FOXL2 expression might be a novel prognostic biomarker in patients with laryngeal squamous cell carcinoma

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Abstract
Objectives: This study aimed to explore the expression profile of the Forkhead box protein L2 gene (FOXL2) and to determine its prognostic value and associated epigenetic and genetic alterations in patients with laryngeal squamous cell carcinoma (LSCC).
Materials and methods: Data for a subset of patients with LSCC (N = 116) were extracted from the head and neck squamous cell carcinoma dataset of The Cancer Genome Atlas and analyzed in relation to FOXL2 expression and survival.
Results: Aberrant FOXL2 expression was an independent prognostic factor for progression-free survival (PFS) (hazard ratio (HR): 2.63, 95% confidence interval (CI): 1.34–5.18) and overall survival (OS) (HR: 2.39, 95% CI: 1.28–4.46). Two gene-body CpG sites (cg10554436 and cg23637494) were moderately and positively correlated with FOXL2 expression. DNA amplification (+2/+1) was common (82/115, 71%) in LSCC, and FOXL2 expression was significantly upregulated in the high-amplification group (+2) compared with copy-neutral (0) cases.
Conclusion: Aberrant FOXL2 expression may be a novel prognostic biomarker for PFS and OS among patients with LSCC. FOXL2 upregulation may be related to gene-body hypermethylation and DNA amplification.

Keywords
Laryngeal squamous cell carcinoma, FOXL2, methylation, copy number alteration, prognosis, survival

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Introduction

Forkhead box protein L2, encoded by the FOXL2 gene, is a member of the forkhead (FOX) family of transcription factors that plays major roles in regulating the expression of genes related to cell division, proliferation, and differentiation. \(^1\) FOXL2 is a single-exon gene that is preferentially expressed in the ovary, endometrium, developing eyelids, and pituitary gland. \(^2\) FOXL2 dysregulation was recently reported to be involved in cancer biology, and a single somatic FOXL2 mutation (c.402>G) was highly prevalent and specific to adult-type granulosa cell tumors. \(^3\) FOXL2 upregulation also enhanced metastases and epithelial-to-mesenchymal transition in chemoresistant stomach cancer, and was associated with significantly shorter survival. \(^4\)

Head and neck squamous cell carcinoma (HNSC) comprises a group of heterogeneous tumors with different anatomic origins, distinct molecular features, and varying prognoses. \(^5\) Laryngeal squamous cell carcinoma (LSCC) is a malignant tumor that originates from the laryngeal framework, and accounts for about 25% of all HNSCs. \(^6\) Despite advances in disease management, LSCC is one of a few malignancies for which the 5-year survival rate has decreased over the past four decades, \(^7\) with an overall 5-year survival rate of only about 60%. \(^7\) The Tumor-Node-Metastasis (TNM) staging system is still the most frequently used classification for prognosis estimation. However, although advanced stage is generally associated with poor survival, some studies failed to identify any independent prognostic value of TNM stage in terms of survival in light of the varying prognoses of LSCC patients with the same TNM stage and receiving the same therapy. \(^8,9\) One possible cause of this phenomenon may be the underlying molecular heterogeneity. \(^5\) There is thus a need to explore reliable prognostic biomarkers that could help to identify patients at high risk of poor survival, to allow the consideration of intensive therapeutic support.

We extracted and analyzed data for patients with HNSC from The Cancer Genome Atlas (TCGA) (TCGA-HNSC) and examined the expression and prognostic significance of FOXL2 and its epigenetic and genetic alterations in patients with LSCC.

Materials and methods

Data acquisition from TCGA-HNSC

We downloaded relevant TCGA data from TCGA-HNSC using UCSC Xena browser (https://xenabrowser.net/hitemap/). The anatomic site parameter (larynx) was applied to extract the LSCC subset. The inclusion criteria were primary LSCC, no neoadjuvant therapy, and available RNA-seq data. The data extracted for analysis included age, sex, pathological stage, longest tumor dimension (mm), surgical margin status, histological grade, radiation therapy, targeted molecular therapy, progression-free survival (PFS), overall survival (OS), gene copy number alterations (CNAs), RNA-seq data (by Illumina HiSeq), DNA methylation (by Infinium HumanMethylation450 BeadChip), and somatic mutation.

CNAs were calculated using the GISTIC2 algorithm, \(^10\) including homozygous deletion (−2), heterozygous loss (−1), copy-neutral (0), low-level copy gain (+1), and high-level amplification (+2). Single-nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs) were considered as somatic mutations.

This was a secondary study based on an online databases. No primary data were collected by any of the authors and ethical permission and consent were therefore not required.
Statistical analysis

Differences between two groups were analyzed by Welch’s t-test. Kaplan–Meier survival curves were generated using the Youden Index of FOXL2 expression determined by receiver operating characteristic curve analysis for OS detection as the cutoff. Survival differences were analyzed by log-rank tests. The prognostic significance of FOXL2 expression was analyzed by univariate and multivariate Cox regression models. Hazard ratios and 95% confidence intervals were estimated between patients with high and low FOXL2 expression. Pearson’s r values were calculated for correlation estimation. A value of $P < 0.05$ was considered statistically significant.

Results

FOXL2 was aberrantly expressed and correlated with poor survival in LSCC

A total of 116 primary LSCC and 12 normal (adjacent) tissues were included. The demographic information for the patients is available from the TCGA-HNSC project. FOXL2 expression was significantly higher in the cancerous compared with the normal tissues in patients with LSCC ($P < 0.01$) (Figure 1a). We also analyzed FOXL2 expression in different clinicopathological groups, and found no significant relationship with sex, pathological stage, or tumor grade stratification (Figure 1b–d). However, FOXL2 expression was significantly higher in patients with progressive disease and in patients who died compared with their respective controls ($P < 0.01$) (Figure 1e, f).

Kaplan–Meier survival analysis showed that PFS ($\chi^2 = 12.34, P < 0.01$) (Figure 1g) and OS ($\chi^2 = 14.78, P < 0.01$) (Figure 1h) were both significantly shorter in the high-FOXL2 expression group compared with the low-expression group. High FOXL2 expression was independently associated with unfavorable PFS ($P < 0.01$), after adjustment for margin status (Table 1). High FOXL2 expression was also an
independent indicator of unfavorable OS ($P = 0.011$), after adjusting for sex, margin status, and histological grade (Table 1). The effect size of the hazard ratio provides clinically useful information for estimating risk. These findings implied that patients with high FOXL2 expression might have a greater than two-fold increased risk of disease progression and death compared with patients in the low-expression group.

**FOXL2 DNA methylation profile**

Using data from the Methylation 450k array, we identified 21 CpG sites with methylation data in patients with primary LSCC (Figure 2a). Using moderate correlation (absolute Pearson’s $\geq 0.4$) as the criterion, two CpG sites, cg10554436 and cg23637494, were positively correlated with FOXL2 expression (Figure 2b). cg10554436 is located in the 5’ untranslated region (UTR), and cg23637494 is located in the 3’ UTR of the FOXL2 gene (Figure 2a). These findings suggest that FOXL2 expression was unlikely to be modulated by promoter methylation in patients with LSCC, but its expression was positively correlated with gene-body methylation.

**Somatic mutations and CNAs in FOXL2 in LSCC**

We examined somatic mutations/CNAs in FOXL2 and its expression profile in patients with primary LSCC. No somatic FOXL2 mutations were observed in the LSCC cases (Figure 2a). The 115 LSCC cases with available FOXL2 CNA data included 24 (+2) cases, 58 (+1) cases, 29 (0) cases, and 4 (−1) cases (Figure 2c). The high-amplification groups (+2) had significantly elevated FOXL2 expression levels compared with the copy-neutral group ($P = 0.011$) (Figure 2c).

### Table 1. Prognostic values of FOXL2 for progression-free and overall survival in patients with laryngeal squamous cell carcinoma.

| Parameter          | Univariate analysis | Multivariate analysis |
|--------------------|---------------------|-----------------------|
|                    | $P$ | HR (95%CI) | $P$ | HR (95%CI) |
| **PFS**            |     |           |     |           |
| Margin status      |     |           |     |           |
| Positive/close (N = 13) vs. negative (N = 84) | $<0.01$ | 3.45 (1.47, 8.13) | $0.05$ | 2.40 (1.00, 5.79) |
| **FOXL2 expression** |     |           |     |           |
| High (N = 54) vs. low (N = 62) | $<0.01$ | 3.05 (1.59, 5.87) | $<0.01$ | 2.63 (1.34, 5.18) |
| **OS**             |     |           |     |           |
| Sex                |     |           |     |           |
| Female (N = 20) vs. male (N = 96) | $<0.01$ | 3.63 (1.84, 7.16) | $<0.01$ | 3.07 (1.52, 6.20) |
| Margin status      |     |           |     |           |
| Positive/close (N = 13) vs. negative (N = 84) | $<0.01$ | 3.57 (1.60, 7.96) | $0.02$ | 2.63 (1.14, 6.05) |
| **Histological grade** |     |           |     |           |
| G1/G2 (N = 79) vs. G3/G4 (N = 33) | 0.03 | 2.19 (1.09, 4.40) | 0.13 | 1.74 (0.85, 3.57) |
| **FOXL2 expression** |     |           |     |           |
| High (N = 54) vs. low (N = 62) | $<0.01$ | 3.05 (1.68, 5.54) | $<0.01$ | 2.39 (1.28, 4.46) |

HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; OS, overall survival.
Discussion

Using LSCC-subset data from TCGA-HNSC, we found that FOXL2 expression was significantly upregulated in cancerous compared with normal tissues. Furthermore, survival analysis revealed that aberrant FOXL2 expression was an independent indicator of shorter OS and PFS in patients with LSCC, suggesting that this gene may have useful prognostic value.

No somatic mutations were detected in the FOXL2 gene in the current LSCC cases, in contrast to the situation in adult-type granulosa cell tumors. We therefore inferred that the mutation status of this gene might be cancer-type-specific. However, the mechanism underlying its dysregulation in LSCC has not yet been reported. FOXL2 was aberrantly methylated in esophageal adenocarcinoma, and...
FOXL2 expression might also be regulated by promoter methylation in non-cancerous tissues. DNA methylation may therefore represent an important epigenetic mechanism for FOXL2 dysregulation. The current study identified two gene-body CpG sites (cg10554436 and cg23637494) that were positively correlated with FOXL2 expression. Recent evidence suggested that gene-body DNA methylation could increase transcriptional efficiency via complex mechanisms, such as inhibiting the initiation of intragenic promoters and supporting the formation of DNA structural conformations, which are required for efficient elongation or splicing. Gene-body methylation might thus increase FOXL2 expression in LSCC; however, further studies are needed to determine the precise mechanism. Amplification-dependent overexpression of FOXL2 is frequent in SCC. The current results also showed that DNA amplification (+2/+1) was common (82/115, 71%) in patients with LSCC, and FOXL2 expression was upregulated in the high-amplification group compared with the copy-neutral cases. DNA amplification might thus be responsible for FOXL2 upregulation in LSCC.

This study had some limitations. We did not explore the downstream targets of FOXL2 in LSCC, or its relative prognostic value compared with previously identified markers. Validation studies are therefore needed to clarify the potential clinical utility of FOXL2 as a biomarker.

Conclusion

Aberrant FOXL2 expression might be a novel prognostic biomarker of PFS and OS in patients with LSCC. Patients with high FOXL2 could have a greater than two-fold risk of disease progression and death compared with patients with low FOXL2 expression. FOXL2 upregulation is probably related to gene-body hypermethylation and DNA amplification.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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