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ORIGINAL ARTICLE

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TGFBR3 is an independent unfavourable prognostic marker in oesophageal squamous cell cancer and is positively correlated with Ki-67

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Summary

The transforming growth factor beta (TGF-β) superfamily plays an important role in cancer development. One aspect of this is that the transforming growth factor beta receptor III (TGFBR3) is frequently overexpressed in some tumours. However, the role of TGFBR3 in oesophageal squamous cell carcinoma (ESCC) has not been explored as yet. In this study, we aimed to determine the role of TGFBR3 in the development and prognosis of ESCC and the correlation between TGFBR3 expression and Ki-67 and p53.

Immunohistochemistry was performed to investigate the expression of TGFBR3 in the tumour tissue microarray consisting of ESCC tissues and matched adjacent normal tissues (n = 80). Only ESCC tissues (n = 20) were also used in our analysis. The association between TGFBR3 expression and clinicopathological characteristics, such as Ki-67 and p53, was analysed by Spearman's rank correlation coefficient analysis. The association between TGFBR3 expression and prognosis of ESCC was analysed using Kaplan-Meier analysis and log-rank tests.

The expression levels of TGFBR3 in oesophageal cancer tissues were markedly higher than in matched adjacent normal tissues. Furthermore, TGFBR3 overexpression was significantly associated with tumour-node-metastasis (TNM) stage, lymph node metastasis (N stage) and Ki-67 expression. However, TGFBR3 overexpression was not significantly related to age, sex or p53. In univariate analysis, overall survival of ESCC patients was significantly associated with high TGFBR3 expression, sex, T stage, N stage and TNM stage. Moreover, ESCC patients with high TGFBR3 expression had poorer overall survival than those with low TGFB R3 expression.

Our findings showed that TGFBR3 was upregulated in the development of human ESCC and high TGFBR3 expression was associated with high expression of Ki-67 and poor prognosis of ESCC. Therefore, TGFBR3 may be a valuable prognostic marker and a novel therapeutic target for ESCC.

KEYWORDS

oesophageal squamous cell carcinoma, prognosis, proliferation, TGFBR3

1 | INTRODUCTION

Oesophageal cancer (EC) is the eighth most common cancer and the sixth most common cause of cancer-related deaths worldwide.[1] Although the detection, diagnosis and treatment of oesophageal squamous cell carcinoma (ESCC) have improved in recent years, the long-term prognosis of ESCC patients remains poor. Standard treatments for ESCC include chemoradiotherapy and surgery. Several studies have reported that proliferation-related proteins, such as Ki-67 and P53, are associated with prognosis of ESCC[2]; however, they are not specific. Thus, a better prognosis marker for ESCC is urgently needed.

The transforming growth factor beta (TGF-β) signalling pathway plays an important role in cell proliferation, differentiation and apoptosis. Previous studies have demonstrated that ligands of the TGF-β superfamily can play different and contrasting roles in human cancers.[3] For example, TGF-β can act as a tumour suppressor in some early-stage human cancers and as a tumour promoter in some late-stage cancers.[4] The three TGF-β isoforms, β1, β2 and β3, exert their effects by binding to type I, II and III TGF-β receptors.[5] Transforming growth factor beta receptor III (TGFBR3) functions as a binding co-receptor for various cytokines of the TGF-β superfamily[6] and is thought to play a role in the pathogenesis of some human cancers.[7] Some reports have suggested that TGFBR3 promotes the growth, migration, invasion and metastasis of colon cancer.[8] In contrast, some evidences indicate that TGFBR3 contributes to reduced angiogenesis, tumour invasion and metastasis, and its expression is decreased or absent in most human cancers including breast,[9] liver,[10] lung,[11] kidney,[12] pancreas[13] and prostate[14] cancers. However, no previous studies have been conducted to determine the role of TGFBR3 in human EC. In this study, we measured TGFBR3 protein expression in ESCC specimens and explored the correlation between TGFBR3 expression and clinicopathological characteristics, such as Ki-67 and p53.

2 | MATERIALS AND METHODS

2.1 | Tumour tissue microarray

Immunohistochemical (IHC) staining was performed on a human oesophageal cancer tissue microarray (TMA; HEso-Squ180Sur-04) containing 80 pairs of ESCC tissues and matched adjacent normal tissues. Twenty samples of only ESCC tissues were purchased from Shanghai Outdo Biotech Co. Ltd. (Shanghai, China). All patients received a pathological diagnosis for ESCC and had not received chemotherapy or radiotherapy before tissue collection.

2.2 | Immunohistochemistry

TGFBR3, Ki-67 and p53 expression in ESCC tissues and matched adjacent normal tissues was examined by IHC. The TMA was dewaxed with xylene, rehydrated with a graded ethanol series, and incubated with 0.3% H_2O_2 to quench endogenous peroxidase activity. Tissue antigens were retrieved by heating in a microwave oven with 10 mM sodium citrate buffer (pH 6.0), and the samples were cooled to room temperature. The TMA was washed with phosphate-buffered saline and incubated overnight at 4°C with antibodies against TGFBR3 (1:100, Abcam), Ki-67 (IR626, Dako) and p53 (IR616, Dako). Anti-TGFBR3 was diluted with 5% sheep serum before use. Ki-67 and p53 primary antibodies were diluted before purchase. After incubation with the primary antibody, the TMA was washed and incubated with secondary peroxidase-conjugated rabbit anti-goat IgG (ZSGB-Bio), washed, incubated for 3 min with 3,3′-diaminobenzidine, and then counterstained with haematoxylin. Finally, the TMA was dehydrated with ascending concentrations of ethanol, mounted with neutral resin, and scored by a pathologist blinded to this study using a bright field microscope (Olympus CX31; Olympus Corporation, Tokyo, Japan) a magnification of 40× or 200×.

TGFBR3 expression was quantified using H-scores, which provide an assessment of staining based on the intensity and percentage of positive cells. The calculation equation is as follows: H-score = $\sum$Pi (i + 1), in which '' represents the intensity of staining, which was scored as zero (no staining), 1 score (weak), 2 scores (moderate) and 3 scores (strong staining). 'Pi' represents the percentage of cells at each intensity (0-100%). The positively stained cells were scored as zero (no staining), 1 score (1%–25% of the whole cells), 2 scores (26%–50% of the whole cells), 3 scores (51%–75% of the whole cells) and 4 scores (76%–100% of the whole cells). The product of 'staining intensity score' and 'staining positive rate score' was used as the total score for grouping. Cells with a score of less than 4 were divided into low expression groups, and cells with a score of 4 or more were divided into high expression group. Specimens were classified into high or low expression groups using the total score of TGFBR3, Ki-67 and p53.

2.3 | Statistical analysis

Statistical analysis was performed using SPSS version 19.0 software (SPSS Inc). TGFBR3 expression levels in 80 pairs of ESCC tissues and matched adjacent normal tissues were analysed using the chi-squared test. Correlations between TGFBR3 expression and clinicopathological characteristics, such as Ki-67 and p53 expression, were evaluated using Spearman's rank correlation analysis.

Kaplan-Meier and log-rank analyses were used to compare the survival rates of patients, and a survival curve (as defined by the ROC curve) was created based on the high and low TGFBR3 IHC scores. Cox regression was used to analyse the correlation between TGFBR3, Ki-67, clinical indicators and pathological features and overall survival of patients with ESCC.

3 | RESULTS

3.1 | TGFBR3 is highly expressed in ESCC cells

The expression levels of TGFBR3, Ki-67 and p53 protein in 80 pairs of ESCC tissues and matched adjacent normal tissues and 20 ESCC tissue samples were evaluated by IHC. The expression of TGFBR3 was significantly higher in cancerous tissues than in matched adjacent normal tissues (Figure 1). TGFBR3 was highly expressed in 67 of 80 ESCC tissues as compared to 19 of 80 matched adjacent normal tissues (Table 1, $P = 2.72E\text{-}14$). Ki-67 was mainly expressed in the nucleus (Figure 2), and its expression was correlated with high expression of TGFBR3 (Table 2, $P = .043$). p53 was mainly expressed in the nucleus (Figure 3), but its expression level did not significantly correlate with the expression of TGFBR3 (Table 2, $P = .956$).

3.2 | High expression of TGFBR3 is associated with ESCC clinicopathological characteristics

Next, we analysed the relationship between the TGFBR3 expression level and clinicopathological characteristics of ESCC. Spearman's rank correlation coefficient analysis (Table 2) showed that high expression of TGFBR3 was significantly associated with the tumour-node-metastasis

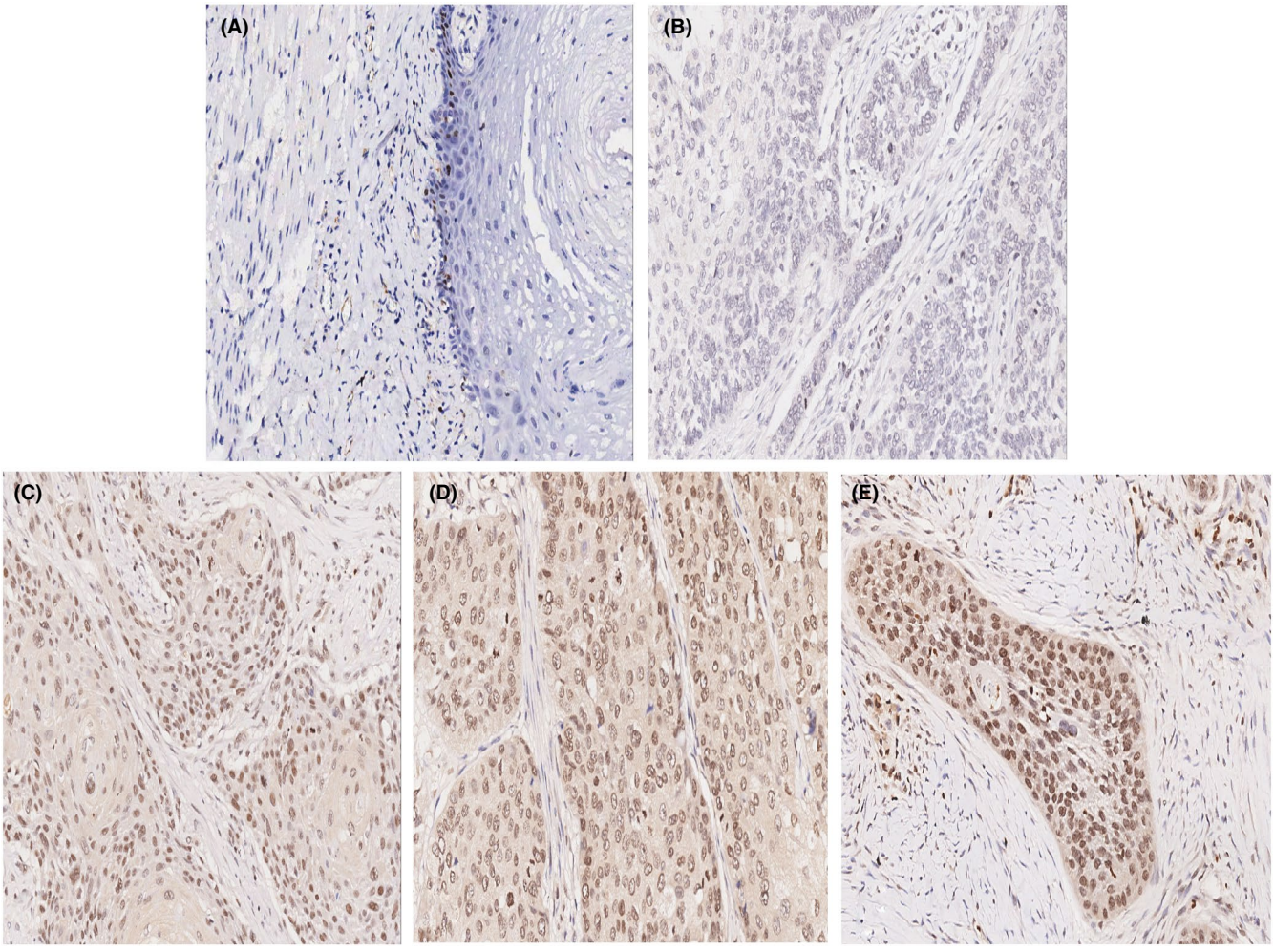

FIGURE 1 The expression of TGFBR3 in ESCC was upregulated. A, Low TGFBR3 expression in matched adjacent normal tissues. B, TGFBR3 expression in ESCC was scored as zero. C, TGFBR3 expression in ESCC was scored as 1. D, TGFBR3 expression in ESCC was scored as 2. E, High TGFBR3 expression in ESCC was scored as 3. 200 × magnification

TABLE 1 Differential expression of TGFBR3 in oesophageal cancer and matched adjacent normal tissues

	TGFBR3 expression			
	High (%)	Low (%)	Chi-square value	*P* value
Oesophageal cancer	67	13	57.92583281	2.72E-14
Matched adjacent normal tissues	19	61		

Note: TGFBR3 was highly expressed in ESCC tissues compared with matched adjacent normal tissues.

*Statistically significant ($P < .05$).

TABLE 2 Correlation between TGFBR3 expression and clinicopathological characteristics

	TGFBR3 expression				
Variables	**High**	**Low**	**Total**	r_s	***P* value**
Age (year)				−0.036	0.719
≤65	44	7	51		
>65	41	8	49		
Sex				−0.057	0.570
Female	23	3	26		
Male	62	12	74		
Grade				0.015	0.881
1	5	1	6		
2	56	10	66		
3	24	4	28		
T stage				0.033	0.752
T1	2	2	4		
T2	11	0	11		
T3	67	12	79		
T4	3	0	3		
Undefined			3		
N stage				0.258	0.010
N0	34	11	45		
N1	29	2	31		
N2	16	1	17		
N3	5	0	5		
Undefined			2		
TNM stage				0.258	0.011
I	2	2	4		
II	34	8	42		
III	47	3	50		
Undefined			4		
Ki67				0.203	0.043
Negative	31	8	39		
Positive	54	7	61		
P53				−0.06	0.956
Negative	46	8	54		
Positive	39	7	46		

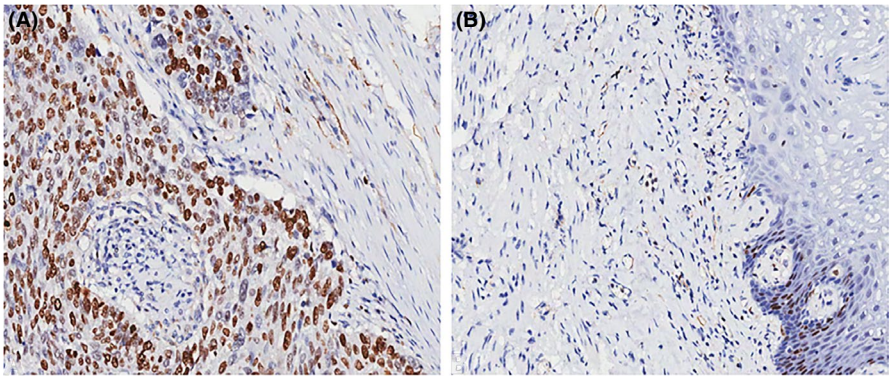

FIGURE 2 The expression of Ki-67 in ESCC and matched adjacent normal tissues. A, High Ki-67 expression in ESCC tissues (nuclear staining). B, Low Ki-67 expression in matched adjacent normal tissues. 200 × magnification

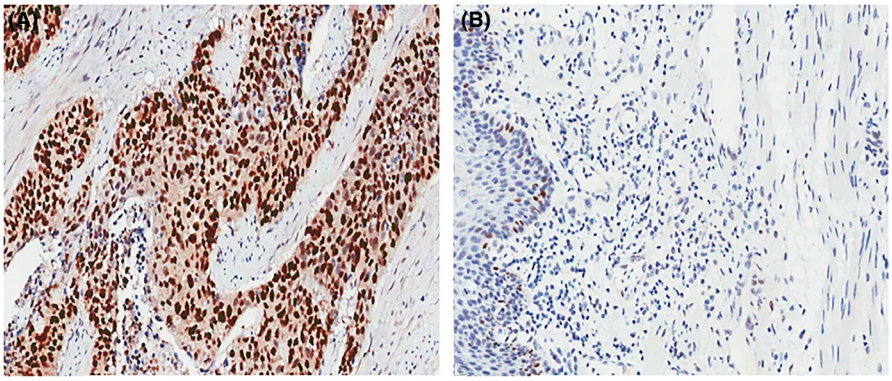

FIGURE 3 The expression of p53 in ESCC and matched adjacent normal tissues. A, High p53 expression in ESCC tissue (nuclear staining). B, Low p53 expression in matched adjacent normal tissues. 200 × magnification

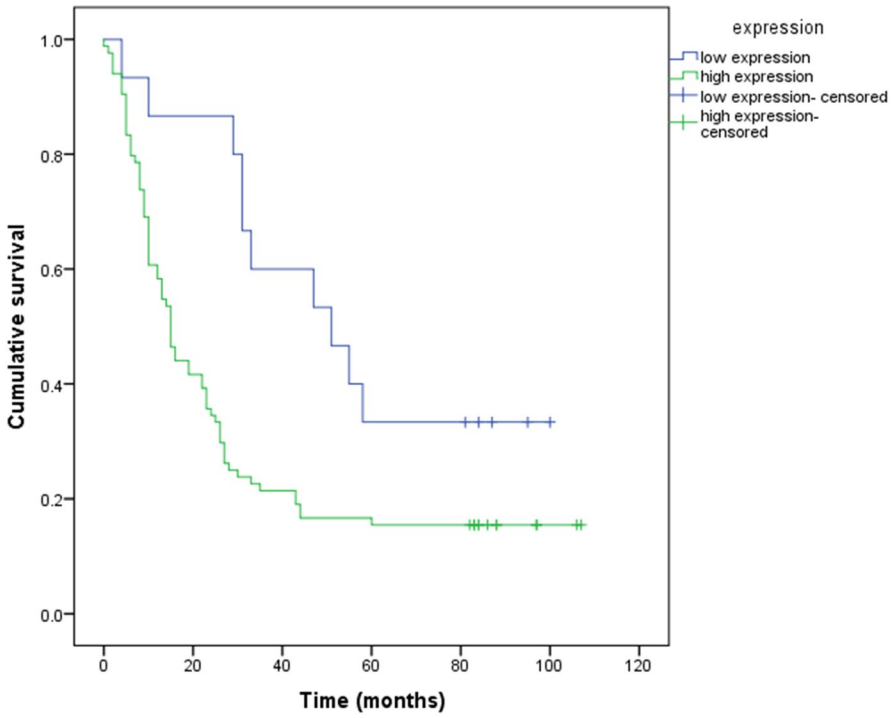

FIGURE 4 Kaplan-Meier survival analysis according to TGFBR3 expression with ESCC (log-rank test). The probability of overall survival of patients is shown

(TNM) stage and the lymph node metastasis (N stage). The level of TGFBR3 expression increased in parallel with advancing TNM and N stages. Other clinical characteristics, such as age ($P = .719$), sex ($P = .570$), T stage ($P = .752$) and grade ($P = .881$), were not related to TGFBR3 expression.

TABLE 3 Univariate analysis of the factors correlated with declined overall survival of oesophageal cancer patients

	Univariate analysis		
Variables	**HR**	**95%CI**	***P* value**
Expression	2.298	1.178-4.483	0.015
Grade	1.029	0.702-1.507	0.885
Sex	1.907	1.101-3.304	0.021
Age	0.888	0.573-1.376	0.594
T stage	2.141	1.288-3.559	0.003
N stage	1.574	1.238-2.000	<0.001
TNM stage	2.635	1.705-4.071	<0.001
Ki67	1.293	0.820-2.038	0.269

*Statistically significant ($P < .05$).

3.3 | High expression of TGFBR3 is associated with the expression of Ki-67, but not p53

Spearman's rank correlation coefficient analysis also showed that high expression of TGFBR3 was significantly associated with the expression of Ki-67 ($P = .043$). In other words, higher the expression levels of TGFBR3 in samples, higher was the expression levels of Ki-67 in those samples. However, high expression of TGFBR3 was not significantly associated with the expression of p53 ($P = .956$).

3.4 | High TGFBR3 expression correlates with decreased overall survival

Kaplan-Meier survival analysis revealed that ESCC patients with high tumour expression of TGFBR3 had decreased overall survival compared to patients with low TGFBR3 levels ($P = .011$, log-rank test; Figure 4).

3.5 | High TGFBR3 expression, sex, T stage, N stage and TNM stage may influence the overall survival of ESCC patients

In univariate analysis (Table 3), overall survival of ESCC was significantly associated with high TGFBR3 expression ($P = .015$), sex ($P = .021$), T stage ($P = .003$), N stage ($P < .001$) and TNM stage ($P < .001$). Therefore, high TGFBR3 expression, sex, T stage, N stage and TNM stage were found to influence the overall survival of ESCC patients.

4 | DISCUSSION

EC is one of the most common and lethal cancers worldwide.[1,15] Because of the poor prognosis, finding new diagnostic and therapeutic biomarkers for EC has become important. Here, we evaluated the potential utility of TGFBR3, Ki-67 and p53 expression as biomarkers for ESCC.

Aberrant TGF-β signal transduction is associated with abnormal embryogenesis, tissue fibrosis, cancer, cardiovascular diseases and autoimmune diseases.[3] In the past decades, many studies have confirmed that TGF-β plays a dichotomous role in human cancer, and it can function as a tumour suppressor in early-stage diseases and as a tumour promoter in late-stage diseases. TGFBR3, the most abundant and widely characterized of the three TGF-β receptors,[6] is a co-receptor that directly binds to a number of ligands in the TGF-β superfamily, including TGF-β1, TGF-β2 and TGF-β3, inhibin, bone morphogenetic protein-2, −4, and −7, and growth differentiation factor-5. In most cases, TGFBR3 binding increases ligand binding to the cognate type I and type II TGF-β receptors, thereby increasing signal transduction via the Smad protein pathway.[7]

Recent studies suggested that TGFBR3 plays a critical role in some human cancers, including breast, prostate, colon and pancreatic cancers. Several studies have also demonstrated that TGFBR3 is significantly upregulated or downregulated in cell lines derived from these cancers as compared to the normal cells. In these studies, high TGFBR3 expression was associated with increased or decreased cell proliferation and invasion.[9-13] However, the expression of TGFBR3 in ESCC has not been examined, and its functions in ESCC remain unclear.

In our study, we found that TGFBR3 was present at significantly higher levels in ESCC tissues than in matched adjacent normal tissues. We also found significant positive correlations between high expression of TGFBR3 and the TNM and N stages. Moreover, the overall survival of patients with high TGFBR3 expression was significantly poorer than that of patients with low TGFBR3 expression.

Ki-67 is a nuclear protein expressed in proliferating cells and is commonly used as a proliferation marker in non-clinical and clinical studies.[16] Ki-67 expression levels are associated with TGF-β1 expression in liver cancer tissues and in the human hepatoma cell line HepG2.[17] In this study, we examined TGFBR3 and Ki-67 expression in 80 pairs of ESCC tissues and matched adjacent normal tissues by IHC (Figures 1, 2). We found that high TGFBR3 expression correlated significantly with high Ki-67 levels. Since Ki-67 has been reported to be a useful marker for tumour proliferative activity,[16] this finding suggests that high TGFBR3 expression in ESCC patients may correlate with high proliferative activity of cancer cells.

p53 acts mainly through the transcriptional regulation of target genes.[18] It has been reported that mutant p53 protein overexpression is associated with poor prognosis in patients with early-stage ESCC, and it is a significantly independent predictor of poor overall survival in early-stage ESCC.[19] Mutant p53 generally subverts tumour-suppressive TGF-β responses, diminishing transcriptional activation of TGF-β target genes.[20] In the current study, we found that p53 was highly expressed in ESCC. However, the high TGFBR3 expression was not significantly correlated with high p53 level, which may be related to insufficient sample size. More studies with larger sample sizes and longer follow-up periods are needed. Additionally, Sengpiel et al examined 103 patients with carcinoma of the oesophagus and gastroesophageal junction and found that p53 mutations were present in 36.9% of the patients, but the survival of patients was not affected by p53 mutation.[21] Further studies are needed to demonstrate the relationship between p53 mutation and TGFBR3 to analyse the critical role of p53 in ESCC prognosis.

In conclusion, we investigated the expression of TGFBR3 in human ESCC and found that levels were elevated in tumour tissues as compared to adjacent normal tissues. We also detected a positive correlation between the expression of TGFBR3 and the proliferation marker Ki-67. Importantly, we identified a significant correlation between high TGFBR3 expression and the low overall survival of ESCC patients. Collectively, our findings suggest that TGFBR3 has tremendous potential as related to the progression of ESCC and may be a new prognostic marker and novel therapeutic target for ESCC.

CONFLICTS OF INTEREST STATEMENT

None declared.

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ETHICAL APPROVAL

All experiments were approved by the Institutional Ethics Committee of The Second Affiliated Hospital of Harbin Medical University, Harbin 150086, Heilongjiang Province, China.

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