

## FXVD3 and TNF $\alpha$ mRNA Expression in Laryngeal Squamous Cell Carcinoma and their Correlation with Clinicopathologic Parameters

Somayeh Dadras\*, Mahboobeh Razmkhah\*, Bijan Khademi\*\*, Mehdi Ansari\*, Ahmad Hosseini\*, Abbas Ghaderi\*,\*\*\*\*

\*Shiraz Institute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, Iran

\*\*Department of Otorhinolaryngology, Shiraz University of Medical Sciences, Shiraz, Iran

\*\*\*Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

### Abstract

**Background:** Because of its effect on speech and swallowing, laryngeal squamous cell carcinoma is a devastating disease which has been shown to have a poor survival rate. Abundant research is being carried out in search of novel biomarkers that can aid the process of diagnosis and treatment of this disease. FXVD3, a modulator of Na/K-ATPase, is presented as a biomarker in some cancers. FXVD3 expression has been shown to be effected reversely by tumor necrosis factor alpha. Tumor necrosis factor alpha is a pro-inflammatory cytokine proposed to play an important role in tumor promotion and progression. In our study we examined FXVD3 and tumor necrosis factor alpha mRNA expressions, their correlation with each other and with clinicopathologic parameters in tumor tissues and lymph nodes.

**Methods:** We assessed 75 tissue samples and 30 lymph node samples of laryngeal squamous cell carcinoma patients and compared them to 9 adjacent normal tissue samples by quantitative real-time polymerase chain reaction.

**Results:** FXVD3 mRNA expression showed no significant difference among different tissues. We observed significantly lower tumor necrosis factor alpha mRNA expression in laryngeal tumor tissues compared to adjacent normal tissues. FXVD3 showed significant correlations with node metastasis (N factor), differentiation grade, and regional metastasis in lymph nodes. FXVD3 and tumor necrosis factor alpha mRNA levels significantly correlated in tumor and normal tissues.

**Conclusion:** FXVD3 might be involved in the dedifferentiation and metastasis process of laryngeal squamous cell carcinoma. This biomarker has contributed to the aggressiveness and progression of the tumor. Verification of the observed results will need evaluation in a larger group of patients.

**Keywords:** Laryngeal squamous cell carcinoma, FXVD3, TNF $\alpha$ , mRNA expression, qRT-PCR

♦Corresponding Author:  
Abbas Ghaderi, MD  
Shiraz Institute for Cancer  
Research, Shiraz University of  
Medical Sciences, Shiraz, Iran  
Tel: 0098-21-2303687  
Fax: 0098-21-2304952  
Email: ghaderia@sums.ac.ir

## Introduction

Head and neck cancer refers to a group of cancers that occur in the upper aerodigestive tract.<sup>1,2</sup> More than 90% of head and neck cancers are of squamous origin,<sup>3,4</sup> which involve the hypopharynx, larynx, oral cavity, oropharynx, nasopharynx, paranasal sinus, nasal cavity, parathyroid, and salivary glands as common anatomical sites.<sup>5</sup> Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer by global incidence.<sup>6</sup> This disease affects more than 500,000 people worldwide each year.<sup>5,7</sup> Comprising approximately 25% of HNSCC incidences, laryngeal squamous cell carcinoma (LSCC) is one of the most common types of head and neck cancers.<sup>3</sup>

Although in recent years molecular biology research has enabled researchers to gain a better insight into this disease, the occurrence and development of the mechanisms involved in the pathogenesis of this cancer are not fully understood.<sup>8,9</sup> A plethora of research has been carried out on a variety of genes to find the ones that have varied expressions in LSCC and determine those which have a possible role in this cancer. These investigations can reveal the underlying mechanisms of initiation, development, and pathogenesis of LSCC and lead to the identification of novel biomarkers. This, in turn, can provide a tool for screening, early detection, new therapeutic strategies, and improved patient outcome and survival.<sup>8,9</sup>

FXVD3 (mammary tumor protein 8kDa, Mat8) is a member of the FXVD family proteins that all share the FXVD motif<sup>10</sup> and are tissue-specific and physiological state-specific regulatory subunits of the Na/K-ATPase.<sup>11</sup> FXVD3 was initially cloned from murine mammary tumors induced by neu and ras oncogenes.<sup>12</sup> Different studies have shown that FXVD3 is overexpressed in some cancers such as breast cancer,<sup>13</sup> prostate cancer,<sup>14</sup> pancreatic ductal adenocarcinoma,<sup>15</sup> gliomas,<sup>16</sup> bladder and kidney urothelial carcinoma,<sup>17</sup> colorectal cancer,<sup>18</sup> and gastric adenocarcinoma,<sup>19</sup> while it is downregulated in lung cancer<sup>20</sup> and

androgen-independent prostate cancer.<sup>21</sup> Zhang et al. have suggested that FXVD3 could be a biomarker and a possible prognostic molecule for urothelial carcinoma.<sup>17</sup> Another study indicated that this gene could be a potential marker for prostate cancer.<sup>14</sup> A recent study showed that the expression of FXVD3 along with two other genes could be a significant predictor of outcome in breast cancer.<sup>22</sup> FXVD3 has been shown to be downregulated by tumor growth factor beta (TGF $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) in cancer cells and normal cells that undergo epithelial-mesenchymal transition (EMT) during induced EMT.<sup>15,23,24</sup> Epithelial-mesenchymal transition is a process that many epithelial tumors undergo during which the epithelial phenotype, which has strong cell-cell junctions and polarity, is replaced by a mesenchymal phenotype with reduced cell-cell interactions, fibroblastic morphology, and increased motility, resulting in facilitated invasion. Tumor growth factor beta has been implicated as a key inducer of the EMT process. Tumor necrosis factor alpha synergizes with TGF $\beta$  and dramatically accelerates EMT.<sup>25</sup>

No study, to our knowledge, has been carried out on FXVD3 and its relation with TNF $\alpha$  in squamous cell carcinoma of the larynx. Therefore our main objective in this study was to investigate the messenger ribonucleic acid (mRNA) expressions of FXVD3 and TNF $\alpha$  in LSCC and examine their correlation with each other. We also assessed the correlation of these two genes with a variety of clinical and pathological parameters.

## Materials and Methods

### *Tissue and lymph node samples*

We obtained all tumor and adjacent normal tissue biopsies from 75 patients diagnosed with LSCC, each of whom underwent a surgical procedure for tumor removal in Nemazee and Khalili hospitals, Shiraz, Iran. This study included all patients diagnosed with LSCC. Exclusion criteria were: patients that received chemotherapy or radiotherapy, those with any immunodeficiencies, and HIV positive patients.

Samples were gathered during a 4-year period from 2009-2012. All patients provided written informed consent for research purpose prior to obtaining the biopsies. Tissue specimens were immediately frozen after surgical removal and kept at -80°C until use. Clinical and pathological information for each of the patients was acquired from their medical records. Clinicopathological information, their subgroups, and the number of cases in each subgroup are shown in Table 1. The age of the patients (74 male and 1 female) ranged from 43 to 83 years with a median of 58 years. All patients had stages 2-4 cancers according to the primary tumor size, regional node metastasis, and distant metastasis [Tumor-Node-Metastasis (TNM)] classification developed by the International Union against Cancer.<sup>6</sup> No patients had stage 1 classification. Tissue samples were obtained from all the 75 patients whereas lymph nodes were obtained from 30 patients, and 9 individuals provided adjacent normal tissue specimens.

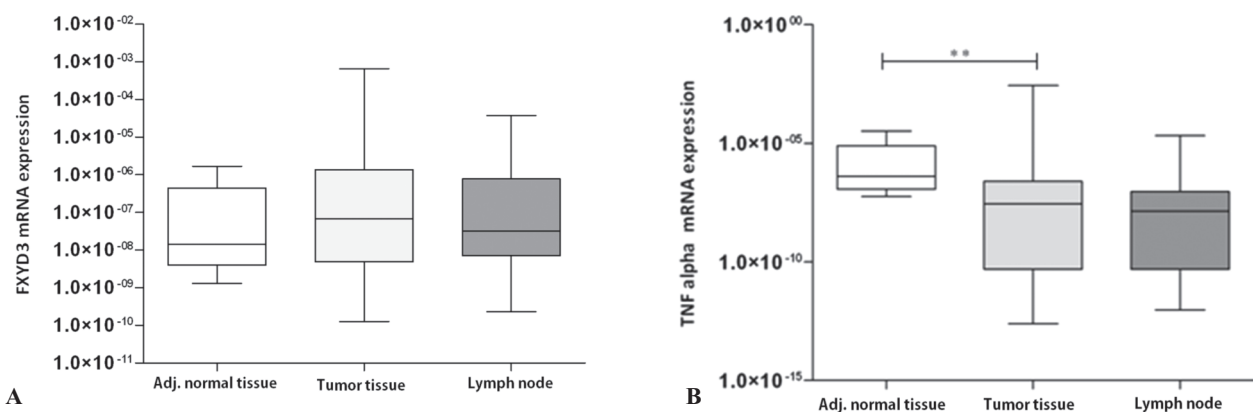
*RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)*

Total RNA was extracted from the specimens using the High Pure RNA Tissue Kit (Roche, Germany) according to the manufacturer's instructions. Reverse transcription was performed on 5  $\mu$ l of total RNA using random hexamers and oligo-dT in a 20  $\mu$ l reaction mix with the First Strand cDNA Synthesis Kit (Fermentas,

EU). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out in a final volume of 20  $\mu$ l in an ABI PRISM 7500 (Applied Biosystems, USA) using a TaqMan Master Mix Kit (Primer Design, ABI, USA) and the specific primers (Bioneer, Korea, Table 2) for FXYD3, TNF $\alpha$ , and 18srRNA as a housekeeping gene for normalization. The microtubes were incubated at 95°C for 10 min for initial denaturation, followed by 40 cycles that consisted of: 95°C for 15 sec, 57°C for 30 sec, and 60°C for 1 min. Real-time PCR efficiencies were calculated by drawing standard curves (from serial dilutions of the positive control) which were acceptable. The specificity of the real-time PCR was confirmed by agarose gel electrophoresis of the PCR products. We used ABI PRISM SDS 2.0 software (Applied Biosystems, USA) to analyze the curves. For relative quantification, the ratio between the expression amount of each gene and 18srRNA was determined.

*Statistical analysis*

We compared the mRNA expression levels of FXYD3 and TNF $\alpha$  in tumor tissues from the different clinicopathologic subgroups (Table 1) and adjacent normal tissues. Expression levels in lymph nodes were compared among subgroups of the clinicopathologic parameters (Table 1) in the lymph nodes and tumor tissues. All analyses were performed with SPSS 17 software (SPSS Inc., Chicago, IL, USA) by the Mann-Whitney U-test.



**Figure 1.** A comparison of relative gene expressions: A) FXYD3 and B) tumor necrosis factor alpha (TNF $\alpha$ ) among three groups of samples: laryngeal tumor tissues, adjacent normal tissues, and lymph nodes. Graphs are shown as vertical box and whiskers which the whiskers plot the minimum and maximum. Results were analyzed by the Mann-Whitney U-test. There was no significant difference observed between different sample groups. \*\*:  $P < 0.01$

The correlation between the two genes was also investigated using non-parametric Spearman's correlation coefficient. *P*-values less than 0.05 were considered statistically significant. Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA) was used for graph depiction.

## Results

### *FXVD3 and tumor necrosis factor alpha (TNF $\alpha$ ) mRNA expression in tumor-adjacent normal tissues, tumor tissues, and lymph nodes and their correlations*

FXVD3 and TNF $\alpha$  expressed in all assessed tissues. FXVD3 upregulated in tumor tissues compared to adjacent normal tissues. Lymph nodes showed a lower expression of FXVD3 than tumor tissues. There was no statistical significance with any of these variations (Figure 1A). We observed significantly lower TNF $\alpha$  mRNA expression in tumor tissues compared to adjacent normal tissues (*P*=0.002). Although TNF $\alpha$  expressed at a lower level in lymph nodes compared to tumor tissues, this difference did not reach a significant level (Figure 1B).

Tumor necrosis factor alpha and FXVD3 showed a strong positive correlation with each other in laryngeal tumor tissues (*P*=0.037) and adjacent normal tissues (*P*=0.007) but no correlation in lymph nodes.

### *FXVD3 clinicopathologic significance in tumor tissues and lymph nodes*

In tumor tissues, FXVD3 mRNA expression did not show any significant correlation with the

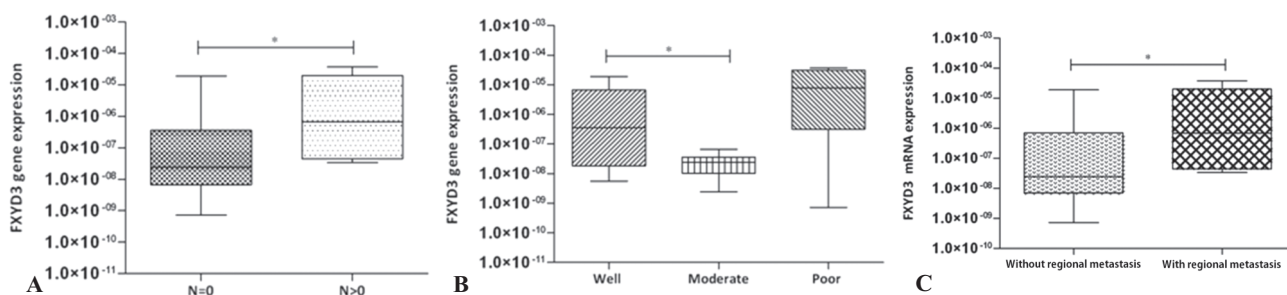
evaluated clinicopathologic parameters.

In the lymph node samples, we categorized the regional node (N) parameter into two groups: N=0 (absence of metastasis in lymph nodes) and N $\neq$ 0 or N=1, 2, and 3 (different degrees of lymph node metastasis). There was a significant difference between the two groups (*P*=0.028). FXVD3 showed an increase in lymph nodes with metastasis compared to those without metastasis (Figure 2A). Lymph node samples from the two subgroups of histological differentiation grade (well- and moderately differentiated) showed a significant difference in FXVD3 expression (*P*=0.03). Lymph nodes with well-differentiated tumor cells expressed higher levels of FXVD3 mRNA compared to those with moderately differentiated ones (Figure 2B). Lymph nodes with poorly differentiated tumor cells were not entered in the analysis because of the low number of cases in this group. Patients with regional metastasis had significantly higher FXVD3 mRNA expression in their lymph nodes compared to those without regional metastasis (*P*=0.028, Figure 2C).

There was no significant association observed between the other clinicopathologic parameters assessed in lymph nodes and FXVD3 mRNA expression levels.

### *Tumor necrosis factor alpha (TNF $\alpha$ ) clinicopathologic significance in tumor tissues and lymph nodes*

There was no significant correlation between TNF $\alpha$  mRNA expression and any of the clinical



**Figure 2.** A comparison of relative gene expression of FXVD3 in lymph nodes A) between subgroups with different degrees of regional node metastasis (N), B) among different differentiation grade subgroups, and C) between two groups of patients (with and without regional metastasis). Graphs are shown as the vertical box and whiskers which the whiskers plot the minimum and maximum. Results were analyzed by the Mann-Whitney U-test. \*: *P*<0.05.

and pathological features assessed in the tumor tissues and lymph nodes of the LSCC patients.

## Discussion

Tumor-Node-Metastasis staging is the main basis for predicting the outcome in patients with LSCC.<sup>9</sup> However, this parameter along with other clinicopathological characteristics such as tumor differentiation grade does not accurately indicate the prognosis of the patients. Thus, as a result, patients with the same stage often have different prognoses.<sup>8,9</sup> For this reason, various investigations are being carried out in search of molecular markers that could also be used as more reliable prognostic tools.

We showed that FXVD3 mRNA expressed in all tissues assessed including tumor-adjacent normal tissues, tumor tissues, and lymph nodes. These mRNA levels did not show any significant difference between the different tissues. This observation was consistent with another study that investigated FXVD3 in normal and cancerous tissues from the urinary bladder, lung, and prostate.<sup>15</sup> It has been shown that FXVD3 mRNA levels do not necessarily reflect the protein levels in different situations. In other words, despite stable mRNA levels, protein production or degradation may have alterations between different states which can cause a difference in protein levels,<sup>20</sup> therefore FXVD3 protein levels should also be measured in different tissues. Other studies have shown that FXVD3 mRNA<sup>15,20,24</sup> and protein<sup>20,24</sup> levels change in cancer compared to normal tissues, which indicates a role for FXVD3 in cancer either as a promoter in cancers that show upregulation of FXVD3<sup>13-19</sup> or a suppressor in cancers with FXVD3 downregulation.<sup>20</sup>

FXVD3 expression has been investigated in a variety of malignancies, among which some correlated with a number of clinical and pathological characteristics: sex,<sup>16</sup> tumor size,<sup>22</sup> multiple site tumors,<sup>16</sup> histological grade,<sup>17,20,22</sup> cancer subtype,<sup>18-20</sup> and tumor location.<sup>18</sup> Here, we observed different correlations of significant importance between FXVD3 and clinicopathologic features that included node metastasis,

differentiation grade, and regional metastasis in lymph node samples. Significantly elevated levels of FXVD3 mRNA in metastatic lymph nodes and also in patients with regional metastasis correlated with tumor aggressiveness and might implicate it in the metastasis process. In this regards FXVD3 might provide an advantage in gaining a mesenchymal phenotype necessary for invasion and metastasis. Significantly elevated FXVD3 mRNA levels existed in well-differentiated tumor cells and showed a decrease with histological dedifferentiation. This seemed consistent with the results of Okudela et al.<sup>20</sup> who studied lung cancer and reported that FXVD3 decreased parallel to histological dedifferentiation. They suggested that FXVD3 might be a potential tumor suppressor whose downregulation could play a role in cancer progression.<sup>20</sup> Our results indicated a direct role for FXVD3 in cellular differentiation to epithelial cells or an indirect role in the dedifferentiation process. Concordantly, previous studies demonstrated that FXVD3 was necessary for the differentiation of human colon cancer cell lines<sup>26</sup> and downregulation of FXVD3 in dedifferentiated breast and lung cancer cells.<sup>20,24</sup>

Tumor necrosis factor alpha is an inflammatory cytokine with an important role in establishing a link between inflammation and cancer.<sup>27</sup> The presence of this cytokine has been reported in the tumor microenvironment of various malignancies where its aberrant expression was implicated as a key factor in cancer development.<sup>28,29</sup> In our evaluations, TNF $\alpha$  mRNA expression existed in all tissues. Laryngeal tumor tissues had a significant decrease in their TNF $\alpha$  mRNA levels compared to normal tissue. It has been shown that low-dose chronic TNF $\alpha$  production is a feature of many cancers during which TNF $\alpha$  promotes cancer growth, invasion, and metastasis through different mechanisms.<sup>28</sup> Chronic low level TNF $\alpha$  exposure can promote tumor progression through angiogenic activities. In contrast, high doses are anti-angiogenic.<sup>30</sup> We could not be certain whether this reduced TNF $\alpha$  mRNA level in tumor tissues accurately indicated its protein level because it has been shown that

TNF $\alpha$  mRNAs can gain enhanced stability in the cancer state<sup>31</sup> which may compensate for the reduction in their amount. Therefore TNF $\alpha$  protein levels in tumor tissues must also be determined. Biberstein et al. showed that immunohistochemical analysis of TNF $\alpha$  did not differ in HNSCC tumor specimens and normal tissues.<sup>32</sup>

In this study, TNF $\alpha$  and FXYD3 correlated significantly with each other in normal and tumor tissues, but not in lymph nodes. FXYD3 mRNA expression has been shown to be affected reversely by TGF $\beta$  in neu induced breast cancers from transgenic mice<sup>23</sup> and different pancreatic cancer cell lines,<sup>15</sup> as well as by TGF $\beta$  and TNF $\alpha$  in Michigan cancer foundation-A10 (MCF-A10) cells (a normal human mammary epithelial cell line).<sup>24</sup> Yamamoto et al. have shown that TGF $\beta$  regulated FXYD3 mRNA expression by transcription factor zinc finger E-box-binding homeobox1/delta-crystallin enhancer-binding factor 1 (ZEB1/ $\delta$ EF1) which binds to specific sites in the FXYD3 promoter region, thus resulting in the downregulation of this gene.<sup>24</sup> The mentioned transcriptional repressor, ZEB1/ $\delta$ EF1, is also induced by chronic treatment of TNF $\alpha$  in MCF-10A cells.<sup>33</sup> The TGF $\beta$  signaling pathway has been shown to have a defect in some cancers and cancer cell lines which causes a loss in its repressive effect on FXYD3 and results in the subsequent over-expression of this gene.<sup>15,23</sup> Our study did not agree with these works because cancerous tissues of the larynx showed a positive correlation between the two genes while the other above mentioned studies reported that TGF $\beta$  and TNF $\alpha$  reversely affected FXYD3 and negatively correlated with this gene.

Overall, FXYD3 expression and its role in cancer development and progression seems to vary in different types of cancer. In LSCC, FXYD3 may be involved in the dedifferentiation and metastasis process of LSCC but verifying the observed results will need evaluation of a larger group of patients. Further studies are needed to determine the relation between the regulatory function of FXYD3 on Na/K-ATPase and its potential roles in carcinogenesis. Although in this

study we have not shown a correlation between clinicopathologic parameters to TNF $\alpha$  mRNA levels, a previous study reported a correlation between whole blood TNF $\alpha$  levels and some clinicopathologic parameters in laryngeal carcinoma.<sup>34</sup> Substantiating these outcomes would need further investigation.

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### Conflict of Interest

No conflict of interest is declared.

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