Investigation of Toll-like Receptor-2, -3 and -4 Gene Expressions in Larynx Squamous Cell Carcinoma

Original Investigation

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Abstract

Objective: Despite all the recent advancements, larynx cancer has shown no improvement in survival rates. The aim of this study was to investigate the expressions of toll-like receptor (TLR)- *2, -3,* and *-4* genes, and determine any relationships with the histopathologic characteristics of the disease.

Methods: This retrospective study included 50 subjects who underwent total or partial laryngectomy with an open surgical method for larynx squamous cell carcinoma. Measurements of TLRs-2, -3, and -4 expression values were taken with quantitative real time-polymerase chain reaction in normal tissue and tumor tissue samples of the patients.

Results: Evaluations were made of TLR-2, -3, and -4 mRNA expressions according to $2^{-\Delta\Delta CT}$ calculations in 50 subjects with larynx cancer. When the tumor tissue was compared with the healthy tissue from the same subjects, reductions were determined in TLR expression in 86%, 84%, and 82%, respectively. This reduction in each gene expression was statistically significant (p<0.001). No statistically significant correlation was determined between the change in TLR-2, -3, and -4 expression and the histopathologic characteristics of the disease.

Conclusion: The data obtained in this study demonstrated that TLR-2, -3, and -4 expressions were reduced in larynx squamous cell cancer. The results of further studies targeting these genes would be useful in the diagnosis and treatment of the disease.

Keywords: Larynx cancer, toll-like receptors, gene expression, squamous cell carcinoma, tumor microenvironment

Introduction

Laryngeal cancers are the second most common malignancy of all head and neck sites and the 5-year survival rate has been reported as 67%. The majority (95%) of these cancers are squamous cell carcinomas (SCCs) (1). Despite all the advancements in healthcare, significant improvement could not be achieved in the survival rates in the past 50 years. Although environmental carcinogens (tobacco, alcohol) contribute significantly to the development of larynx SCC, it is

thought that partial inadequacy of the natural immune response to the tumor could lead to the progression of the disease.

Significant risk factors that reduce survival in laryngeal cancers are the local invasion status (e.g., lymphatic invasion, vascular invasion, perineural invasion, cartilage invasion), spread to surrounding tissues, and local and/or distant metastasis. Tumor development and invasion of surrounding tissues occur as a result of complex events in which several mechanisms interact such as apoptosis inhibition in the tumor micro surroundings, angiogenesis, and proliferation activation (2).

Toll-like receptors (TLRs) are a class of receptors expressed from the epithelial and endothelial cells of the human immune system cells. Ten human TLRs, each of which recognizes a certain microbial component, have been identified (3). The function of each TLR is extremely complex and varied and affected by several factors. Various TLRs play different roles in carcinogenesis (2). The main role of TLRs in tumor formation is still not fully known. They may be related to cancer progression or inhibition. Improvement of the inflammatory process with TLRs plays a key role in the processes that both increase tumor progression and induce anti-tumor reactions. Just as TLRs can prevent apoptosis, they have also been reported to have pro-apoptotic activity through a series of mechanisms (4).

This study aimed to obtain insight about how the *TLR-2, -3* and *-4* genes play a role in laryngeal cancer carcinogenesis and to evaluate the relationship between the expression level of these genes and tumor histopathological features.

Methods

Ethical Considerations

The İstanbul University-Cerrahpaşa, Cerrahpaşa Medical Faculty Ethics Committee approved the study, and the study was conducted in line with the Declaration of Helsinki. All patients and volunteers provided their written informed consent. Approval for the study was granted by the Clinical Research Ethics Committee of İstanbul University-Cerrahpaşa, Cerrahpaşa Medical Faculty (decision date: 15.11.2017, decision no: 93777809-604.01.01-429320).

Patients

This retrospective study evaluated 50 subjects with confirmed laryngeal SCC. Tumor tissues and adjacent non-cancerous laryngeal mucosal tissues of patients who underwent total or partial laryngectomy between 2016 and 2018 at the Otolaryngology and Head and Neck Surgery Department of the İstanbul University-Cerrahpaşa, Cerrahpaşa Medical Faculty were analyzed. Non-cancerous tissue samples were taken by incisional biopsy from the intact laryngeal tissue

farthest from the tumor. Subjects who had received preoperative chemotherapy and/or radiotherapy were excluded from the study. All of the samples were human papilloma virus-negative. Inclusion criteria were first time diagnosed laryngeal SCC patients over 18 years of age. Clinical and histopathological features of all subjects' tumors were recorded. Localization, stage, grade, and invasion characteristics (lymphatic invasion, vascular invasion, perineural invasion, cartilage invasion) of the tumors were determined according to the histopathology reports. Grade and stage of the tumors were defined according to the guidelines published by the 2017 American Joint Committee on Cancer (5).

RNA Isolation and cDNA Synthesis

To quantitate mRNA expression of *TLR-2, -3,* and *-4* genes, total RNA was extracted from the tumor and adjacent non-cancerous laryngeal mucosal tissues using the PureLink RNA Mini Kit (Life Technology, NY, USA) according to the kit protocol. Following RNA isolation, RNA measurements were taken with Nano Drop ND1000 (Thermo Fisher Scientific, USA). A total of 200 ng isolated RNA was reverse-transcribed using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Quantitative Real Time-polymerase Chain Reaction (qRT-PCR) Analyses

The mRNA expression levels of TLR-2, -3 and -4 were quantified by qRT-PCR using the Light Cycler 480 II device. qRT-PCR was performed using 200 ng cDNA, PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, USA) and 10µ M forward and reverse primers. The glucose-6-phosphate dehydrogenase (*G6PD*) gene was used as a housekeeping gene for normalization of mRNA levels of target genes. The sequences of the primers are presented in Table 1. qPCR reactions were performed at least three times. PCR cycling conditions were as follows: 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 sec, 61 °C for 30 sec and 72 °C for 30 sec, and a final 10 sec at 50 °C. Quantitative RT-PCR data of mRNA levels were calculated using the comparative CT method (also known as the $2^{-\Delta\Delta CT}$ method) (6). Expression levels were evaluated according to the assumptions that the $2^{-\Delta\Delta CT}$ value was between 0.9 and 1.1, greater than 1.1 and smaller than 0.9. Values between 0.9 and 1.1 were considered as unchanged, i.e., meaning there was no difference at gene expression level between the tumor and the normal tissue of the patient. Values greater than 1.1 were considered as increased, and values smaller than 0.9 were considered as decreased.

Statistical Analysis

Data obtained in the study were analyzed statistically using IBM SPSS version 21.0 software. Descriptive statistics of numerical variables were expressed as mean ± standard

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deviation, minimum and maximum values, and categorical variables were expressed as number (n) and percentage (%). For the statistical evaluation of the data obtained, first the normality test was performed. In the comparisons of tumor tissue and normal tissue, data not showing normal distribution were assessed using the Wilcoxon signed-rank test. Spearman's rho correlation test was used in the comparisons of TLR-2, -3, and -4 gene expressions with each other, and the chi-square test was used in the comparison of clinicopathologic data. A value of p<0.05 was accepted as statistically significant.

Results

The study was carried out with 50 subjects with laryngeal SCC. Of the 50 subjects, 47 were male and 3 were female with a mean age of 60.56±9.67 (range, 34–84) years; 12 consumed alcohol and 45 smoked. Early-stage tumor (T1, T2) was determined in six subjects and advanced stage (T3, T4) in 44 subjects. There was blood vessel invasion in 25 (50%), lymphatic invasion in 43 (86%), perineural invasion in 22 (44%), and thyroid cartilage invasion in 22 (44%) subjects.

Table 1. Primer series of *TLR-2*, *-3*, and *-4* genes

Gene	Primer series (5' → 3')
TLR-2	Forward primer: 5'-GGTGTCGGAATGTCACAGGACA-3'
	Reverse primer: 5'-GCATCATAGCAGATGTTCCTGCT-3'
TLR-3	Forward primer: 5'-TGGAGCCAGAATTGTGCCAGA-3'
	Reverse primer: 5'-GGATTGAGTTGGACATGAGATGGA-3'
TLR-4	Forward primer: 5'-TGCGTGGAGGTGGTTCTTAAT-3'
	Reverse primer: 5'-AAGCTATAGCTGGCTAAATGCCTG-3'
G6PD	Forward primer: 5'-ATGCCTTCCATCAGTCGGATACA-3'
	Reverse primer: 5'-ATAGCCCACGATGAAGGTGTTTTC-3'

TLR: Toll-like receptor, G6PD: Glucose-6-phosphate dehydrogenase

The TLR-2, -3, and -4 gene expression levels were analyzed in the tumor and adjacent non-cancerous laryngeal tissue samples taken from the 50 subjects by normalizing with G6PD housekeeping gene. Except for one subject, TLR-2, -3, and -4 mRNA expressions were determined in all tumor and adjacent non-cancerous laryngeal tissue samples. In one subject, TLR-2 mRNA was not determined in the non-cancerous tissue so the data of that subject were not included in the evaluation.

In this study with 50 subjects, CT values of target gene (TLR-2, -3 or -4) were normalized with CT values of internal control *G6PD* gene. After normalization, as described by Schmittgen and Livak (6), expression of all target genes were presented as fold change ($2^{-\Delta\Delta CT}$). All fold change rates for TLR-2, -3, and -4 are shown in Table 2 (0.07, 0.04 and 0.04, respectively). In the tumor tissue samples, TLR-2 gene expression level was determined to have decreased in 86% (42/49), TLR-3 in 84% (42/50), and TLR-4 in 82% (41/50) compared to the non-cancerous tissue samples. The expression levels of TLR-2, -3, and -4 genes in tumor tissues were lower than those of the non-cancerous tissues and these decreases were determined to be statistically significant (p<0.001) (Figure 1). On the other hand, no statistically significant correlations were found between any of the histopathological parameters and TLR-2, -3 and -4 gene expression levels (Table 3).

Discussion

TLRs are found in immune system cells, and they recognize the surface proteins of micro-organisms (7). TLRs also have a role in the control of the immune system, tissue homeostasis, and the regulation of cell death (8). Depending on the intracellular localization, type, amount, and presence of ligands, TLRs have a two-way effect in cancer development, either by supporting the tumor or by promoting an anti-tumor response (7).

qRT-PCR is a widely applied technique that provides quantitative analyses of gene expression. Experimental comparison between normal and abnormal tissues can be made with the qRT-PCR technique. Wide dynamic range, remarkable sensitivity and sequence specificity are

Table 2. Expression levels of *TLR-2*, *-3*, and *-4* genes in laryngeal tissues of 50 patients

		CT Mean ± SD	G6PD CT Mean ± SD	ΔCT Mean ± SD	ΔΔCT Mean ± SD	$2^{-\Delta\Delta CT}$	*p-value
TLR 2	Tumor	25.96±1.7	24.20±2.29	1.76±2.25	3.82±3.51	0.07	<0.001
	Normal	26,56±1.57	28.76±2.53	-2.03±2.44	0	1	
TLR 3	Tumor	27.41±1.84	24.20±2.29	3.21±2.6	4.71±5.02	0.04	<0.001
	Normal	27.26±3.47	28.76±2.53	-1.50±4.45	0	1	
TLR 4	Tumor	32.01±2.76	24.20±2.29	7.81±2.9	4.83±4.28	0.04	<0.001
	Normal	31.74±2.27	28.76±2.53	2.98±3.01	0	1	

SD: Standard deviation, TLR: Toll-like receptor, CT: Threshold cycle, *Statistical analysis was tested with the paired sample t-test
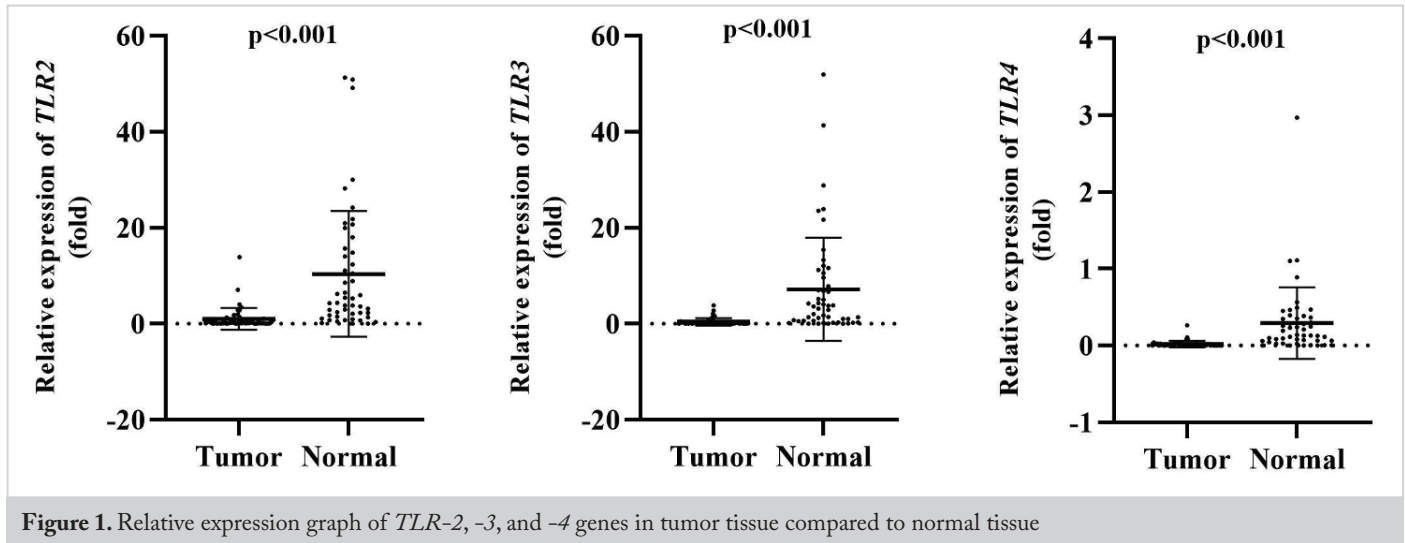
Figure 1. Relative expression graph of *TLR-2*, *-3*, and *-4* genes in tumor tissue compared to normal tissue

Table 3. Relationships of histopathological parameters with TLR-2, 3, and 4 gene expressions

Clinical and pathological parameters		TLR-2 expression				TLR-3 expression				TLR-4 expression			
		Increased	Decreased	Unchanged	*p-value	Increased	Decreased	Unchanged	*p-value	Increased	Decreased	Unchanged	*p-value
Tumor localization	Glottic	2 (4.1)	13 (26.5)	1 (2)		2 (4)	15 (30)	0 (0)		3 (6)	13 (26)	1 (2)	
	Transglottic	3 (6.1)	13 (26.5)	0 (0)	0.494	4 (8)	12 (24)	0 (0)	0.353	3 (6)	13 (26)	0 (0)	0.666
	Supraglottic	1 (2)	16 (32.7)	0 (0)		1 (2)	15 (30)	1 (2)		2 (4)	15 (30)	0 (0)	
Stage	T1	0 (0)	1 (2)	0 (0)		0 (0)	1 (2)	0 (0)		0 (0)	1 (2)	0 (0)	
	T2	0 (0)	5 (10.2)	0 (0)		0 (0)	5 (10)	0 (0)		0 (0)	5 (10)	0 (0)	
	T3	1 (2)	6 (12.2)	0 (0)	0.968	1 (2)	6 (12)	0 (0)	0.956	1 (2)	6 (12)	0 (0)	0.935
	T4	5 (10.2)	30 (61.2)	1 (2)		6 (12)	30 (60)	1 (2)		7 (14)	29 (58)	1 (2)	
Grade	Grade 1	0 (0)	1 (2)	0 (0)		0 (0)	1 (2)	0 (0)		0 (0)	1 (2)	0 (0)	
	Grade 2	4 (8.2)	23 (46.9)	1 (2)	0.885	5 (10)	24 (48)	0 (0)	0.713	4 (8)	24 (48)	1 (2)	0.874
	Grade 3	2 (4.1)	18 (36.7)	0 (0)		2 (4)	17 (34)	1 (2)		4 (8)	16 (32)	0 (0)	
Blood vessel invasion	Present	4 (8.2)	20 (40.8)	1 (2)		4 (8)	20 (40)	1 (2)		5 (10)	19 (38)	1 (2)	
	Absent	2 (4.1)	22 (44.9)	0 (0)	0.418	3 (6)	22 (44)	0 (0)	0.538	3 (6)	22 (44)	0 (0)	0.423
Lymphatic invasion	Present	4 (8.2)	37 (75.5)	1 (2)		5 (10)	37 (74)	1 (2)		6 (12)	36 (72)	1 (2)	
	Absent	2 (4.1)	5 (10.2)	0 (0)	0.343	2 (4)	5 (10)	0 (0)	0.461	2 (4)	5 (10)	0 (0)	0.583
Perineural invasion	Present	3 (6.1)	17 (34.7)	1 (2)		3 (6)	18 (36)	1 (2)		4 (8)	17 (34)	1 (2)	
	Absent	3 (6.1)	25 (51)	0 (0)	0.459	4 (8)	24 (48)	0 (0)	0.522	4 (8)	24 (48)	0 (0)	0.473
Cartilage invasion	Present	2 (4.1)	18 (36.7)	1 (2)		2 (4)	20 (40)	0 (0)		3 (6)	18 (36)	1 (2)	
	Absent	4 (8.2)	24 (49)	0 (0)	0.459	5 (10)	22 (44)	1 (2)	0.431	5 (10)	23 (46)	0 (0)	0.494

TLR: Toll-like receptor, *Statistical analyses were done with the chi-square test

the most important advantages of this technique over other techniques (9). The test is based on measuring the increase in fluorescent signal corresponding to the amount of DNA produced during each PCR cycle. A single PCR reaction is characterized by the PCR cycle in which fluorescence first rises above threshold background levels (threshold cycle, CT). Therefore, the higher the mRNA concentration of the target gene, the lower the CT value. If a comparison is

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to be made between two tissues, the relative mRNA levels are calculated using the comparative $2^{-\Delta\Delta CT}$ method and valued as the gene expression level ratio between the two tissues (6). *G6PD* gene mRNA levels are used as controls for normalization.

There are a few reference studies that have examined TLR family members in head and neck cancers, especially in laryngeal cancer (10-17). With the inclusion of 50 subjects with laryngeal cancer, this study is one of the most extensive in the literature. The results of this study showed that TLR-2, -3, and -4 mRNA expressions were present in both the cancer tissues and the healthy tissues of the same subjects. Moreover, TLR-2, -3, and -4 mRNA expressions in the tumor tissues were statistically significantly lower than in healthy tissues. These results have both similar and conflicting points with those of previous studies that have investigated the TLR-2, -3, and -4 levels in patients with head and neck cancer (10-17).

TLR-3, which is a member of the TLR family with intracellular location, has the predominant characteristic of being tumor-suppressing. In a previous study, TLR-3 activation has been shown to initiate anti-tumor immunity involved in dendritic cell maturation, natural killer (NK) cell activation, and type I interferon signaling. Administration of Poly(I:C) (TLR-3 analogue) increases tumor infiltration of NK cells, leading to NK-dependent tumor regression (18). In some cancers, including endometrial cancer and acute lymphoblastic leukemia, the absence of TLR-3 has been observed to cause tumor growth (19). Chew et al. (20) determined a positive relationship between TLR-3 expression in liver cancer and patient survival rates. In another study by Salaun et al. (21), it was shown that apoptosis in the breast cancer cell line could be directly started by TLR-3. A study conducted on head and neck cancer cell lines in 2010 reported that TLR-3 was the cause of direct apoptosis (10). In another study by Luo et al. (17), TLR-3 activation was shown to inhibit tumor growth in oral SCC in a xenograft mouse model.

In the literature only a study by Pries et al. (11) reported in 2008 that c-MYC oncoprotein expression was reduced when TLR-3 expression was reduced with siRNA, and therefore, suggested that TLR-3 had an effect on tumor cell proliferation. In our study, a significant reduction of 84% was determined in TLR-3 mRNA expression in the tumor tissues of the subjects with laryngeal cancer compared to healthy tissues (p<0.001). The results of our study are consistent with those of the other studies in the literature, except for the above-referred one. We concluded that *TLR-3* gene showed a tumor suppressor characteristic at the mRNA level in larynx cancer.

A review of the studies conducted on TLR-2 and -4, which unlike TLR-3 are located on the cell surface, showed an organ-specific contribution with a tumor-suppressing effect in some cancers and with an oncogenic effect in some. TLR-4 has been determined to have a tumor-supportive role in colon and liver cancers, and by contrast, to prevent cancer development in some cancers such as lung and breast (22-25). In a 2009 study by Szczepanski et al. (12), TLR-4 expression was shown to be increased in head and neck cancers. The higher expression has been found especially in tongue cancers compared with larynx and other oral cavity cancer tissues. In a study by Mäkinen et al. (13) who investigated the tongue cancer cell cultures and the tissues of six patients with tongue cancer, TLR-4 expression was found higher in tumor cells, and higher expression was determined in the nuclei in healthy tissues and the cytoplasm in cancerous tissues. Mäkinen et al. (14) conducted another study on 30 patients with primary, recurrent, or metastatic tongue cancer. In primary tumors, the nucleus localization of TLR-4 was found to be higher than recurrent and metastatic tumors. Although there are few studies in the literature supporting the tumor suppressor effect of TLR-4, unlike other TLRs, TLR-4 activation is regulated by MyD88 as well as toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF). This stimulation induces the activation of antigen presenting cells, mainly contributing to the increase in T-cell proliferation (26). In addition, it is known that lipid A, the biologically active part of lipopolysaccharides, binds to TLR-4 and promotes the inhibition of tumor growth (27). In our study, TLR-4 expression was seen to be reduced in 82% of the cancer tissues (p<0.001). When compared to the other studies in the literature, it was seen that the previous studies had been conducted with peripheral blood samples taken from subjects or with different methods in cell cultures. Therefore, it would not be correct to make direct comparisons.

TLR-2, which is another cell surface TLR family member, may promote or suppress tumors, just like TLR-4. Tumor-supportive characteristics of TLR-2 are seen in lung cancer (28). Wang et al. (15) reported that TLR-2 mRNA expression was higher in tumor tissues in 22 patients with laryngeal cancer. In the study by Mäkinen et al. (13), increased expression of TLR-2 and -4 in tongue cancer was seen to be positively correlated with deeper tumor invasion. In contrast to these findings, Park et al. (16) found that TLR-2 expression did not affect oral squamous carcinoma cell invasion. Moreover, TLR2 deficiency leads to increased cell proliferation and reduced apoptosis during colitis-associated cancer development (29). In another study, researchers found that a TLR-2 variant with a deletion in the promoter region increased the risk of hepatocellular carcinoma in patients with chronic hepatitis C (30). In our study, TLR-2 expression was reduced in 85.7% of the 50 subjects with laryngeal carcinoma (p<0.001). This result demonstrated the tumor-suppressing characteristic of TLR-2 in laryngeal cancer.

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As a result of the studies, it has been seen that TLR-3 had predominant anti-tumor activity (10, 17-21). This, however, does not apply to TLR-2 and -4. Depending on the tumor type, these two receptors sometimes indicate pro-tumorigenic effects, and sometimes the opposite effect. In different studies this reciprocal effect has been seen even in the same tumor type (13, 16). Although the authors have put forward various theories about these outcomes the reason has not been fully revealed. According to the literature data, TLRs may show reciprocal effects depending on the cell type. The most plausible theories related to this are quantitative differences in the expression of various TLRs on tumor cells and inflammatory cells, mutating or polymorphism of TLRs on tumor cells. The results of our study support the literature for the anti-tumor activity of TLR-3 in laryngeal carcinoma, and new data and discussion have been put forward for TLR-2 and -4.

The limitations of our study were that it was retrospective and the expression level in the tissue was not taken into account when evaluating gene expression levels with qRT-PCR. Non-cancerous tissue samples were obtained from the laryngeal tissue in the furthest region from the cancerous tissue, constituting the control group. Another limitation was that the control group was not obtained from subjects with intact larynx. While a significant decrease in gene expressions has been detected, prospective studies are needed to investigate its expression at the protein stage in tissue.

Conclusion

Despite the recent advancements, laryngeal cancer is one of the diseases with a rarely falling 5-year survival rate. Recent studies have shifted to research on the role of cellular immunity, in particular, and on the tumor micro-environment. In the current study, we examined TLR-2, -3, and -4 from the TLR family in subjects with laryngeal cancer as these have been determined to play a role in cancer progression in several types of tumors such as those in the oral cavity, the colon, the prostate, the breasts, the skin, and the lungs. TLR-2, -3 and -4 mRNA expressions were determined to be significantly low in larynx cancer tissue samples compared to the healthy surrounding tissues. There are clues and signs that TLR-2, -3 and -4 assume a tumor suppressor role in laryngeal cancer. With the future development of agents directed at these receptors, it can be considered that tumor behavior, resistance, and sensitivity to medical interventions and cancer progression could be changed.

Main Points

- Depending on the intracellular localization, type, amount, and presence of ligands, TLRs have a two-way effect in cancer development either by supporting the tumor or by promoting an anti-tumor response.
- We found TLR-2, 3, and 4 expressions decreased in larynx squamous cell carcinoma tissues compared to normal tissues.
- Decrease in TLR-2, 3 and 4 may contribute to the progression of laryngeal cancer.
- In particular, therapies targeting the TLR-3 gene may be used in the treatment of laryngeal cancer in the future.

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