritic and mast cells, being important invasion and metastasis promoters: MMP expression is regulated by TGF-β, while MMPs activate latent TGF-β in the extracellular matrix, together sustaining the tumor progression [20]. The gene *MMP2* was significantly overexpressed in tumor, in patients 2 and 5.

PDGFβ – Plateled-derived growth factor beta – a member of the platelet-derived growth factor (PDGF) family. These growth factors are mitogens for cells of mesenchymal origin. Low oxygen tension, thrombin or other cytokines induce PDGF synthesis; it may function in autocrine stimulation of tumor cells, regulation of interstitial pressure and angiogenesis [22]. PDGF is a proangiogenic cytokine, usually overexpressed in tumors [7]. The gene *PDGFB* was significantly overexpressed in tumor, in patients 2 and 5.

PDGFRα and **PDGFRβ** – are plateled-derived growth factor receptors (alpha and beta polypeptides), stromal tyrosine kinase receptors for PDGF family members. PDGFRα plays a role in organ development, wound healing, and tumor progression. Mutations in the *PDGFRA* gene have been associated with a variety of cancers. PDGFRβ is frequently upregulated in

tumor stroma and blood vessels [7]; it is involved in autocrine stimulation of tumor cells, tumor angiogenesis and regulation of tumor interstitial fluid pressure [7, 23]. Both, the genes *PDGFRA* and *PDGFRB* presented a similar expression to *PDGFB* gene: a significant overexpression in tumor, in patients 2 and 5.

VEGFs – is a group of five Vascular Endothelium Growth Factors (ligands): VEGFA, VEGFB, VEGFC, VEGFD and PIGF (Placenta Growth Factor), produced by many cell types, involved in different biological responses such as angiogenesis, lymphangiogenesis, permeability, inflammatory cell recruitment and fatty acid uptake. VEGF group is also a major driver in tumor angiogenesis [24]. **VEGFA** is a crucial node of the angiogenic molecules network. **VEGFB** sustains inflammation induced angiogenesis [25]. The expression of **VEGFC** in embriogenesis appears especially in those regions where lymph vasculature is forming. Its secretion is stimulated also by inflammatory processes; it induces survival, proliferation and migration of vascular endothelium cells [25], angiogenesis, lymphangiogenesis [25, 26], invasion and metastasis of lymph nodes [25]. Our results have shown an individual pattern of the gene expression dynamics corresponding to these three VEGF factors: in patient 1, only ***VEGFA*** gene is overexpressed in tumor; in patient 2, only ***VEGFC*** gene has an increased expression level in tumoral tissue; in patient 3, all three genes appeared underexpressed; in patients 4 and 5 ***VEGFA*** and ***VEGFC*** were overexpressed, with a maximum value in patient 5. ***VEGFB*** appeared underexpressed in tumor compared to peritumoral tissue, in all 5 patients. Yet, the CT values for mRNA levels of ***VEGFA*** and ***VEGFB*** were lower than 30 (**Table 2**), demonstating a strong expression of these two genes also in tumor and in peritumoral tissue, in all patients.

CXCL10 – CXC motif chemokine ligand 10 - its transcription is induced by interferon gamma (IFN-γ), thus, CXCL-10 is known also as IFN-γ - induced protein (IP-10) [21]. This protein is expressed in thymus and also by NK cells, being very common in blood plasma [11, 12]. It has antitumor activity by recruiting immunomodulatory T lymphocytes at the tumor site [12], and also angiostatic effect [11, 27]. In four of the five patients (1, 2, 4 and 5) ***CXCL10*** appeared significantly overexpressed in tumoral tissue, with a maximum value in patient 2.

IFN-γ – Interferon gamma is a cytokine produced mainly by T cells (CD4+ and CD8+ T lymphocytes), and by NK cells. It activates macrophages [28] and stimulates them to augment production of TNF [29]. The antitumor effects of IFN- γ were attributed to its capacity to inhibit tumor vascularization in early stages of tumorigenesis [30]. In patients 1 and 3, the ***IFNG*** gene was underexpressed in tumor, and in patients 2, 4 and 5, it was strongly overexpressed (with an extremely high value in patient 2).

IL-12 – Interleukin 12 is produced by mononuclear phagocytes and dendritic cells. It stimulates the production of interferon γ (IFN-γ) by NK cells and T lymphocytes; IL-12 enhances the cytolytic functions of activated NKs and T CD8+ lymphocytes [29]. By these actions, IL-12 has an important antitumoral role [31, 32]. IL-12A (knows also as Natural Killer Cell Stimulatory Factor 1, Cytotoxic Lymphocyte Maturation Factor 1, or P35) is the subunit α of interleukin 12 [21]. The ***IL12A*** gene product has an angiogenesis inhibitory effect [33]. In patients 1, 2, 4 and 5, ***IL12A*** gene has been detected as overexpressed in the tumor compared to the peritumoral tissue.

TNF α – Tumor Necrosis Factor alpha, also known as cachexin or cachectin, is synthesized by many cell types among which are: macrophages, neutrophils, eosinophils, mast cells, NK cells, B- and T lymphocytes, fibroblasts, and also by tumoral cells, mediating inflammatory-, autoimmune- and tumoral processes [34]. TNF is involved in sistemic inflammation. At the inflammation site, it stimulates synthesis of genotoxic molecules (e.g. ROS) [35]. Dysregulation of TNF production has been implicated in a variety of human diseases,

including cancer [36]. In the endothelial cells, TNF can act as angiostatic, by inducing apoptosis (through caspase 3 activation) [37]. This cytokine reduces tumor interstitial pressure [7] and induces thrombosis in the tumor blood vessels [29]. In three of our patients (2, 4 and 5) the ***TNFA*** gene was strongly overexpressed.

TNFSF15 is a cytokine belonging to the TNF ligand family (TNF ligand SuperFamily, member 15); it is also called VEGI (Vascular Endothelial Cell Growth Inhibitor) [21]. TNFSF15 is expressed on the surface of endothelial cells, it mediates local inflammatory processes, growth processes, septic shock, activates the NF-kB transcription factor and c-Jun Kinase [37], inhibits angiogenesis by activating caspase 3 and suppress cell growth in colon neoplasia [37-39]. Overexpressing of VEGF inhibits expression of ***TNFSF15*** gene, and tumor angiogenesis occurs [38]. The expression dynamics of this gene appears correlated with the expression of ***TNF***: in patients 2, 4 and 5, it is strongly expressed in tumor compared to peritumoral tissue.

The expression profiles of the analysed genes, in each of the five patients, are presented in the **Figure 1**.

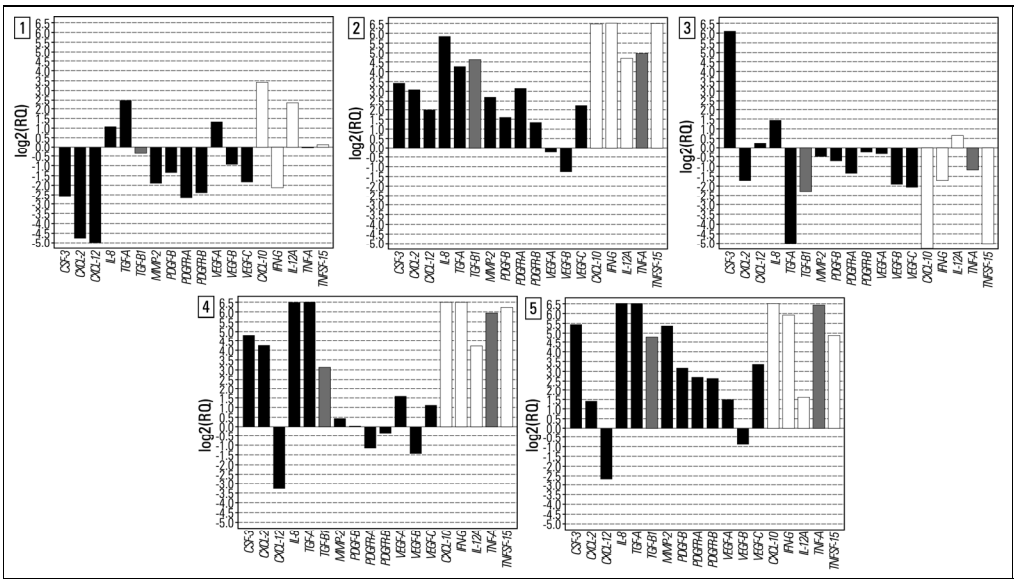

Figure 1. Gene expression levels in tumoral versus peritumoral tissue.
The relative quantification (RQ) values for the analysed genes. 1 – 5, Patients.
Dark gray columns – angiogenic genes; light gray columns – angiogenic / angiostatic (dual character) genes; white columns – angiostatic genes.

4. Discussion

All the five analysed patients presented advanced stages of oropharyngeal and laryngeal neoplasia ($T_3N_2M_0$ - $T_3N_3M_0$), still without distant metastases (**Table 1**); all of them were exposed, for a long time, to the major risk factors for this type of cancer: alcohol and tobacco. Associate pathologies – chronic tonsillitis, chronic bronchitis, chronic sinusitis and gastroesophageal reflux (**Table 1**) – indicate that inflamation of the upper aerodigestive tract is an important predisposing factor for inducing and maintaining a local malignant transformation.

From the 18 genes (as the corresponding mRNAs) analysed in this study, the most important reflecting the presence of an inflammatory process are IL8, TNF and TNFSF15. In

all 5 investigated patients, mRNA corresponding to IL8 cytokine appeared with significantly higher mRNA levels in tumoral tissue compared to the peritumoral one. The CT values (**Table 2**) for the *IL8* gene are most of them under 30, which signifies that this gene is strongly expressed both in peritumoral and tumoral tissues. The gene *TNF* was strongly overexpressed in tumor, in patients 2, 4 and 5; its expression was correlated with that of *TNFSF15* gene which was significantly overexpressed in tumoral tissue compared to the tumor surrounding tissue, in the same three patients. The expression pattern of these three genes confirm that inflammation is an important component in inducing and sustaining the development of oropharyngeal and laryngeal neoplasia.

In patient 1, most of the analysed genes appeared underexpressed in tumor. The angiogenic capacity of the tumor is reflected by enhanced mRNA levels for *IL8*, *TGFA* and *VEGFA* genes; among the genes with an angiostatic effect, *CXCL10* and *IL12A* were significantly overexpressed in tumor compared to the peritumoral tissue. This patient died in the first year after surgery.

In patient 2, almost all the analysed genes, excepting *VEGFA* and *VEGFB*, appeared strongly overexpressed in tumor; two of the angiostatic genes presented huge RQ values: *CXCL10* (RQ~1135) and *IFNG* (PQ~2107!). From all the 5 investigated patients, this one is, at present, is alive and in a good condition (with no treatment indication, yet under medical observation).

In patient 3, a stronger expression, especially of angiogenic genes, was recorded in the peritumoral tissue (CT values $\Re$ 30) compared to the tumor. The only genes overexpressed in tumoral tissue were *CSF3* and *IL8* (both angiogenic); *IL12A* (angiostatic) presented a modest overexpression tendency (RQ 1, 56). This patient is alive but with relapsed disease.

In patient 4, almost all of the analysed genes (excepting *CXCL12* and *VEGFB*, which have a high expression in both tissues) have a strongly enhanced expression in tumor compared to peritumoral tissue. These aspects could reflect a powerful tumor vascularization process, however insufficient, reported to the growth rate of the tumor, generating intra-tumoral necrotic areas. The RQ values of mRNAs corresponding to *IL8*, *TNF* and *TNFSF15* genes show a major expression enhancement of these genes in the tumoral tissue, reflecting an important local inflammatory process. The patient died two years after surgery.

In patient 5, the gene expression analysis revealed a similar situation to the patient 4: strongly enhanced mRNA levels in almost all of the analysed genes, in the tumoral tissue, with notable RQ values for *IL8* and for *TGFA*; the exceptions were *CXCL12* and *VEGFB*, which appeared highly expressed in both tissues. The patient died three years after surgery.

The five gene expression profiles reveal major variations between patients (**Figure 1**), suggesting a heterogeneity of molecular mechanisms that lead to the tumor development. This aspect reflects the multifactorial character and uniqueness of the neoplastic disease in each patient: the genetic and epigenetic background, the balance between different cellular components of the immune system, complex molecular interactions, environmental factors. This intertumoral angiogenic heterogenity was noticed also by Hasina [40].

5. Conclusions

The implication and importance of the inflammatory process in inducing and sustaining of oropharyngeal and laryngeal neoplasia is highlighted by the significantly increased of mRNA levels corresponding to 3 inflammatory cytokines – IL8, TNF and TNFSF15 – in tumoral tissue compared to the peritumoral one.

Our results revealed that each tumor is a genetic and morpho-physiological unique entity, due to a global characteristic gene expression profile which represents its identity „signature”. However, oropharyngeal and laryngeal carcinomas share some common features:

(I) they are among the most aggressive cancers, with a high mortality rate; (II) these tumors have high proliferation rates, due to their potential to develop a dense intratumoral microvasculature and to inhibit the immune system of the host; (III) the persistant inflammatory process – due to chronic diseases (e.g. chronic sinusitis, chronic tonsillitis, gastroesophageal reflux), associated with high risk factors like alcohol and tobacco consumption – is a strong stimulating factor in initiating and sustaining the tumoral growth, in oropharyngeal and laryngeal carcinomas.

6. Acknowledgments

The authors are grateful to *Professor Octavian Popescu* for his support and helpful suggestions comming at the right time during this study, and also for his kindness in facilitating the collaboration with the scientists from the Molecular Biology Center of the Institute of Interdisciplinary Researches in Bio-Nano-Sciences, from „Babes Bolyai" University, Cluj Napoca.

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