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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/heatmap/). 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

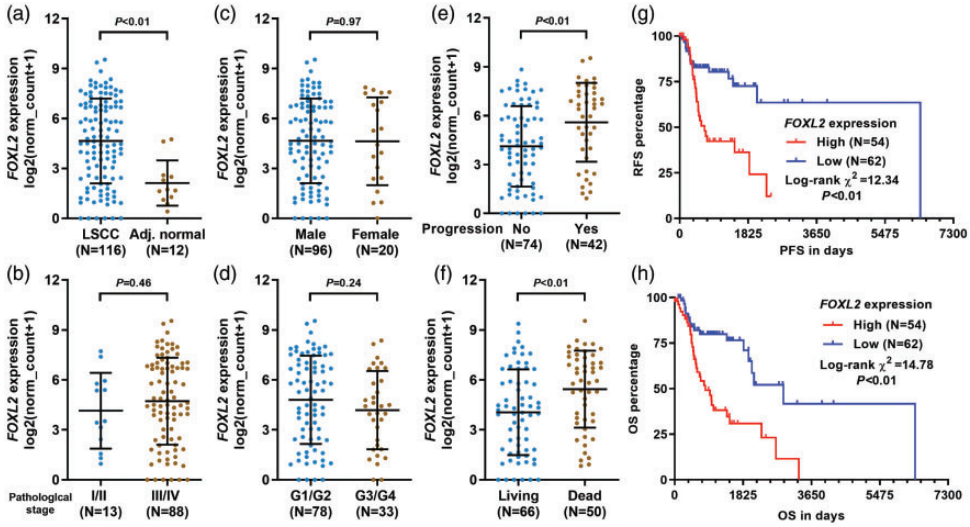


Figure 1. *FOXL2* dysregulation in patients with LSCC. (a) Plot chart showing *FOXL2* expression in LSCC (N = 116) and adjacent normal (N = 12) tissues. *FOXL2* expression in patient groups divided by pathological stage (b), sex (c), grade (d), disease-progression (e), and living/dead status (f). Kaplan–Meier curves of PFS (g) and OS (h) in patients with LSCC. LSCC, laryngeal squamous cell carcinoma; PFS, progression-free survival; OS, overall survival.

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

	Univariate analysis				Multivariate analysis			
Parameter	*P*	HR	95%CI (lower/upper)		*P*	HR	95%CI (lower/upper)	
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.

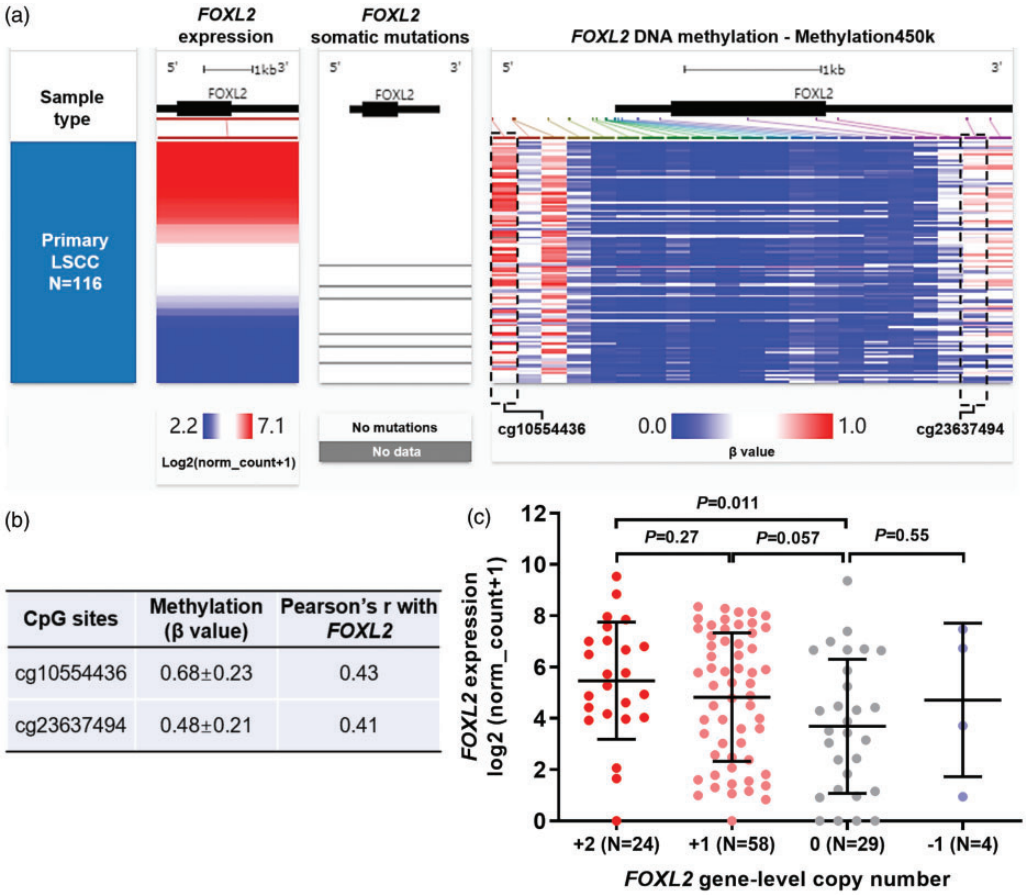
independent indicator of unfavorable OS ($P = 0.011$), after adjusting for sex, margin status, and histological grade (Table 1). The effect size of the hazard ratio provides clinically useful information for estimating risk. These findings implied that patients with high *FOXL2* expression might have 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 ≥ 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).



CpG sites	Methylation (β value)	Pearson's r with *FOXL2*
cg10554436	0.68±0.23	0.43
cg23637494	0.48±0.21	0.41

Figure 2. *FOXL2* DNA methylation, somatic mutations, and CNAs in LSCC. (a) Heatmap demonstrating methylation of 21 CpG sites and somatic mutations (single nucleotide polymorphisms and small insertion/deletions) in *FOXL2* locus. (b) Two CpG sites showed moderate correlation (absolute Pearson's r≥0.4) with *FOXL2* expression. Their locations in the *FOXL2* gene are indicated in (a). (c) Plot chart comparing *FOXL2* expression in LSCC patients with different gene-level copy numbers. CNA, copy number alterations; LSCC, laryngeal squamous cell carcinoma.

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.[3] 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,[11] and

FOXL2 expression might also be regulated by promoter methylation in non-cancerous tissues.[12] 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.[13] 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.[14] 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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