

Differentially expressed genes related to lymph node metastasis in advanced laryngeal squamous cell cancers

ÖMER BAYIR¹, MEHMET DOĞAN AŞIK², GÜLESER SAYLAM¹, FERDA ALPASLAN PINARLI³, EMEL ÇADALLI TATAR¹, ÜNSAL HAN⁴, ENDER ŞİMŞEK² and MEHMET HAKAN KORKMAZ^{1,5}

¹Department of Otolaryngology, Head and Neck Surgery, University of Health Sciences, Dışkapı Yıldırım Beyazıt Training and Research Hospital, Ankara 06110; ²Department of Medical Biology, School of Medicine, Ankara Yıldırım Beyazıt University, Ankara 06010; ³Myogen Laboratory and Health Services, Ankara 06520;

⁴Department of Pathology, University of Health Sciences, Dışkapı Yıldırım Beyazıt Training and Research Hospital, Ankara 06110; ⁵Department of Otolaryngology, Head and Neck Surgery, School of Medicine, Ankara Yıldırım Beyazıt University, Ankara 06010, Turkey

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Abstract. Understanding the molecular mechanisms and gene expression in laryngeal squamous cell carcinoma (LSCC) may explain its aggressive biological behavior and regional metastasis pathways. In the present study, patients with locally advanced LSCC tumors were examined for differential gene expression in the normal mucosa (non-tumoral mucosa), tumors and lymph node tissues. The aim was to identify possible predictive genes for lymph node metastasis. A total of 16 patients who had undergone total laryngectomy with neck dissection for advanced LSCC were randomly selected from a hospital database: Eight of the patients had lymph node metastasis (Group 1) and the other eight patients did not have metastasis (Group 2). Overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) were analyzed. For each patient, paraffin-embedded tissue samples were collected from non-tumoral mucosa, tumoral lesions and lymph node tissues. RNA was extracted from the tissue samples and used for complementary DNA synthesis, and microarray analysis

was subsequently performed on each sample. Gene expression levels were determined in each specimen, and Groups 1 and 2 were compared and statistically analyzed. The microarray results for lymph node metastasis-positive and -negative groups, indicated the differential expression of 312 genes in the lymph nodes, 691 genes in the normal mucosal tissue and 93 genes in the tumor tissue. Transgelin (TAGLN) and cofilin 1 (CFL1) were identified as possible target genes and validated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The RT-qPCR results for TAGLN and CFL1 supported the microarray data. OS, DFS and DSS times were longer in Group 2 than in Group 1 (P=0.002, 0.015 and 0.009, respectively). In addition, TAGLN and CFL1 were associated with DFS and DSS. On the basis of these results, it is suggested that TAGLN and CFL1 expression may play an important role in the pathogenesis of regional metastasis and poor prognosis in advanced LSCC.

Introduction

Head and neck cancers (HNCs) are the sixth most frequently occurring type of solid cancer. Most HNCs (90%) originate from mucosal tissues and are known as head and neck squamous cell carcinoma (HNSCC) or upper aerodigestive system cancer (1,2). The incidence and sub-anatomical distribution of HNSCC may vary according to the geographic location and ethnicity of the population. Its etiology includes exposure to various carcinogens, smoking, alcohol consumption, poor oral hygiene and viral infections (3,4). Several types of cancer are linked to genetic factors, and in addition to environmental factors, genetic predisposition may also play a role in HNSCC.

Oral cavity cancer is the most frequently diagnosed subgroup of HNSCC worldwide. According to the 2014 World Cancer Report, the numbers of new cases of HNSCC and associated mortality were 700,000 and 370,000, respectively (5). Among these, the numbers of laryngeal cancer cases were 157,000 and 83,000, respectively (5). Similarly, the mortality rate for the 60,000 patients newly diagnosed with HNSCC in

Correspondence to: Dr Ömer Bayır, Department of Otolaryngology, Head and Neck Surgery, University of Health Sciences, Dışkapı Yıldırım Beyazıt Training and Research Hospital, 20 Şehit Ömer Halisdemir, Dışkapı, Ankara 06110, Turkey
E-mail: bayiromer@hotmail.com

Abbreviations: CFL1, cofilin 1; cDNA, complementary DNA; Cq, quantification cycle; DFS, disease-free survival; DSS, disease specific survival; HNC, head and neck cancer; IVT, *in vitro* transcription; HNSCC, head and neck squamous cell carcinoma; LSCC, laryngeal squamous cell carcinoma; OS, overall survival; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TAGLN, transgelin

Key words: laryngeal squamous cell cancer, gene expression profile, microarray, tansgelin, cofilin-1

the USA in 2017 was 30%, and most of these cancers developed from the oropharynx and oral cavity (6). In Turkey, the most frequently diagnosed HNSCC is laryngeal cancer with an incidence of 7/100,000 between 2010-2014, and it is the eighth most common cancer in Turkish men (7).

Host and tumor factors may serve a role in the clinical behavior of laryngeal cancer, including the histological grade, localization, extension, tumor size and lymph node metastasis (8,9). Lymph node metastasis is an important risk factor for prognosis that is mostly associated with the localization and T stage of the tumor. Lymph node metastasis markedly reduces the survival rate; however, the curative treatment of laryngeal cancer with lymph node metastasis is possible (10-12). The only parameter available to guide the selection of treatment modality is the Tumor-Lymph Node-Distant Metastasis (TNM) stage. Notably, no histological or biological parameters are currently used when making treatment decisions. Certain genetic factors may also play a role in the pathogenesis and prognosis of LSCC (13). Despite increased genetic and molecular knowledge, no clinical application that defines the treatment and prognosis of laryngeal cancer has yet been established (13).

Improved understanding of the molecular mechanisms underlying LSCC lymph node metastasis and the identification of potential molecular targets would be favorable. Interpreting the associations between the differentially expressed genes in advanced stages may facilitate the search for predictive markers that could help in the determination of potential treatment routes. The present study was designed to detect possible genetic alterations in a homogeneous group of patients with locoregionally advanced LSCC who underwent total laryngectomy and neck dissection. Patients with and without lymph node metastasis were selected to examine the differential gene expression in the normal mucosa (non-tumoral mucosa), tumor and lymph node tissues. The main purpose of the study was to identify the commonly expressed genes in this homogenous group of Turkish patients with locoregionally advanced laryngeal cancer. A further aim was to determine the predictive role of these genes in lymph node metastasis and overall prognosis.

Materials and methods

Ethics. The present study was performed after obtaining approval from the Local Ethics Committee of the Dışkapı Yıldırım Beyazıt Training and Research Hospital, University of Health Sciences (18/42/14; Ankara, Turkey).

Tissue samples and patients. A total of 16 patients who had undergone total laryngectomy and neck dissection for locoregionally advanced LSCC at the Dışkapı Yıldırım Beyazıt Training and Research Hospital (Ankara, Turkey) between January 2013 and January 2016 were randomly chosen from the hospital's database. Their medical records, follow-up data and formalin-fixed paraffin-embedded tissue samples of the normal mucosa, tumors and lymph nodes were obtained. Patients were excluded from the study if they had a history of cancer, the presence of tumor-positive surgical margins or any other connective tissue diseases. Eight patients with histologically positive neck lymph nodes were assigned to Group 1, and eight patients with negative lymph nodes were assigned to

Group 2. All the specimens were re-examined by experienced head and neck pathologists, and the patients were classified with stage 3 or 4 cancer according to the TNM Classification of Malignant Tumors, 7th edition (14).

Gene array experiments were conducted on three different tissue specimens from each patient, namely tumor tissue, lymph nodes and normal mucosal tissue surrounding the tumor. The mucosal tissue samples were collected ≥ 1 cm from the tumor margins. This is consistent with previous studies in which mucosal specimens morphologically free of carcinoma *in situ* or dysplasia were evaluated as normal mucosal tissue (15,16). All metastasis-negative lymph nodes were evaluated for micrometastases using serial sections. Moreover, 2-mm tissue specimens were collected from the centers of the tumor and lymph nodes, with and without metastasis, from the paraffin blocks.

Nucleic acid isolation and microarray analysis. Tissues from the paraffin blocks were treated with a PureLink™ FFPE RNA Isolation Kit (Thermo Fisher Scientific, Inc.) to isolate RNA. Using this kit, following deparaffinization, the samples were treated with proteinase K and centrifuged in spin columns to remove cell debris according to the manufacturer's protocol. Isolated total RNA was eluted from the spin columns and stored at 4°C.

The Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) was used to obtain complementary DNA (cDNA) from total RNA for further analysis. The prepared solution including the cDNA, Oligo(dT), hexamer, RNase inhibitor, dNTPs and reverse transcriptase was incubated for 1 h at 16°C and 10 min at 65°C. After the incubation with double-stranded cDNA, amplified RNA (aRNA) was synthesized by the *in vitro* transcription (IVT) method using a GeneChip® 3'-IVT Express Kit (cat. no. 901229; Affymetrix; Thermo Fisher Scientific, Inc.). A solution was prepared comprising IVT biotin label, buffer, enzyme mixture and double-stranded cDNA, and the IVT reaction was performed at 40°C for 16 h. The labeled aRNA was subsequently purified using aRNA-binding magnetic microbeads in a binding buffer, and after washing, the RNA was eluted with elution buffer. After elution, the labeled aRNAs were incubated with Mg²⁺ ions for aRNA fragmentation. The aRNA fragments were hybridized with a GeneChip PrimeView Gene Expression Array (cat. no. 901838; Affymetrix; Thermo Fisher Scientific, Inc.) for 16 h at 45°C, and streptavidin phycoerythrin dye was used to stain the array. After staining, the array was scanned with the Affymetrix GeneChip Scanner 3000 (Thermo Fisher Scientific, Inc.) to obtain raw data. The raw data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE201777.

Statistical analysis. Statistical analyses were performed using IBM SPSS for Windows version 22.0 (IBM Corp.). Numerical variables are expressed as the mean \pm standard deviation. Categorical variables are presented as numbers and percentages. Overall survival (OS), disease-free survival (DFS) and disease-specific survival (DSS) probabilities were estimated using the Kaplan-Meier product limit estimator. The differences between independent groups, according to the survival curves, were compared using the log-rank test. P<0.05 was

Table I. Patient characteristics.

| Case | Sex | Age, years | Alcohol/smoking | Site | pT | pN | Pathologic stage | NED, LRR, DM | Mortality | Cause of death |
|---------|-----|------------|-----------------|--------------|----|----|------------------|--------------|-----------|----------------|
| Group 1 | | | | | | | | | | |
| 1-1 | M | 55 | N/P | Transglottic | 4 | 2c | 4 | LRR | Dead | LRR |
| 1-2 | M | 58 | N/P | Supraglottic | 4 | 1 | 4 | DM | Dead | DM |
| 1-3 | M | 49 | N/P | Transglottic | 4 | 3 | 4 | NED | Dead | OR |
| 1-4 | M | 49 | N/P | Transglottic | 4 | 2c | 4 | LRR+DM | Dead | LRR+DM |
| 1-5 | M | 51 | N/P | Transglottic | 4 | 2c | 4 | LRR+DM | Dead | LRR+DM |
| 1-6 | M | 54 | N/P | Transglottic | 4 | 1 | 4 | LRR+DM | Dead | LRR+DM |
| 1-7 | M | 57 | N/P | Transglottic | 3 | 1 | 4 | NED | Dead | OR |
| 1-8 | M | 44 | N/P | Transglottic | 4 | 2c | 4 | LRR | Dead | LRR |
| Group 2 | | | | | | | | | | |
| 2-1 | M | 72 | N/P | Transglottic | 4 | 0 | 4 | NED | Alive | - |
| 2-2 | M | 52 | P/P | Supraglottic | 4 | 0 | 4 | LRR | Dead | LRR |
| 2-3 | M | 56 | N/P | Transglottic | 4 | 0 | 4 | NED | Alive | - |
| 2-4 | M | 57 | N/P | Transglottic | 3 | 0 | 3 | NED | Dead | OR |
| 2-5 | M | 63 | N/P | Transglottic | 4 | 0 | 4 | NED | Alive | - |
| 2-6 | M | 55 | P/P | Transglottic | 4 | 0 | 4 | NED | Alive | - |
| 2-7 | M | 51 | N/P | Transglottic | 4 | 0 | 4 | NED | Alive | - |
| 2-8 | M | 62 | P/P | Transglottic | 3 | 0 | 3 | NED | Dead | OR |

M, male; N, negative; P, positive; pT, pathological tumor stage; pN, pathological lymph node metastasis stage; NED, no evidence of disease; LRR, locoregional recurrence; DM, distant metastasis; OR, other reasons.

considered to indicate a statistically significant result. After the GeneChips were scanned with the Affymetrix scanner, the raw data were analyzed using Transcriptome Analysis Console 4.0 software (Affymetrix; Thermo Fisher Scientific, Inc.). During these analyses, the Robust Multi-chip Analysis algorithm was used to make background adjustments and perform quantile normalization. Summarization indicated that all samples passed quality control checks. No additional filtering was applied to the data. After analysis, the differentially expressed genes between groups that had a fold change of >2 and $P < 0.05$ were considered statistically significant.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) validation. Gene functions were investigated and gene expression levels were compared between metastasis-negative and -positive samples. Two genes were found to be significantly associated with metastasis. For RT-qPCR, RNAs were isolated with RiboEx™ (cat. no. 301-001; GeneAll®) and Hybrid-R (catalog no: 305-101; GeneAll) kits. Later, cDNA was synthesized from the RNA using a WizScript™ cDNA Synthesis Kit (cat. no. W2211; Wizbio). The reverse transcription reaction was performed in 3 steps. In the first step, samples were incubated for 10 min at 25°C, followed by incubation for 120 min at 37°C in the second step. In the third step, samples were incubated at 85°C for 5 min. The following primers were used: Transgelin forward, 5'-GGGGTTAGAGAATAGTGA AGTAGGAGTA-3' and reverse, 5'-ACACTCACAAAACCTTCTCAAAACT-3' (17); cofilin1 forward, 5'-GGTGCTCTTCTGCCTGAGTG-3' and reverse, 5'-TCTTGACAAAGGTGGCGTAG-3'; and β -actin

forward, 5'-CATCCTCACCTGAAGTACC-3' and reverse, 5'-TGAAGGTCTCAAACATGATCTG-3'. These primers were purchased from Oligomer Biotechnology and used with a WizPure™ qPCR Master (SYBR) kit (cat. no: W1711; Wizbio). The qPCR analysis was performed with initial denaturation at 95°C for 300 sec, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60 sec. By subtracting the housekeeping gene (actin) quantification cycle (Cq) values from the Cq values obtained for each sample, the relative expression was calculated as ΔCq . For the survival analysis, ΔCq values >-2 were accepted as low levels of expression and any ΔCq values <-2 were accepted as high expression values. The gene expression levels were identified by comparing the ΔCq of metastasis-negative and -positive samples, and calculated as $2^{-\Delta\Delta Cq}$ or fold-change values (18).

Results

Patient characteristics and survival analysis. The mean age of the 16 patients was 56.2 ± 5.9 years (range, 44-72 years). The patients were all men and were followed up for a mean period of 47.8 ± 25.2 months. In Group 1, one patient had a supraglottic tumor, seven patients had transglottic tumors, and all patients had stage 4 tumors. All patients in Group 1 died; six patients died due to locoregional recurrence (LRR) and/or distant metastasis and two patients died due to other reasons. In Group 2, one patient had supraglottic tumor and seven had transglottic tumors; two patients had stage 3 tumors, and the others had stage 4 tumors. Five patients in Group 2 were alive, one died due to LRR and two died due to other reasons. The

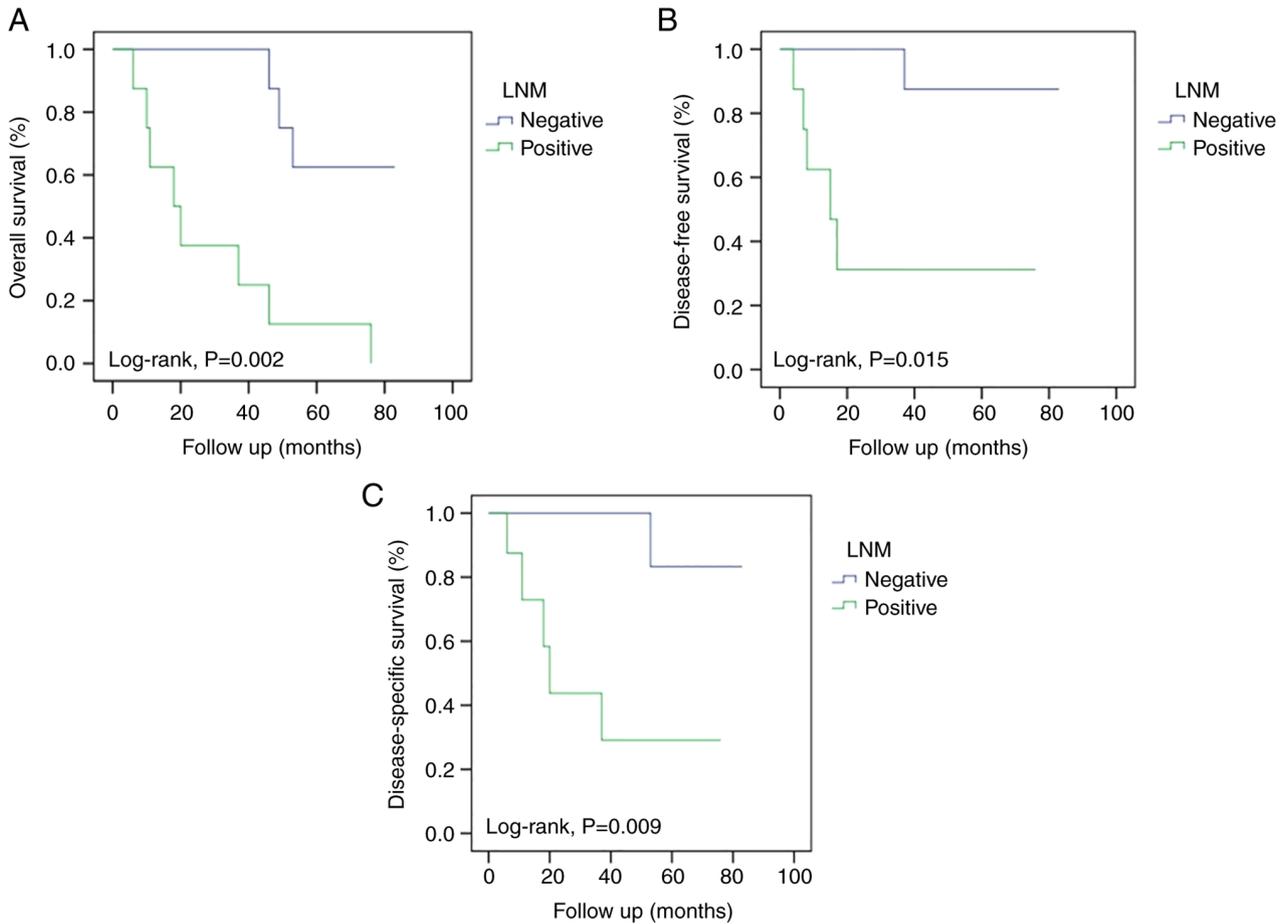


Figure 1. Kaplan-Meier survival curves of the patients. (A) Overall survival, (B) disease-free survival and (C) disease-specific curves. Lower (green) curves represent patients with LNM (Group 1) and upper (blue) curves represent patients without LNM (Group 2). LNM, lymph node metastasis.

patient characteristics summarized in Table I. The 5-year OS rates were 12.5 and 62.5% in Groups 1 and 2, respectively ($P=0.002$). The DFS rates were 31.3% in Group 1 and 87.5% in Group 2 ($P=0.015$). The DSS rates were 29.2% in Group 1 and 83.3% in Group 2 ($P=0.009$). The survival rates were higher in Group 2 than in Group 1, as shown by the survival curves in Fig. 1.

Microarray and qPCR analyses. The average expression values of various RNAs in the three types of tissue were compared between metastasis-positive and -negative patients. The comparisons indicated that 68 genes were differentially expressed in these tissues. Among these, 18 were ribosomal proteins and 15 were associated with mitochondrial pathways. A third group of genes were classified based on protein synthesis and proliferation. Other genes that were identified but not included in these groups included transgelin (TAGLN) and cofilin 1 (CFL1) (Table SI; Fig. 2).

The lymph node, mucosal and tumor tissues yielded similar results when compared between metastasis-negative and -positive patients. When lymph node tissues were investigated in the lymph node metastasis-positive and -negative groups, 312 genes showed a ≥ 2 -fold expression difference (Table SII). A volcano plot of this analysis indicated that all the differentially expressed genes were downregulated in metastasis-negative tissues. In addition, a hierarchical analysis indicated similar

signal distributions between the groups (Fig. 3). When the normal mucosal tissues were investigated, 691 genes showed a ≥ 2 -fold difference in expression (Table SIII). The volcano plot of this analysis indicated that 24 of the genes were upregulated in the metastasis-negative tissues. Moreover, the hierarchical analysis indicated similar signal distributions between the groups (Fig. 4). When the tumor tissues were investigated, 93 genes showed a ≥ 2 -fold difference in expression (Table SIV). The volcano plot of this analysis indicated that 11 of the genes were upregulated in the metastasis-negative group. Hierarchical analysis indicated similar signal distributions between the groups (Fig. 5).

The analysis of average expression in the three tissues revealed the upregulation of TAGLN and CFL1 in Group 1, and these genes were then selected for RT-qPCR validation (Fig. 6). The microarray data for the mucosa and lymph node tissues revealed the differential expression of CFL1 and TAGLN according to lymph node metastasis status. By contrast, the microarray data for the tumor tissue did not show a significant difference in the expression of these genes. The PCR $\Delta\Delta Cq$ values of TAGLN also indicated significant differences between the mucosa and lymph nodes, and the $\Delta\Delta Cq$ values of CFL1 in the mucosa and tumor tissue validated the microarray data.

The microarray analysis of the average of the three tissue types indicates a 3.29-fold difference between the lymph node metastasis-negative and -positive groups for TAGLN

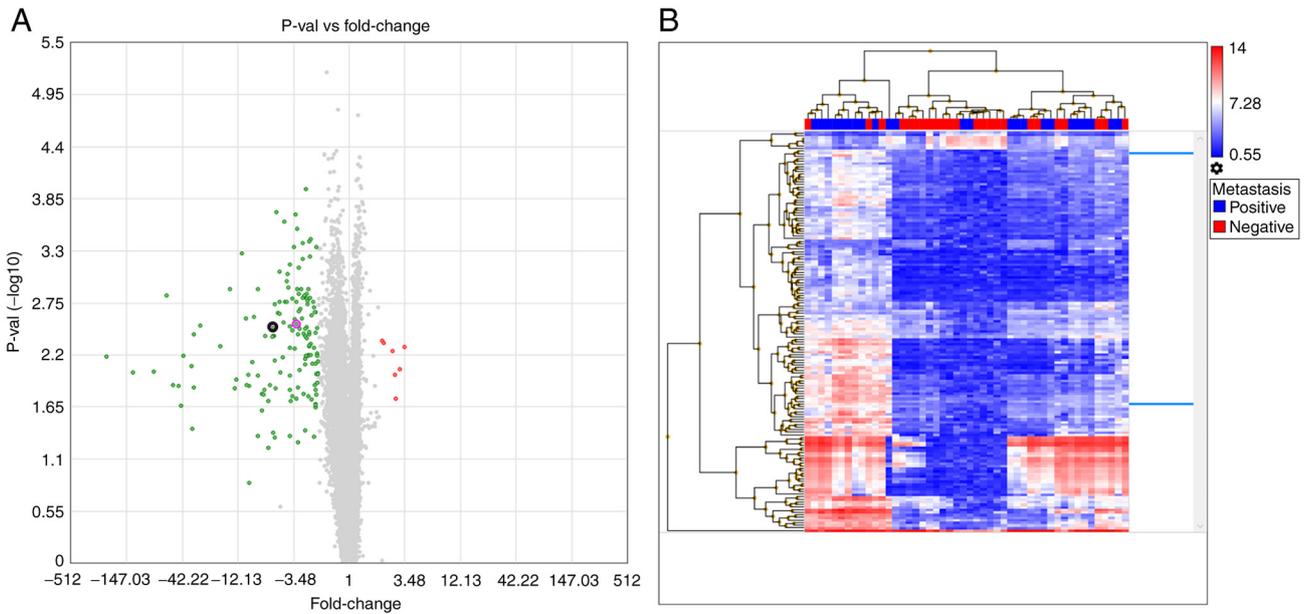


Figure 2. Differential expression analysis between metastasis-negative and -positive patients in tumor, lymph and normal mucosal tissues. (A) Volcano plot and (B) hierarchical gene analysis. In the volcano plot, the red circles indicate cofilin 1 and transgelin genes. Fold change values are for the metastasis-negative group relative to the metastasis-positive group. Transgelin is indicated with purple circle and cofilin 1 indicated with black circle.

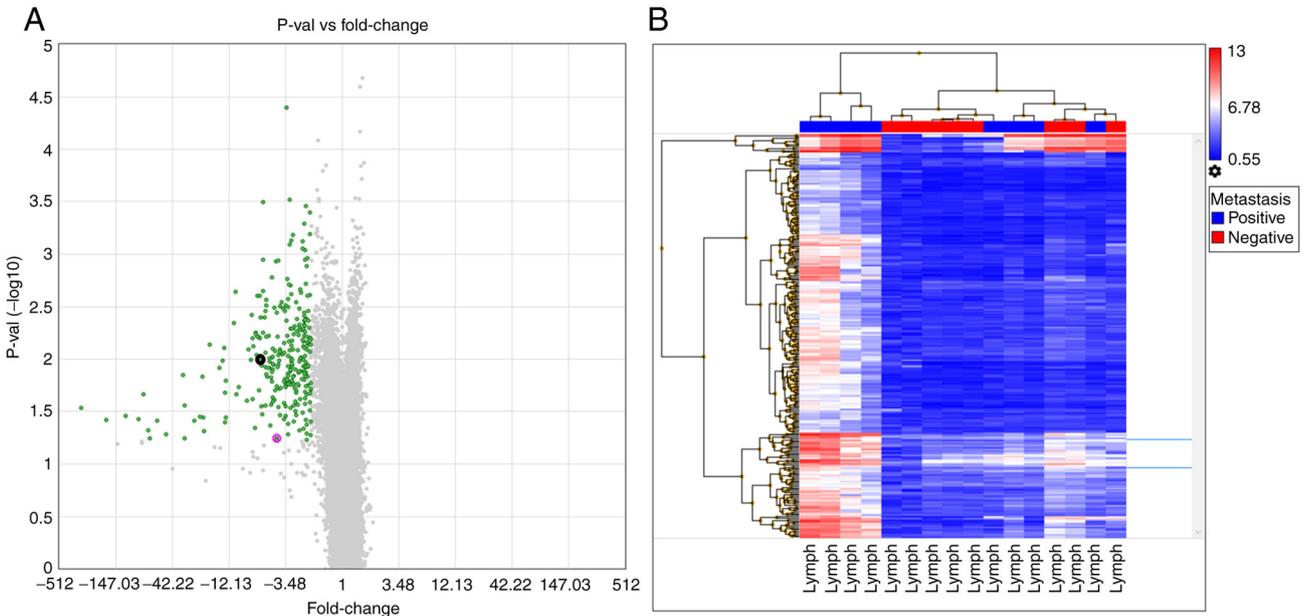


Figure 3. Differential expression analysis between metastasis-negative and -positive patients in lymph tissues. (A) Volcano plot and (B) hierarchical gene analysis. In the volcano plot, the black circles indicate cofilin 1 and purple circle indicate transgelin genes. Fold change values are for the metastasis-negative group relative to the metastasis-positive group.

(Fig. 2A). When examined individually (the most abundant probe was chosen to demonstrate differences if there was more than one probe targeted for a gene), the mucosal tissue showed an 11.06-fold increase in TAGLN expression in Group 1 compared with Group 2 (Fig. 4A) and the lymph tissue showed a 4.23-fold increase (Fig. 3A); however, no significant difference in TAGLN was observed in the tumor tissues (Fig. 5A). The PCR data validated these microarray results, with fold differences in the mucosa, lymph node and tumor tissues of 22.87-, 154.64- and 2.17-fold, respectively. By contrast, the

CFL1 microarray analysis revealed a 5.53-fold increase in Group 1 compared with Group 2 for the average in the three tissues (Fig. 2A), a 7.91-fold change in the mucosa (Fig. 4A) and a 6.06-fold change in lymph node (Fig. 3A) tissues. Additionally, no significant difference in CFL1 between the groups was observed in the tumor tissues (Fig. 5A). The PCR data for CFL1 validated the results for mucosal tissues with a 2.03-fold change.

OS, DFS and DSS analyses indicated that survival between the lymph node metastasis-positive and -negative groups

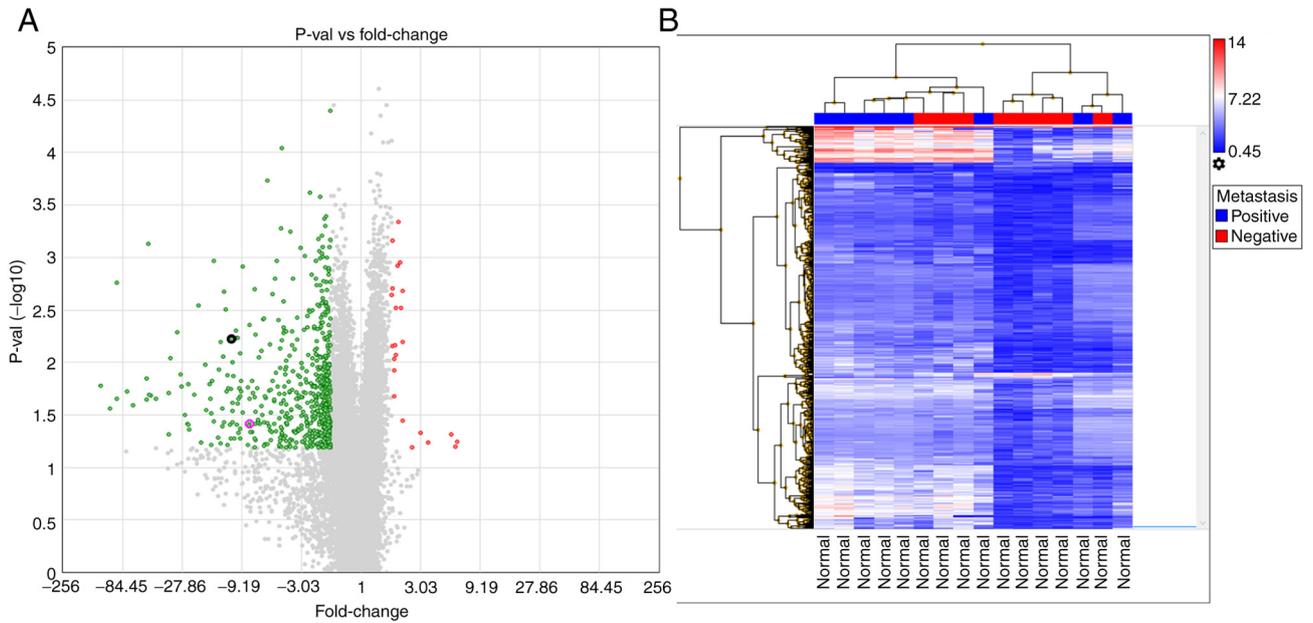


Figure 4. Differential expression analysis between metastasis-negative and -positive patients in normal mucosal tissues. (A) Volcano plot and (B) hierarchical gene analysis. In the volcano plot, the black circles indicate cofilin 1 and purple circle indicate transgelin genes. Fold change values are for the metastasis-negative group relative to the metastasis-positive group ('Variable charge, X-linked 2' has two identical point for two different probes. Please see Table SIII).

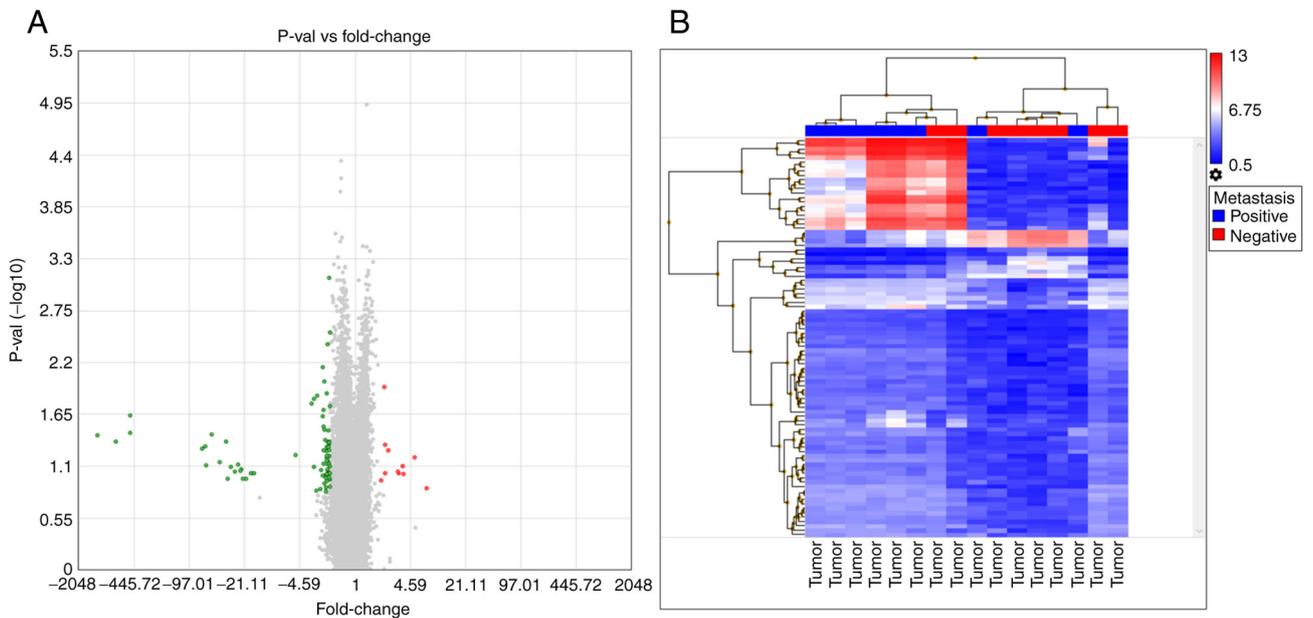


Figure 5. Differential expression analysis between metastasis-negative and -positive patients in tumor tissues. (A) Volcano plot and (B) hierarchical gene analysis. Fold change values are for the metastasis-negative group relative to the metastasis-positive group.

was differed significantly. In addition, survival comparison between patients with high- and low-level TAGLN gene expression indicated an association between high TAGLN expression and decreased OS, DFS and DSS in all tissues. However, a significant association was only observed with high TAGLN expression in the lymph nodes. By contrast, higher CFL1 expression levels in tumor and mucosal tissues were associated with increased survival. However, statistically significant differences based on the expression of CFL1 were only observed for DFS and DSS in the tumor tissues (Table II).

Discussion

In several types of cancer, it has been shown that there are various genetic markers that may predict regional metastasis. However, only a few studies have reported these differences in LSCC. In a case-control study, it was suggested that the presence of 'risk alleles' of the nucleotide excision repair genes ERCC excision repair 1 (ERCC1), ERCC5, ERCC6 and RAD23 homolog B could significantly increase the risk of laryngeal cancer in patients with a history of smoking and alcohol intake (19). The expression of Notch pathway proteins

Table II. Survival analysis according to lymph node status and gene expression.

| Variable | OS | | DFS | | DSS | |
|-----------------------------|-----------------------------|---------|-----------------------------|---------|-----------------------------|---------|
| | Mean survival time (95% CI) | P-value | Mean survival time (95% CI) | P-value | Mean survival time (95% CI) | P-value |
| Lymph node status | | | | | | |
| Negative | 71.1 (60.4-81.8) | 0.002 | 77.3 (66.7-87.8) | 0.015 | 78.3 (70.0-86.7) | 0.009 |
| Positive | 30.8 (14.7-46.8) | | 31.1 (8.4-53.8) | | 38.7 (19.0-58.4) | |
| TAGLN tumor tissue | | | | | | |
| High | 48.5 (32.6-64.4) | 0.879 | 53.5 (33.7-73.3) | 0.605 | 58.0 (40.8-75.0) | 0.561 |
| Low | 56.8 (29.1-84.4) | | 58.8 (29.5-88.0) | | 63.0 (39.2-86.8) | |
| TAGLN mucosal tissue | | | | | | |
| High | 43.8 (28.4-59.1) | 0.178 | 47.0 (26.6-67.3) | 0.093 | 52.0 (34.4-69.5) | 0.087 |
| Low | 70.3 (57.1-83.4) | | 79.0 (79.0-79.0) | | 79.0 (79.0-79.0) | |
| TAGLN lymph node | | | | | | |
| High | 33.7 (19.1-48.2) | 0.018 | 32.4 (11.8-52.9) | 0.005 | 39.6 (22.3-56.9) | 0.005 |
| Low | 71.3 (60.6-82.0) | | 83.0 (83.0-83.0) | | 83.0 (83.0-83.0) | |
| CFL1 tumor tissue | | | | | | |
| High | 56.0 (38.0-74.0) | 0.270 | 74.7 (59.3-90.1) | 0.021 | 75.0 (60.2-89.8) | 0.039 |
| Low | 43.6 (23.2-64.0) | | 33.1 (11.8-54.5) | | 43.6 (24.9-62.2) | |
| CFL1 mucosal tissue | | | | | | |
| High | 59.4 (43.6-75.3) | 0.412 | 63.9 (41.4-86.3) | 0.397 | 69.0 (52.5-85.5) | 0.411 |
| Low | 43.3 (24.6-62.1) | | 49.2 (27.1-71.3) | | 52.0 (31.7-72.4) | |
| CFL1 lymph node | | | | | | |
| High | 49.3 (24.4-74.1) | 0.622 | 52.0 (24.8-79.2) | 0.644 | 53.5 (27.2-79.8) | 0.643 |
| Low | 49.0 (34.3-63.7) | | 53.8 (34.2-73.3) | | 58.7 (42.5-75.0) | |

High, $\Delta Cq < -2$; low, $\Delta Cq \geq -2$; OS, overall survival; DFS, disease-free survival; DSS, disease-specific survival; CI, confidence interval; TAGLN, transgelin; CFL1, cofilin1.

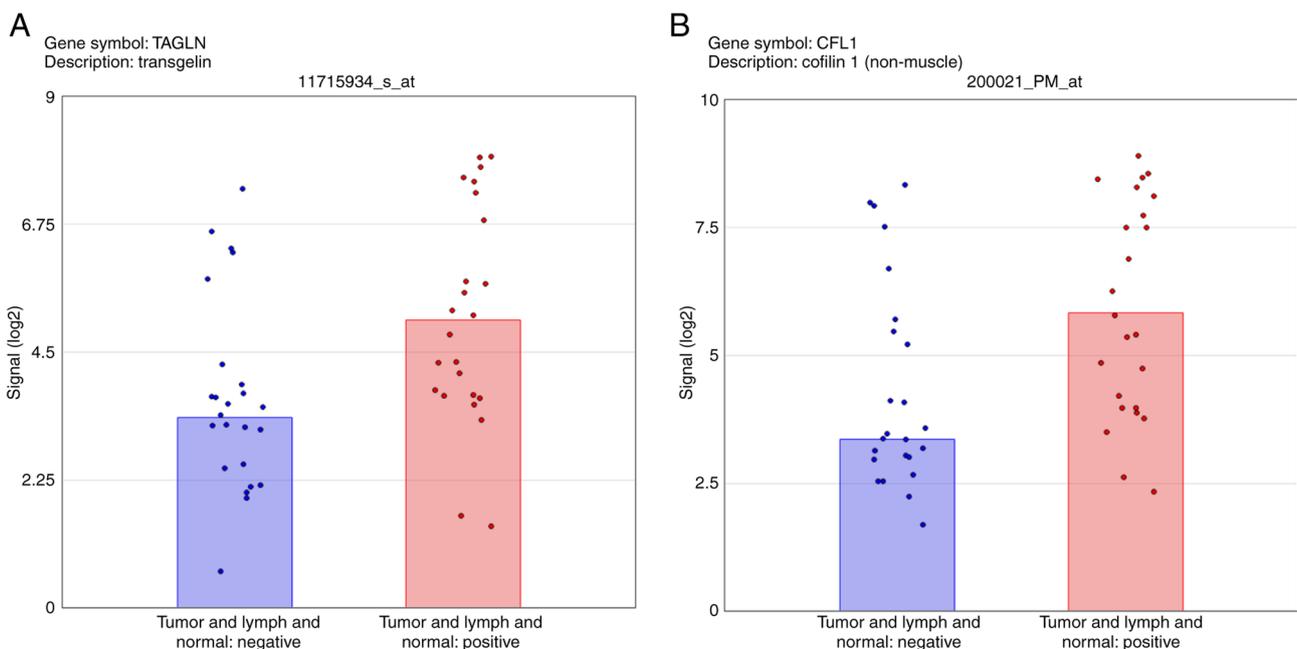


Figure 6. Differentially expressed genes in the tumor, lymph and normal mucosal tissues of patients with metastasis-negative and -positive laryngeal squamous cell carcinoma. (A) Cofilin 1 and (B) transgelin RNA expression levels.

has been shown to play a similar role in laryngeal cancer prognosis (20). The overexpression of EGFR, which is well known to occur in HNSCC, may play a role in poor prognosis and resistance to treatment in LSCC (21,22). It has also been reported that lower expression levels of membrane-associated protein 17 (MAP17) in LSCC are associated with poorer OS and laryngoesophageal dysfunction-free survival rates (23), while conversely, the upregulation of MAP17 and H2AX phosphorylation are associated with improved survival rates (24). Another study suggested that the increased interaction between endoplasmic reticulum protein 57 and STAT3 may contribute to radioresistance in LSCC (25). Yang *et al* (26) concluded that the overexpression of diaphanous related formin 1 (DIAPH1) regulates apoptosis via the ataxia telangiectasia and Rad3-related/p53/caspase-3 signaling pathway in LSCC. The authors suggested that DIAPH1 acts as an oncogene and is a potential therapeutic target. Li *et al* (27) suggested that the low expression of cell adhesion molecule 1 and contactin associated protein 2 genes and high expression of folate receptor γ and kynureninase genes may be indicators of chemosensitivity in LSCC. Another study indicated that the downregulation of zinc finger E-box-binding homeobox 2 protein, which is associated with proliferation, migration, invasion, cell cycle progression, apoptosis and epithelial-mesenchymal transition (EMT), could play a promising role in LSCC treatment (28). In addition, the DNA methylation of CpG islands, HOX transcript antisense RNA, CLKF-like MARVEL transmembrane domain containing 3, MYC target 1, zinc finger protein 667 (ZNF667)-antisense RNA 1 and ZNF667, has been revealed to cause epigenetic alterations in LSCC (29-32). Zhang *et al* observed that the downregulation of dachshund family transcription factor was associated with advanced clinical stage in patients with LSCC (33). In another study, the upregulation of CDR1 antisense RNA was shown to be related to tumor progression (34). Although several studies have focused on the genomics and proteomics of LSCC, there are no effective predictive molecular targets or markers for LSCC (13,35-39). Moreover, it is not possible to predict lymph node metastasis using the currently available genetic information in LSCC (40).

The literature suggests several potential biomarkers (19-34). CFL1 is an important protein that contributes to cell migration processes and serves a crucial role in actin filament dynamics as well as in oxidant-induced apoptosis (41). The results of the present study suggest that CFL1 may serve as a biomarker for LSCC. Lu *et al* (42) reported that CFL1 plays an important role in the development of prostate cancer and lymph node metastasis, while Polachini *et al* (43) affirmed that CFL1 modulates cell invasion in oral cavity squamous cell cancers. In addition, Madak-Erdogan *et al* (44) found that upregulated CFL1 expression was associated with tumor aggressiveness and poor prognosis in patients with estrogen receptor α -negative breast cancer. Furthermore, Zhang *et al* presented results indicating that TAGLN and CFL1 genes are involved in the development of esophageal squamous cell cancer, leading to the suggestion that CFL1 can be used as a biomarker in the early diagnosis of this type of cancer (45). Although the microarray differential expression analyses and gene expression-dependent DFS and DSS results for CFL1 in the present study showed similar significance to those in previous studies, the PCR analysis did not indicate a strong association. These results suggest that it

is necessary to analyze a larger group of patients to understand whether CFL1 is suitable for use as a lymph node metastasis biomarker.

According to the results of the present study, TAGLN is another gene that may contribute to the clinical behavior of LSCC. No study has established a relationship between TAGLN and LSCC. However, TAGLN has been shown to have effects on numerous different types of cancer. TAGLN, also known as smooth muscle 22 α , is an actin-binding protein abundantly expressed on smooth muscle cells (46). It has been reported that the increased expression of TAGLN may trigger the development of metastasis and affect the prognosis of colorectal cancer (47,48). Xu *et al* (49) suggested that TAGLN can be used in the postoperative follow-up period to screen for recurrence in colorectal cancer, and as a prognostic marker. Furthermore, Dvorakova *et al* (50) demonstrated that TAGLN expression was higher in breast cancer patients with lymph node metastasis compared with those without metastasis. In addition, Wu *et al* (51) suggested that TAGLN may be a potential biomarker and therapeutic target gene in lung adenocarcinoma, while Bu *et al* (52) argued that TAGLN overexpression in tissues and salivary secretion is an independent prognostic factor in oral cavity cancer and has the potential to be used as a reliable biomarker. A clear relationship between TAGLN and LSCC was revealed by the results of the present study, which showed that higher TAGLN expression was associated with poorer prognosis. These results were confirmed by microarray, PCR and survival analyses. In order to invade and metastasize, it is necessary for cancerous cells to exhibit certain properties, including reorganization of the actin cytoskeleton, an increase or change of metalloproteinases in the extracellular fluid, and focal adhesion signaling (53,54). Additionally, EMT is an important process occurring in cancer cells with metastatic properties, which plays a major role in resistance to cancer treatment (55). Lin *et al* (56) suggested that TAGLN expression in different tissues and tumors is consistent with its involvement in EMT, by which tumor cells exhibit a more aggressive phenotype. In accordance with these data and the results of the present study, the overexpression of TAGLN has the potential to be an important poor prognostic factor for cancers of epithelial origin, including LSCC.

In the present study, the DFS and DSS analyses suggested that changes in CFL1 and TAGLN expression may have important effects on survival. Increased TAGLN expression in lymph node tissues was indicative of poor survival. The increase in TAGLN expression in the mucosal tissue of patients in the metastasis-positive group, 11.06-fold in the microarray and 22.87-fold in the RT-qPCR results, appears to be a strong predictor of regional metastasis. The survival analysis supported the microarray and RT-qPCR results and strengthened the potential of TAGLN as a novel biomarker for poor prognosis and metastasis. Similarly, the mean survival time of patients with high CFL1 expression in the tumor tissue was longer than that of patients with low tumor CFL1 expression, which indicates that CFL1 may be a useful biomarker for good prognosis. However, these results require confirmation in a different cohort and a larger sample group. Moreover, protein-level analyses should be included in further studies to reveal the significance of these genes at the protein level. Another limitation of the present study is the inclusion of

the samples from paraffin-embedded tissues; it is speculated that fresh tissues may give a higher resolution for expression level analysis.

In conclusion, TAGLN and CFL1 may serve important roles in the mechanism of regional lymph node metastasis in advanced LSCC. The downregulation of CFL1 and upregulation of TAGLN are associated with regional metastasis. However, their combined effect and their relationship with metastasis merit further discussion and investigation in further studies. Although this relationship and its effects are not yet fully understood, the findings of the present study may increase the possibility of obtaining an early diagnosis for metastatic advanced LSCC and predicting a poor prognosis for the disease. If the present results are confirmed by the analysis of large populations, these genes may be used as biomarkers for the prediction of future regional metastasis. Further studies with a larger group of patients should be conducted to understand the mechanisms and clinical value of these two genes in LSCC. Moreover, these data could be collected at different stages of lymph node metastasis to elucidate the combined effects of TAGLN and CFL1.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available in the Gene Expression Omnibus repository (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201777>).

Authors' contributions

ÖB, MDA, GS and FAP were responsible for the study concept and ÖB, MDA, GS, FAP and UH were responsible for study design. EÇT, UH, EŞ and MHK supervised the study. ÖB, MDA and GS provided resources and ÖB, MDA, UH and FPA provided materials. ÖB, MDA, GS, UH, FPA, EÇT, EŞ and MHK performed data collection and/or processing. ÖB, MDA, GS, UH, FPA and MHK contributed to the analysis and/or interpretation of the data. ÖB, MDA, UH and FPA searched the literature. ÖB and MDA wrote the manuscript and EÇT, FPA and MHK critically reviewed it. All authors read and approved the final version of the manuscript. ÖB and MDA confirm the authenticity of all the raw data.

Ethics approval and consent to participate

This study was performed at Dışkapı Yıldırım Beyazıt Training and Research Hospital after approval by the local Ethics Committee (18/42/14) and was conducted in compliance with the principle of the Declaration of Helsinki. Written

informed consent was obtained from all patients or their family members.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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