



Expression of roundabout receptor family members 1 and 2 in laryngeal squamous cell carcinoma and correlation with clinical and pathological parameters

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ABSTRACT

Objectives: This study aims to investigate whether there is a role of ROBO-1, ROBO-2 and TGM-3 gene expression levels in the development of laryngeal cancer.

Patients and Methods: The study was completed with 29 patients who underwent total or partial laryngectomy due to squamous-cell laryngeal cancer. Gene expression analysis was performed by quantitative real-time polymerase chain reaction (qRT-PCR). Expression ratios were transformed into fold changes and reported as relative expression. Obtained fold changes were compared between different tumor grades in addition to normal tissue samples.

Results: The ROBO-1, ROBO-2 and TGM-3 genes were expressed at a lower level than the control group.

Conclusion: Our study results showed that there was no correlation between ROBO-1, ROBO-2 and TGM-3 gene expression and development of laryngeal cancer.

Keywords: Gene expression; laryngeal carcinoma; Roundabout 1; TGM-3.

Laryngeal cancer represents the second most common malignancy of the head and neck worldwide.^[1,2] Head and neck cancer is the sixth most common cancer in the world.^[2] Laryngeal cancer accounts for about 1.2% of all cancers and 1.1% of all deaths due to cancer, with about 150,677 new cases in a year [2.2 ASR (W): Age-world-standardized incidence rate] and a ratio of men to women of 6.2:1 worldwide. Especially for laryngeal cancer, the five-year survival rate depends on the anatomical site and stage of head and neck cancer patients

in Europe (26 to 63%). The five-year survival rate is 60-90% in stage 1 cancer and decreases at higher stages to 34-60%, 44-74% and 32-56% for supraglottic, glottic and subglottic cancers respectively.^[2,3] The major risk factors for head and neck cancer are tobacco smoking and alcohol consumption.^[3,4] Additional risk factors include passive smoking, human papillomavirus (HPV) infection, low body mass index, and family history of cancer.^[3-9] The Roundabout (ROBO) gene encodes a transmembrane receptor that was initially identified in *Drosophila*. Six years

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later the Drosophila Slit protein was identified as the ligand for the ROBO receptor.^[10,11] The SLIT-ROBO signaling cascade includes the Slit family of secreted proteins (Slit1, 2, and 3) and their corresponding receptors (ROBO1, 2, 3, and 4). Although first characterized in neuronal development, Slit2 was recently found to interact with ROBO1 to mediate repulsive cues in myogenesis, leukocyte chemotaxis, and endothelial cell migration.^[10] Intense investigation found that this pathway also plays a role in other biological processes including angiogenesis.^[12] Slit2 has been widely identified in various human cancers, and the interaction between Slit2 and ROBO1 is thought to induce tumor angiogenesis. In addition, Slit2 has been identified as a regulator of lymphangiogenesis. The full-length Slit2 protein is a 200 kDa secreted ligand that is cleaved into two smaller fragments, a 140 kDa N-terminal product (N-Slit2) and a 50-60 kDa C-terminal product (C-Slit2). N-Slit2 remains tightly bound to the cell membrane, whereas C-Slit2 is readily diffusible. Little is known about the amino acid motifs involved in the interaction between N-Slit2 and the cell membrane, or whether further enzymatic processing might be necessary to generate additional N-terminal fragments *in vivo*. However, many studies have found that N-Slit2 binds to ROBO, which is a single-pass transmembrane receptor.^[10] In addition, Slit2 has been implicated in breast cancer metastasis and ROBO1 has been implicated in hepatocellular carcinoma.^[13,14] Transglutaminases (TGs) are a big class of ubiquitous enzymes that catalyze post-translational modifications of proteins. The formation of these enzymes is catalyzed by the cross-linking of glutamyl residues of a protein/peptide substrate to lysyl residues of a protein/peptide co-substrate.^[15-18] The main activity of TGase 3 is a soluble enzyme expressed predominantly in differentiating keratinocytes in the epidermis.^[19] TGase 3 is 77 kDa length and the protein is widely expressed and is important for epithelial barrier formation. It is a zymogene, requiring activation by proteolytic cleavage, and supposed to be responsible for the later stages of the forming of the epidermis. In the epidermis, TGase 3 is present only in the upper granular layer.^[20,21] TGM-3 sequences outside of transglutaminase isoenzymes

of the family, differences were known, to explain the role of TGM-3 isozyme functional to determine their substrate preference and related information is very important to learn. The aim of the study is to determine whether there is a role of ROBO-1, ROBO-2 and TGM-3 in laryngeal squamous cell cancer.

PATIENTS AND METHODS

Initially 32 patients were enrolled in the study. Three cases were excluded from the study due to some technical reasons. Twenty-nine patients diagnosed with laryngeal squamous cell carcinoma by histopathological examination and who underwent total or partial laryngectomy in Faculty of Medicine Department of ORL and Head and Neck Surgery, were included in the study between November 2010 and November 2011. Patients who received other primary therapies such as radiotherapy or chemotherapy for head and neck cancer were excluded. Tissue samples were obtained from both healthy adjacent mucosa and the tumor tissue itself during surgery. They were immediately placed in storage at -80°C until the RNA extraction procedure. The study protocol was approved by both the Ethical Committee of the Istanbul Faculty of Medicine (October 14, 2011 No: 03) and The Scientific Research Projects Coordination Unit of Istanbul University (Project number: 21131). We gave information about the study to the patients and obtained written consent. The study was completed in 29 patients whose mean age \pm standard deviation was 59.5 ± 12.5 years. Clinicopathological data of patients are shown in Table 1.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the tissue samples using Roche High Pure RNA Tissue Kit (Cat. No. 12033674001 Roche, GmbH, D-40724 Hilden, Germany) according to the instructions of the manufacturer. RNA samples were quantified using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA), and their integrity was checked electrophoretically. First strands of the cDNA samples were synthesized by using RT PCR (Cat. No. 11483188001 Roche, GmbH, D-40724 Hilden, Germany). Gene expression analysis was

Table 1. Clinicopathological data of patients

	n	Mean±SD	Tumor stage	n
Age (year)		57.0±10.7	T ₁	1
Smoking			T ₂	9
Yes	27		T ₃	13
No	2		T ₄	6
Alcohol			N stage	
Yes	3		N ₀	21
No	26		N ₁₋₃	8
Tumor differentiation			Extra-capsular spread of the lymph nodes	
Well	12		Yes	6
Moderately	14		No	23
Poorly	3		Cartilage invasion	
Tumor localization			Yes	11
Supraglottic tumor	19		No	18
Glottic tumor	10		Lymphatic vessel invasion	
Regional lymph node metastasis			Yes	12
Yes	8		No	17
No	21			

SD: Standard deviation.

performed by quantitative reverse transcription (qRT)-PCR (LightCycler 1.5, Roche, Germany). The PCR reaction started with a denaturation step at 95°C for 10 seconds (1 cycle), followed by 45 cycles at 95°C for 10 seconds, 55°C for 5 seconds

and 72°C for 20 seconds. Subsequently, a melting curve program was applied with continuous fluorescence measurement. A standard curve for ROBO-1 ROBO-2 and TGM-3 templates was generated through serial dilutions of PCR

Table 2. The fold change of ROBO-1, ROBO-2 and TGM-3 genes

Gene	Log2 fold change	95% CI	Fold change	95% CI	p
ROBO-1	-1.77	-1.089 - -0.62	0.56	0.47 - 0.65	0.003799
ROBO-2	-2.10	-1.59 - -0.66	0.48	0.33 - 0.63	0.020999
TGM-3	-1.20	-1.12 - -0.26	0.83	0.46 - 1.20	0.480100
Tumor differentiation					
ROBO-1	-2.04	-1.43 - 0.71	0.49	0.37 - 0.61	0.006236
ROBO-2	-5.39	-3.18 - -1.88	0.19	0.11 - 0.27	0.009487
TGM-3	-3.40	-16.60 - 0.59	0.29	0.00001 - 0.66	0.425495
Cartilage invasion					
ROBO-1	-3.84	-2.39 - -0.81	0.26	0.19 - 0.33	0.002173
ROBO-2	-2.76	-3.47 - -0.66	0.36	0.09 - 0.63	0.085589
TGM-3	-1.59	-1.05 - -0.35	0.63	0.48 - 0.78	0.016022
Tumor stage (T₁+T₂, T₃+T₄)					
ROBO-1	-1.91	-1.05 - -0.83	0.52	0.48 - 0.56	0.000079
ROBO-2	-2.05	-1.15 - -0.91	0.49	0.45 - 0.53	0.000030
TGM-3	-2.03	-1.12 - -0.94	0.49	0.46 - 0.52	0.000014

ROBO: Roundabout; CI: Confidence interval.

products. Each reaction was performed duplicate. The β -Actin (ACT β) gene was used as reference for normalization of the gene expression levels.

Statistical analysis

The relationship between the ROBO-1, ROBO-2 and TGM-3 clinicopathologic parameters were analyzed using the Pearson's chi-square test or Fisher's exact test. Expression ratios were transformed into fold changes and reported as relative expression. Obtained fold changes were compared between different tumor grades in addition to surrounding tissue samples. Significant fold changes were determined with t-test using PASW for Windows version 17.0 software program (SPSS Inc., Chicago, IL, USA). All tests were two-sided, and P values less than 0.05 were considered statistically significant. The results were analyzed by the threshold cycle (Ct)

numbers as fold-changes and calculated by the $2^{-\Delta(\Delta CT)}$ method [$2^{\text{geneT-N(Ct)}}$ / $2^{\beta\text{-ActinT-N(Ct)}}$] (N, matched surrounding tissue; T, tumor tissue).

RESULTS

ROBO-1, ROBO-2, TGM-3 genes are expressed in various cancer types. These genes are play an important role in cell division, angiogenesis, tumor differentiation, but this task has not been clarified yet. In our study, were compared the expression levels of three genes which plays a role in signal transduction pathways. We determined ROBO-1, ROBO-2, TGM-3 gene expression levels of laryngeal cancer patients. These results are shown in Table 2. According to our results ROBO-1 gene than in surrounding tissue approximately two-fold, ROBO-2 gene by 2.5 times, TGM-3 gene is 1.25 times were found to be less expression. We determined, ROBO-1 ROBO-2 and TGM-3

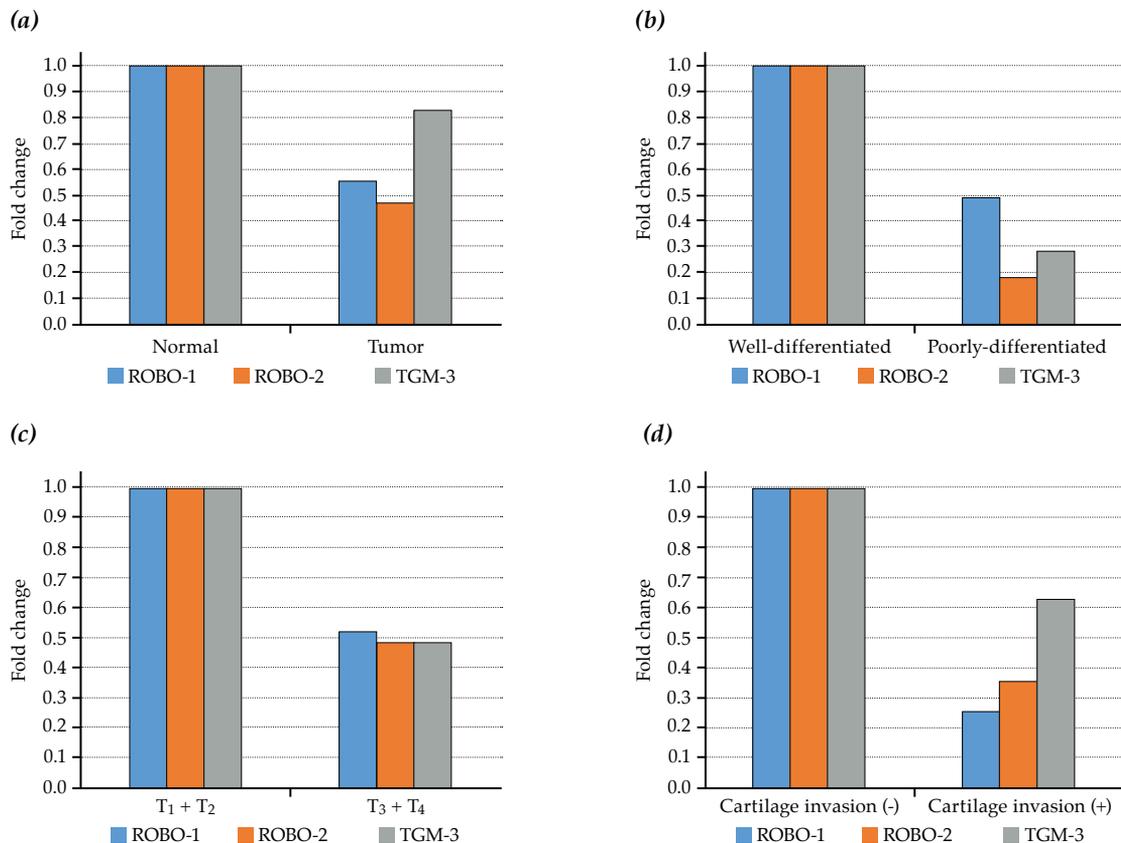


Figure 1. (a) Bar charts of ROBO-1, ROBO-2, TGM-3 genes fold changes in tumor and surrounding tissue. (b) Bar charts of ROBO-1, ROBO-2, TGM-3 genes fold changes in well differentiation and poorly differentiation tumor tissue. (c) Bar charts of ROBO-1, ROBO-2, TGM-3 genes fold changes in T₁+T₂ and T₃+T₄ tumor tissue. (d) Bar charts of ROBO-1, ROBO-2, TGM-3 genes fold changes in cartilage invasion (-) and cartilage invasion (+) tumor tissue.

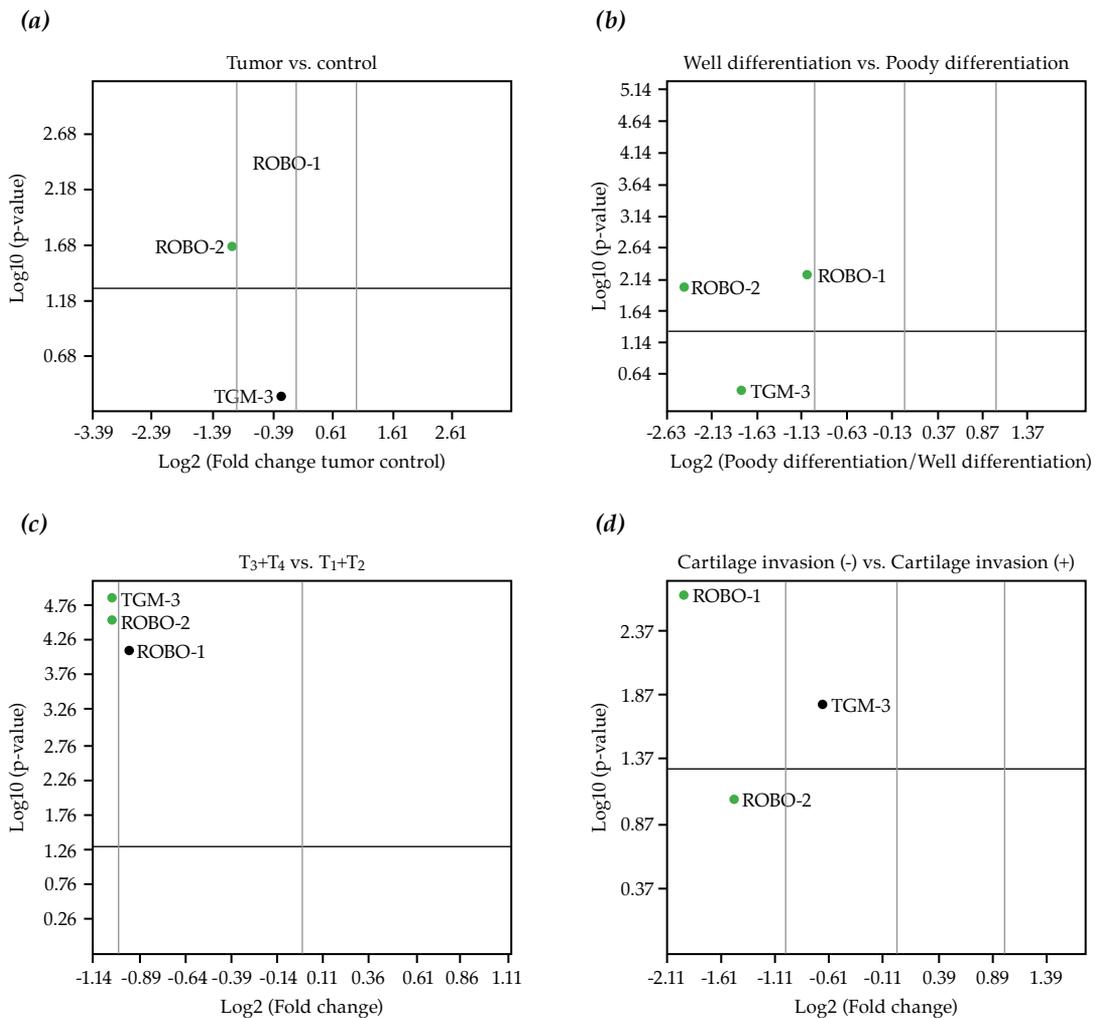


Figure 2. (a) Box plots of ROBO-1, ROBO-2, TGM-3 genes fold changes in tumor and surrounding tissue. (b) Box plots of ROBO-1, ROBO-2, TGM-3 genes fold changes in well differentiation and poorly differentiation tumor tissue. (c) Box plots of ROBO-1, ROBO-2, TGM-3 genes fold changes in T₁+T₂ and T₃+T₄ tumor tissue. (d) Box plots of ROBO-1, ROBO-2, TGM-3 genes fold changes in cartilage invasion (-) and cartilage invasion (+) tumor tissue.

gene expression tumor tissues differentiation in terms of the results of comparison in the ROBO-1 gene in the tumor tissue approximately two-fold, ROBO-2 gene five times, TGM-3 gene three times less, lymphatic invasion, in terms of by comparing ROBO-1 gene in the tumor tissue of about two-fold, ROBO-2 gene, six-fold, TGM-3 gene is eight-fold less, cartilage invasion terms by comparing ROBO-1 gene in the tumor tissue are about four times, ROBO-2 gene 3 levels, TGM-3 gene two times less, T₁ + T₂, and T₃ + T₄ terms by comparing ROBO-1 gene in the tumor tissue of about two-fold, ROBO-2 gene 2 levels, TGM-3

gene two times less as expressed. Bar charts of the results are shown in Figure 1 and box plots of the results are shown in Figure 2.

DISCUSSION

Laryngeal cancer represents the second most common malignancy of the head and neck worldwide. Larynx plays an important role in human speech and communication.^[1] Smoking is the very important risk factor for developing laryngeal carcinomas.^[22] Investigators are having been initiated clinical trials of gene therapy since the end of 1990s. Much interest has focused

on the clinical potential of this new modality to treat cancer, especially on the use of tumor suppressor genes.^[23,24]

Slit protein, a secreted glycoprotein, which regulates axon guidance, branching and neural migration during development of the central nervous system. Expression of genes of the Slit-ROBO signaling pathway was detected at the venous pole of the heart, suggesting an important role in the formation of this area.^[25]

Field cancerization is an important concept for head and neck cancer diagnosis and treatment options for understanding and determining. Cancer begins with multiple cumulative epigenetic and genetic alterations that sequentially transform a cell or a group of cells in a particular organ.^[26]

Recent studies have demonstrated a role for Slit-ROBO signaling in leukocyte chemotaxis and tumor angiogenesis.^[27] Slits and their receptors have been identified to be associated with angiogenesis in several studies. The Slit-ROBO signaling complex acts as a key ligand-receptor interaction in the development of neurons, blood vessels, and some organs.^[28] Although biochemical studies have defined the domains mediating ROBO-Slit interaction as ROBO Ig1-2 and Slit D2. Robo1 Ig1 domain is the primary binding site for Slit2. A sequence alignment of metazoan homologues reveals that the Slit2 D2-binding residues are highly conserved. ROBO1, to the medium to block the effect of secreted Slit2 from microencapsulated cells.^[28]

TGases are aberrantly expressed in a wide range of degenerative diseases affecting many cell and tissue types.^[29] While TGases 1, 2, 3, 5, 6, and 7 are known to be expressed in epithelia, to date only TGase 1, TGase 3 and TGase 5 are proven participants in cornified cell envelope assembly. This conclusion is based largely on *in vitro* cross-linking of several cornified cell envelope precursors, and comparisons with cross-linked glutamine and lysine residues used *in vivo*.^[30]

TGM3, encoded by the TGM3 gene, is widely expressed in the small intestine, brain, skin and mucosa. In the skin and mucosa, TGM3

is predominantly expressed in the suprabasal layers of the stratified squamous epithelium. It has been demonstrated that TGM3 is essential for epidermal terminal differentiation and formation of the cornified cell envelope through cross-linking structural proteins such as involucrin, loricrin and small proline-rich proteins.^[31,32]

It has been documented that the activated accounts for up to 75% of the total TGase activity in mammalian epidermis although TGase 3 mRNA represents less than 2% of the TGase transcripts. So, this is important to know the mechanism underlying the regulation of TGM3 expression in the process of terminal differentiation of epithelial cells.^[33]

Recent studies have revealed that the down-regulation of the TGM3 gene is closely linked with a variety of human cancer types, including laryngeal carcinoma, esophageal and oral squamous cell carcinoma (OSCC).^[34-39]

However, the biological function and molecular mechanism of the TGM3 gene in cancer initiation and progression have not been reported. In addition, whether the TGM3 gene might be a valuable diagnostic or therapeutic biomarker for cancer, especially for HNC, needs to be further investigated.^[34]

Although such paracrine pathways involving Slit-ROBO interactions have been extensively studied, the biological significance and molecular mechanisms of autocrine Slit-ROBO signaling are largely unexplored Zhou et al.^[40] reported previously reported elevated expression of Slit2 in human colorectal carcinoma tissues and cell lines.^[41]

We examined ROBO-1, ROBO-2, TGM-3 gene expression levels of laryngeal cancer, the study according to our results ROBO-1 gene than in normal tissue approximately two-fold, ROBO-2 gene by 2.5 times, TGM-3 gene is 1.25 times were found to be less pronounced Avcı et al.^[42] in their study in liver cancer tissue and the ROBO-1 ROBO-2 genes more expressed in the tumor tissue compared to normal tissue is indicated.

Latil et al.^[43] study in prostate cancer tissue is supporting Avcı et al.^[42] They suggest that ROBO genes (ROBO-1, ROBO-2) are unregulated in prostate tumor tissue than normal tissue.

This condition can be explained that ROBO-1 and ROBO-2 genes that probably came from a common neuronal substructure or impaired the level of expression during embryonic stem cell differentiation. However, the above mentioned studies that suggest increased expression levels of these genes are in contrast to our study.

The results of Liu et al.^[44] study reduced expression of TGM3 may play an important role in esophageal carcinogenesis. Our study results also support Liu et al.^[44] study and it is evaluated that TGM-3 gene expression is lower in laryngeal cancer tumor tissues than normal tissues.

Negishi et al.^[39] suggest that TGM3 expression was markedly decreased in oral squamous cell carcinoma and that the reduced expression of TGM3 was clearly correlated with loss of histological differentiation. In our study TGM-3 gene three times less expressed in tumor tissues differentiation state.

Liu et al.^[44] evaluated expression of TGM3 correlated significantly only with histological grade of esophageal squamous cell carcinoma. Significant inverse correlation existed between the intensity of TGM3 expression and histological grade and no significant correlation was found between abnormal expression of TGM3 and lymph node metastasis and depth of invasion. He et al.^[38] suggest that TGM-3 expression in the tumor tissues significantly lower than normal tissues. TGM-3 gene expression could play an important role in the formation and development of laryngeal cancer. Liu et al.^[44] TGM-3 gene low expression indicated in laryngeal carcinomas. Our compared to the results of their study, TGM-3 gene in laryngeal cancer tumor tissue was determined to be less pronounced.

Declaration of conflicting interests

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