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Thioredoxin Reductase Activity and Its Tissue Distribution in the Pathologic Specimens of Patients with Laryngeal Squamous Cell Carcinoma

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Abstract

Background: Squamous Cell Carcinoma (SCC) is the second most common malignancy of the respiratory tract. Recently, researchers believe that thioredoxin system is effective in the cancerization of some tissues. Thus, this study has been conducted with the aim of measuring of thioredoxin reductase (TrxR) enzyme activity and tissue distribution in the pathologic specimens of patients with laryngeal SCC.**Materials and Methods:** This study was performed on 40 pathologic blocks (20 healthy and 20 tumoral) from 20 patients with laryngeal SCC who were candidates for laryngectomy surgery. The TrxR enzyme activity was measured by the commercial kit. Also, the tissue distribution of TrxR was determined by immunohistochemical staining and the percentage of staining cells (SC%) and staining intensity were calculated. Data were analyzed by using SPSS13 and significant level was set at P≤0.05.**Results:** The average the TrxR enzyme activity in the healthy and tumoral tissues was 0.004±0.003µM/min/ml and 0.006±0.003µM/min/ml, respectively (ranged 0.0009 to 0.0104 vs. 0.001 to 0.011). However, there was no relationship between the TrxR enzyme activity in the tumoral and healthy tissues (P=0.084). The total score of IHC staining in the healthy tissue was 4.45±1.09 whereas the total of these scores in the tumoral tissue 6.25±0.63. The both scores of SC% and staining intensity in the tumoral tissue was significantly higher than the healthy tissue (P<0.001).**Conclusion:** Based on the results, although the TrxR enzyme activity has not the significant differences in tumoral tissue compare to healthy tissue, but the tissue distribution in tumoral tissue was higher than healthy tissue. **[GMJ.2016;5(3):153-159]**

Keywords: Laryngeal; Squamous Cell Carcinoma; Thioredoxin Reductase Enzyme

Introduction

Laryngeal cancer is the second malignancy of upper respiratory- gastrointestinal tract and also the second most common malignancy of the respiratory tract with the incidence of 12,000 new cases per year and mortality rate of 3500 people each year only in the United States [1,2].

Although a variety of malignancies can occur in the larynx, squamous cell carcinoma (SCC) constitutes 85% to 95% of laryngeal cancers, which originates from the epithelial layer of the larynx [1-3].

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The SCC destroys the basic membrane to invade the underlying tissues.

Clinical symptoms developed in laryngeal SC depend on the place from which the primary tumor originates. The location of the primary tumor in SCC can be in glottic, supraglottic and subglottic areas [1, 4]. Successful management of laryngeal malignancy requires accurate diagnosis and staging of the disease, proper evaluation of the patient's demands and selection of the most appropriate treatment method for each patient together with accurate follow-up after treatment. With the advent of new surgical techniques, development of radiotherapy methods and new chemotherapy drugs, treatment options have become wider and more complex. However, these treatments have still many complications. The risk of their side effects on sound quality and integrity of breathing and swallowing tract still exists [1]. Therefore, it seems essential to find a way that leads to complete remission, preserving the function of the larynx and minimizing the treatment-related complications and injuries.

Based on the results obtained from new studies, researchers believe that the thioredoxin (Trx) system which catalyzes oxidation-reduction reactions in mammals is effective in the cancerization of some tissues. The Trx together with thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH) forms the Trx system. Oxidized thioredoxin (Trx-S2) is reduced by NADPH and with the help of selenoenzyme thioredoxin reductase (STR). The electron produced by NADPH reduces an active disulfide (FAD) and in this way goes to N-terminal of reduced active disulfide in one of TrxR infrastructures and is finally transferred to the active site of terminal selenothiol that exists in the sequence Gly-Cys-Sec-Gly in C-terminal of all TrxR isoforms. Since electrons are transferred to Trx that is the location of the reduction of the disulfides of proteins or other substrates [5-7], reduced Trx catalyzes the reduction of the disulfide bond in many proteins. Additionally, this enzyme plays different roles including the involvement in cell growth, defense against oxidative stress, control of apoptosis and creating cancer in mammalian cells [8, 9].

The TrxR's RNA reductase which is required for DNA production. Further, this system has a major role in cell reduction signaling through controlling the activity of many translation factors such as NF-kB, p53, Ref-1, HIFα, PTEN, AP-1 and glucocorticoid receptors. Reduced Trx can bind to apoptosis signal-regulating kinase (ASK1) and make it inactive and in this way, it can regulate and control ASK1-dependent apoptosis. It has been found that the expression of Trx system proteins in many diseases such as cancer, diabetes, cardiovascular diseases, neurodegenerative disease, and rheumatoid arthritis undergoes a change [5, 9, 10].

According to the studies conducted, Trx system is effective in escape from apoptosis, unlimited proliferation, continuous angiogenesis, and tissue invasion. Also, it has a role in insensitivity to growth inhibitory signals and independence in growth signals [8]. The main function of Trx is to maintain the protein activity so that the activity of translation factors to bind to DNA is preserved, and the amount of enzyme activity is controlled [5, 7].

Many studies show that Trx has a paradoxical role in the prevention of the development of cancer and its protection. The Trx is not a mutation factor, but the Trx system is somewhat involved in antioxidant defense and probably in cancer prevention through eliminating carcinogenic antioxidants or repairing the oxidized proteins. In a similar way, repairing damages in mutant DNA by the repair system of nucleotide and ribonucleotide reductase-dependent on Trx system can prevent cancer [8, 11]. It soon became apparent that Trx can stimulate the proliferation and growth of cancer cells through intracellular reactions [8].

However, even if Trx is expressed in large amounts in numerous types of cancer cells, this connection does not mean that intracellular Trx is a direct cause of mitosis. Thus, concerning the current knowledge, it can be concluded that Trx system clearly protects cell growth, but there is no credible evidence indicating that thioredoxin can be considered as a real cancer-causing factor [8].

The relentless spread of knowledge about the paths relating the Trx system to cancer illu-

minates other amazing communication mechanisms. This knowledge ultimately leads to advances in treatment and prevention of cancer and this regard, thioredoxin reductase and selenium have a key role [2, 8]. Currently, many studies have focused on the structure and function of TrxR, particularly concerning the drugs that are used today in the treatment of inflammation and cancer [5]. Hence, study and identification of the impact of TrxR on the development of cancer seem a crucial matter. To this end, this study aimed to measure the TrxR activity and its tissue distribution in pathologic specimens of patients with laryngeal SCC.

Materials and Methods

Sampling

In this case- control study, 20 patients suffering from laryngeal SCC, who referred to Qaem and Imam Reza hospitals of Mashhad in 2014, were the candidates for laryngectomy operation, have taken informed consent form before attending the research. Patients with a history of any cancer treatment (as radiotherapy on the head and neck), a history of previous surgery on the larynx, alcoholic consumers, smoking, allergic person, and not verified tumor in the sample by pathological examination were excluded from the study. Case samples included two similar samples of the tumoral tissue (one for pathology tests and another for biochemical test) of each patient with laryngeal SCC. Control samples comprised two samples of the healthy tissue adjacent (verified by a specialist in operating room) to the tumor with a margin of at least 2 cm of tumoral tissue, which were separated from the patients along with the tumoral tissue during the surgery.

After the operation, a healthy sample and a tumoral sample from each patient were separately put into formalin and were sent to the pathology laboratory of Qaem Hospital of Mashhad to determine the tissue distribution of TrxR enzyme through immunohistochemical (IHC) staining and examine whether the sample is healthy or tumoral.

The another healthy and tumoral samples from each patient were washed with phosphate buffered saline (PBS).

The samples were quickly transferred to the biochemistry laboratory of Medical School of Mashhad University of Medical Sciences while being carried in special containers containing PBS together with ice container to perform the standardization process (homogenization) for measuring the activity level of TrxR enzyme.

Determine the Tissue Distribution of TrxR Enzyme

For determining the tissue distribution of TrxR enzyme, thioredoxin IHC staining kit: Trx (FL-105):sc-20146 (Santa Cruz Inc.- United States) was applied according to manufacturers' instruction. For the positive control sample, normal cervical mucus was used and for the negative control sample, the primary antibody was removed from testing stages.

Then, the stained slides were examined under a light microscope (Nikon-Japan) made in with 40 and 100 times magnification and were compared with positive and negative control samples. Afterwards, the results of the percentage of staining cells (SC%) were semi-quantitatively classified from 1 to 4 (1% to 25%= score 1, 26% to 50%= score 2, 51% to 75%= score 3, and 76% to 100%= score 4). Staining intensity was rated from 1 to 3 (weak [light brown] = score 1, average [oak brown] score 2 and intense [dark brown] score 3). The overall score of Trx was determined in the form of the total score of staining intensity and SC%.

Measurement of the Trx Enzyme Activity

Measuring of the Trx enzyme activity was done using Thioredoxin Reductase Colorimetric Assay Kit (Caymans Chemical Company-United States) (Item No.10007892). The sample was sliced into tiny pieces. Then, the tissue was homogenized in 5-10 mL of cold buffer (50 mM potassium phosphate and containing 1mM EDTA) for per gram of tissue. Next, this product was centrifuged at 10,000g for 15 min at 4°C. Afterward, the supernatant was used to analyze enzyme activity was used to analyze enzyme activity. This experiment is based on the reduction of 5, 5-dithio-bis (2-dinitrobenzoic acid) (DTNB) and converting it

to 5-thio-2- DTNB was followed at 412 nm using a spectrophotometer.

Statistical Analysis

The data was analyzed using the SPSS16 software. The normality of the distribution of quantitative variables was initially determined using Kolmogorov-Smirnov test. After specifying the normality, T and T-paired tests were applied to compare the enzyme activity in tumoral and healthy tissues. Moreover, Wilcoxon Signed Ranks Test was used to compare the staining percentage, staining intensity and their total in tumoral and healthy tissues, given that the scoring system was applied to them. The significant level was set at $P \leq 0.05$.

Results

In this study, the 12 men and 8 women within the age range of 43 to 87 years and with the mean age of 62.4±10.7 years participated in the study. The average the TrxR enzyme activity in the healthy and tumoral tissues was 0.004±0.003µM/min/ml and 0.006±0.003µM/min/ml, respectively (ranged 0.0009 to 0.0104 vs. 0.001 to 0.011). However, there was no relationship between the TrxR enzyme activity in the tumoral and healthy tissues (P=0.084).

The total score of IHC staining in the healthy tissue was 4.45±1.09 whereas the total of these scores in the tumoral tissue 6.25±0.63 (Table-1). The score of SC% in the tumoral tissue was significantly higher than the healthy tissue (P<0.001, Figure-1). Additionally, staining intensity in two tumoral and healthy tissues had a statistically significant relationship with each other (P =0.001).

Discussion

Cancer is a disease that has caused great concern in public health in many countries of the world. Various factors are effective in the development of this disease. Identification of these factors can be helpful in its prevention and treatment [12-14]. In recent years, one of the factors whose role in developing cancer has received the attention of many researchers is SRT enzyme and research has been carried out to identify the relationship between this enzyme and cancer development [15-17]. Since a similar study has not been so far conducted to determine the relationship between this enzyme and SCC, this research has carried out to measure the activity level and tissue distribution of TrxR in the laryngeal tumoral tissue.

Based on epidemiological studies, it has been revealed that given the increase in the population of women smokers over the past 60 years, the difference in the rate of the incidence of laryngeal cancer between men and women has currently reached from 15:1 into 4:1 [1]. In this study, this amount between men and women has been 3 to 2 [18, 19].

Based on IHC staining, the distribution of TrxR enzyme in the laryngeal SCC tissue is significantly higher compared to its adjacent healthy tissue. Besides, it was found in this study that despite the higher TrxR enzyme activity in the laryngeal SCC tissue more than the healthy tissue, there was no relationship between this disease and TrxR enzyme activity. This could be due to small sample size.

In the study conducted by Gallegos *et al*., tissue level and activity rate of Trx, TrxR, and their mRNA in colorectal cancer and its adjacent normal mucus, some hematological cancers and solid tumors were examined.

Their results revealed that mRNA tissue level of Trx in colorectal cancer compared to its adjacent healthy tissue has increased by 3 to 100 times and the TrxR activity in the tumoral tissue of colorectal cancer compared to its adjacent healthy tissue has increased, on aver-

Table1. The Percentage of Staining Cells (SC%) and Staining Intensity of Tumoral and Healthy Tissues Based on the IHC Assay. Data Was Presented in Mean±SD.

	Healthy tissue	Tumoral tissue
Staining intensity score	1.3±0.57	2.4±0.5
SC%	3.15±0.74	3.85±0.36
Total score	4.45±1.09	6.25±0.63

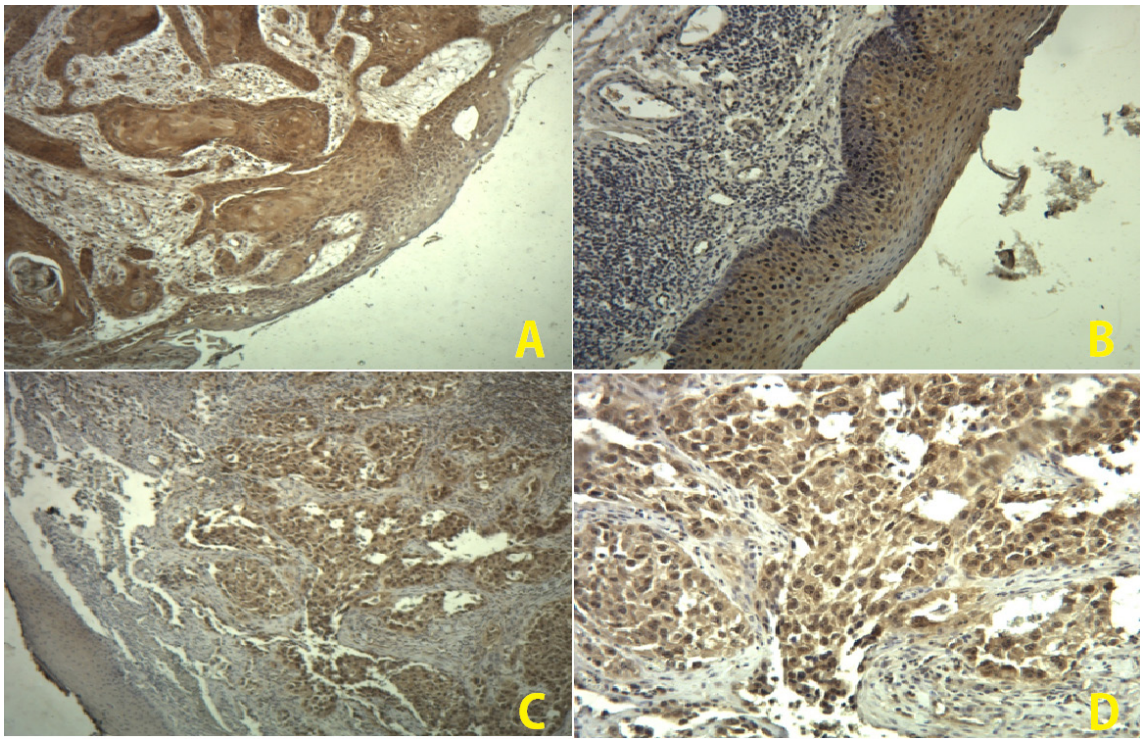

Figure 1. Immunohistochemical staining of Trx in laryngeal cells. (×40 magnification) **A:** Tumor cells with severe staining in 95% of the cells and weak staining in 30% of non-dysplastic mucous cells. **B:** Normal mucosal cells with moderate staining in 50% of the cells. **C:** Non- tumoral mucosal cells with weak staining in 10% of the cells and tumoral cells with severe staining in 100% of the cells. **D:** Tumoral cells with moderate staining in 95% of the cells.

age, by 2 times. Also, they demonstrated that Trx secretion by tumoral cells may lead to the stimulation of cancer cells growth [20].

According to the results of present study, it was revealed that the amount of thioredoxin reductase enzyme has a significant relationship with staging in SCC. In the study performed by Raffel J *et al*., that investigated the expression rate of Trx in normal colonic mucus, adenomatous polyps and primary and metastatic cancers of the colon, it was determined that the expression rate of Trx rises with an increase in Dukes staging. However, this relationship was not statistically significant. Additionally, it was observed in this study that a significant association exists between the expression rate of Trx and patient's survival rate. It was concluded in this research that an increase in Trx expression occurs in advanced stages of colorectal cancer, and the rate of Trx expression can be an independent marker of a patient's prognosis [21].

Also, in the study carried out by Kakolyris *et al*., it was concluded that there is a significant relationship between the lack of Trx expression and absence of lymph nodes in the involved area. Moreover, high level of proliferation is accompanied by a high rate of Trx expression [22].

In the studies conducted on prognosis in cancer treatments, it has been reported that the expression rate and activity of Trx can develop resistance against the effects of anti-cancer drugs. For instance, in a study, a relationship has been demonstrated between the high levels of Trx in the tumoral tissues of the bladder and prostate cancers with resistance to cisplatin, doxorubicin, mitomycin C, and etoposide [23]. Besides, in another research, the relationship between high levels of Trx in adult T-cell leukemia and resistance to Adriamycin has been mentioned [24]. In other studies conducted, a relationship has been demonstrated between high levels of Trx in the tumor-

al tissue of breast cancer and hepatocellular carcinoma with resistance to docetaxel and cisplatin [25, 26]. Creating drug resistance by Trx system can be related to the role of Trx in cell survival and its anti-apoptosis function or may be associated with the collection of reactive oxygen species (which are developed during chemotherapy or radiotherapy and cause apoptosis) by Trx [27, 28]. However, drug resistance created in any form raises this issue that the Trx system can be an attractive target for anti-cancer drugs since with the inhibition of thioredoxin, its anti-apoptotic activity is reduced and this makes a reduction in tumor growth and progression. As a result, resistance to other chemotherapy drugs decreases.

The Trx system is considered as an electron donor for ribonucleotide reductase, which is expressed in large amounts in cancer cells, and can cause an increase in malignant growth rate through genetic rearrangement, gene amplification, complete loss of growth control and resistance to treatment. Most cancer cells have high levels of TrxR and Trx expression. Nevertheless, some of the malignant cells have low or non-measurable levels of Trx and probably in these cells, glutaredoxin or possibly unknown electron donors have a role in ribonucleotide reduction and DNA construction. An important goal of future research could be to determine the nature of electron donor (Trx or glutaredoxin), based on which we can apply suitable inhibitors of TrxR or glutaredoxin system [5]. Thus, considering the limitations of this research, it is recommended that in future, studies with greater sample size be performed in the field of measuring the enzyme activity.

Conclusion

Finally, it was observed that the rate of expression and incidence and tissue distribution of TrxR enzyme in the tumoral tissue of laryngeal SCC compared to its adjacent healthy tissue is significantly higher. Furthermore, doing further research on potential effects of Trx system on tumor growth, invasion, the impact of environmental, pharmaceutical factors, and dietary supplements on it can open a wide window to us not only in the treatment of cancers but also in the treatment of many inflammatory and infectious diseases.

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Conflict of Interest

The authors declared no conflict of interest.

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