

Neutrophil gelatinase-associated lipocalin and its receptor: independent prognostic factors of oesophageal squamous cell carcinoma

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ABSTRACT

Aim Previous studies have shown that neutrophil gelatinase-associated lipocalin (NGAL) is overexpressed in oesophageal squamous cell carcinoma (ESCC) and closely associated with the invasiveness of ESCC cells. Recently, NGAL receptor (NGALR) was identified from ESCC cells, and was also found to be increased in ESCC. The purpose of this study was to reveal the clinical significance of NGAL and/or NGALR in ESCC.

Methods Tissue microarray was performed to detect expression of NGAL and NGALR in 222 ESCC specimens. Pearson χ^2 test was used to analyse correlations between NGAL and/or NGALR expression and clinicopathological features. Kaplan—Meier survival curves and the Cox proportional hazards regression model were used to evaluate the effect of NGAL and/or NGALR expression on prognosis of patients with ESCC.

Results NGAL and NGALR were highly expressed in ESCC. χ^2 test results showed no significant correlations between NGAL or NGALR expression and clinicopathological features. However, NGAL/NGALR coexpression correlated with histological differentiation grade ($p=0.033$). Survival analysis showed that positive expression of NGAL or NGALR was significantly associated with a poor prognosis for patients with ESCC ($p=0.000$ or $p=0.002$). Patients with positive expression of both NGAL and NGALR had a shorter survival time than those with negative expression of both ($p=0.048$). Multivariate analysis showed that both NGAL and NGALR were independent prognostic factors.

Conclusion These results indicate that both NGAL and NGALR may be involved in the progression of ESCC and can be considered as independent prognostic factors of ESCC.

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin 2, a member of the lipocalin family, was originally found as a protein stored in specific granules of human neutrophil.¹ A marked increase in NGAL has been observed in many inflammatory diseases, such as respiratory infection, Crohn disease and myocarditis.^{2–4} An increase in NGAL protein in serum and urine has been found to be an early and specific biomarker of renal damage and clinical prognosis.⁵ Similarly to other lipocalins, NGAL also participates in growth, development and differentiation of different human tissues as early as embryonic phases, indicating an important role in the regulation of physiological cell multiplication.⁶ Strikingly, raised

NGAL has been observed in various cancers, including colorectal neoplasm, pancreatic cancer, primary breast cancer, ovarian cancer and cholesteatoma.^{7–11} Our previous studies have shown that overexpression of NGAL plays an important role in malignant transformation of human immortalised oesophageal epithelial cells and enhances differentiation and invasion of oesophageal squamous cell carcinoma (ESCC) cells.^{12–13}

Since a specific cell-surface receptor for NGAL (NGALR) was isolated from murine FL5.12 cells, which mediated iron transportation of NGAL,¹⁴ the coexpression pattern of NGAL and NGALR has shown the tip of the iceberg. Devireddy *et al* found that NGAL was dramatically upregulated in BCR-ABL+ mouse cell lines and peripheral blood from patients with chronic myelogenous leukaemia blast crisis. Significantly, NGALR expression was repressed by BCR-ABL.¹⁴ We have found that NGALR expression in ESCC is significantly higher than in normal oesophageal epithelium.^{15–16} However, the relationship between expression of NGAL and/or NGALR and the prognosis of patients with ESCC, and their coexpression pattern are still unknown. Therefore, the purpose of this study was to qualitatively analyse the expression patterns of NGAL and NGALR in ESCC, by using immunohistochemistry and tissue microarray technology, and to determine their associations with the prognosis of patients with ESCC.

MATERIALS AND METHODS

Specimen collection

For this retrospective study, 222 formalin-fixed, paraffin-embedded archival specimens from patients with primary ESCC were collected at the Central Hospital of Shantou City from 1987 to 1999. The patient group consisted of 150 men and 72 women with a median age of 53 years (range 30–84). None of the patients received radiotherapy or chemotherapy before surgery. Information on gender, age, stage of disease and histopathological features was retrieved from the medical records, and is summarised in table 1. This study was approved by the ethics committee of the Central Hospital of Shantou City. Written informed consent to the use of resected samples for research was obtained from all patients undergoing surgery.

Construction of tissue microarrays

Representative regions of each tissue were selected from H&E-stained sections and marked on

Table 1 Basic and clinical characteristics of the 222 patients studied

Characteristic	No	5-year survival rate (%)	p Value
Age (years)			
<55	108	37.3	0.598
≥55	114	40.0	
Gender			
Female	72	45.4	0.358
Male	150	36.7	
Location of tumour			
Upper	6	66.7	0.635
Middle	146	35.4	
Lower	70	43.2	
Tumour size			
<5 cm	112	46.7	0.02*
≥5 cm	110	31.9	
Histology			
G1, well-differentiated	57	54.2	0.002**
G2, moderate differentiation	139	37.5	
G3, poor differentiation	26	17.3	
TNM stage			
I and II	99	57.5	0.000**
III and IV	123	24.3	
Surgery			
Radical	195	42.1	0.000**
Palliative	27	20.8	

*p<0.05; **p<0.01.

TNM, tumour, node, metastasis.

individual paraffin blocks. Samples were chosen from specimens with enough tissue available, so that the availability of tissue for correlative studies would not be compromised. Two tissue cores were obtained from each specimen measuring 1.8 mm in diameter and ranging in length from 1.0 to 3.0 mm depending on the depth of tissues in the donor block. Each core was precisely arrayed into a new paraffin block. These microarrays were serially sectioned (4 µm) and stained with H&E to verify tissue sampling and completeness. Unstained sections were baked overnight at 56°C in preparation for immunohistochemistry.

Immunohistochemistry and scoring

Immunohistochemical manipulation and validation of antibodies were as described previously.¹⁶ Slides were incubated with rat anti-human NGAL monoclonal antibody (R&D Systems, Minneapolis, Minnesota, USA) or rabbit anti-human NGALR polyclonal antibody (Beijing Biosynthesis Biotechnology, Beijing, China). Then slides were analysed by application of the SuperPicTure Polymer Detection kit and Liquid DAB Substrate kit (Zymed/Invitrogen, San Francisco, California, USA). The percentage of positive tumour cells was determined semiquantitatively by assessing the whole tumour section, and each sample was assigned to one of the following categories: 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The intensity of immunostaining was determined as 0 (no staining), 1+ (weak staining), 2+ (medium staining) or 3+ (strong staining). An immunoreactive score was calculated by multiplying the percentage of positive cells and the staining intensity. In the case of heterogeneous staining intensities within one sample, each component was scored independently, and the results were summed. With this system, the maximum score was 12. For statistical analysis, the scoring was applied as follows. We presumed that only if the expression levels of NGAL and NGALR reach a certain threshold will their protein products execute certain pathological functions in ESCC cells. So only

moderately to strongly positive were considered as absolutely positive cases, which might reflect the underlying pathological state of high expression of NGAL and NGALR. Therefore, cases with scores of 0–4, 5–8 and 9–12 were defined as negative (–), weakly positive (+) and strongly positive (++), respectively.

Statistical analysis

Associations between NGAL and/or NGALR expression and clinicopathological features were analysed by χ^2 test. The significant effects of NGAL and/or NGALR expression levels on patient survival time were examined using Kaplan–Meier curves and log-rank test. The influence of each variable on survival was assessed by the Cox proportional hazards model. All statistical analyses were performed using SPSS for windows (V.13.0). The accepted level of significance was p<0.05.

RESULTS

Patients' characteristics

The distributions and 5-year survival rate for the patients with ESCC are shown in table 1. Briefly, the 5-year survival rate of female and male patients was 45.4% and 36.7%, respectively. According to the histological differentiation grade, 57 tumours were well differentiated (G1), 139 moderately differentiated (G2), and 26 poorly differentiated (G3). According to the *UICC-TNM Classification for Pathological Stage of ESCC* (6th edition, 2002), there were 99 patients with 57.5% 5-year survival rate at stages I and II, and 123 patients with 24.3% 5-year survival rate at stages III and IV.

Strong expression of NGAL and NGALR in ESCC

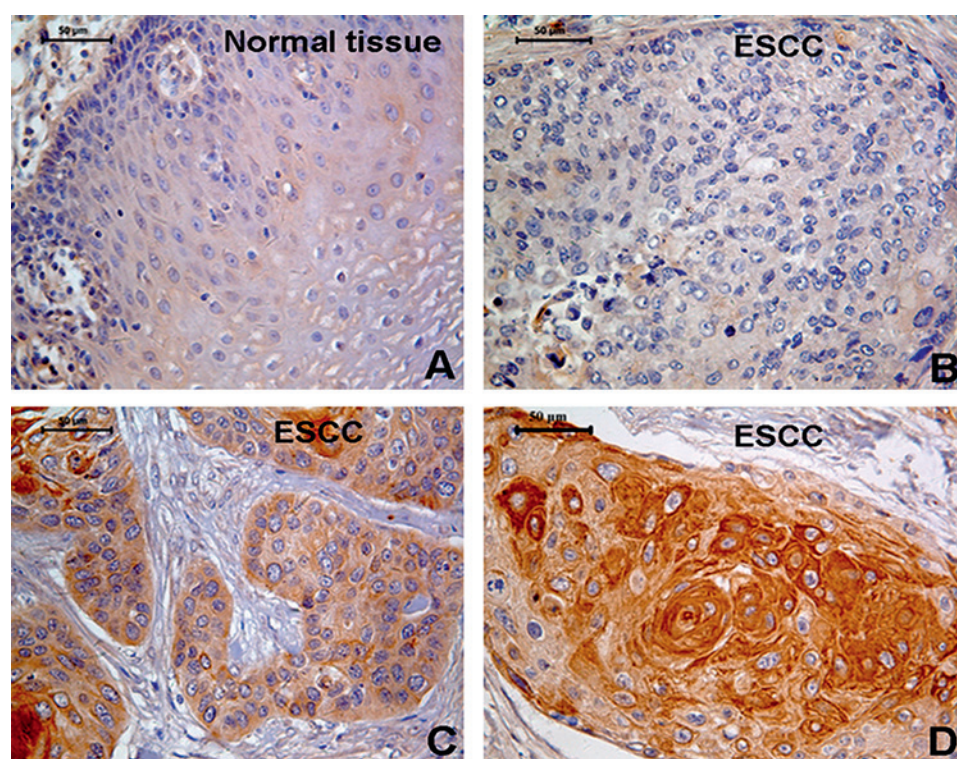
Owing to problems with tissue microarray manipulation, of 222 cases of ESCC specimens collected for immunohistochemical staining, NGAL was successfully detected in only 202 cases and NGALR was identified in 205. NGAL/NGALR staining was successful in 131 cases. The clinical characteristics of these three situations are shown in online supplementary tables S1–S3, respectively, which are also consistent with the characteristics of all the cases (table 1). In accordance with our previous reports, both NGAL and NGALR were highly expressed in ESCC in this study.^{13 15 16} NGAL immunoreactivity in normal adult oesophageal epithelial tissue was weak or undetectable. However, strong cytoplasmic staining of NGAL was observed in ESCC (figure 1). Intense diffuse membranous and/or cytoplasmic staining of NGALR was detected in ESCC, whereas absent/weak membranous staining of NGALR was observed in oesophageal epithelial tissue of normal adults (figure 2).

Associations of NGAL and/or NGALR expression with clinicopathological features in ESCC

Of the 202 cases of successful NGAL staining, positive NGAL expression was found in 101 cases (50%). In the 205 cases for NGALR, the positive rate was 24.9% (51 out of 205). No significant associations were observed when correlations between expression levels of these two proteins and various clinical features were investigated (online supplementary tables S4 and S5).

As previously reported, NGAL plays an important role in ESCC,^{12 13} and its role of transferring iron is mediated by NGALR.¹⁴ However, the clinical significance of coexpression of NGAL/NGALR in ESCC remained to be defined. For the convenience of statistical analysis, we defined the expression levels of these two proteins as follows: 0–4 points as '–' and 5–12 points as '+'. We then tentatively classified the

Figure 1 Immunohistochemical staining of neutrophil gelatinase-associated lipocalin (NGAL) in normal and cancerous oesophageal epithelial tissue. NGAL has weak or undetectable immunoreactivity in normal oesophageal epithelium (A). Negative (B), weakly positive (C) and strongly positive (D) immunoreactivity of NGAL was observed in the oesophageal tumour tissues. Scale bar, 50 μ m.



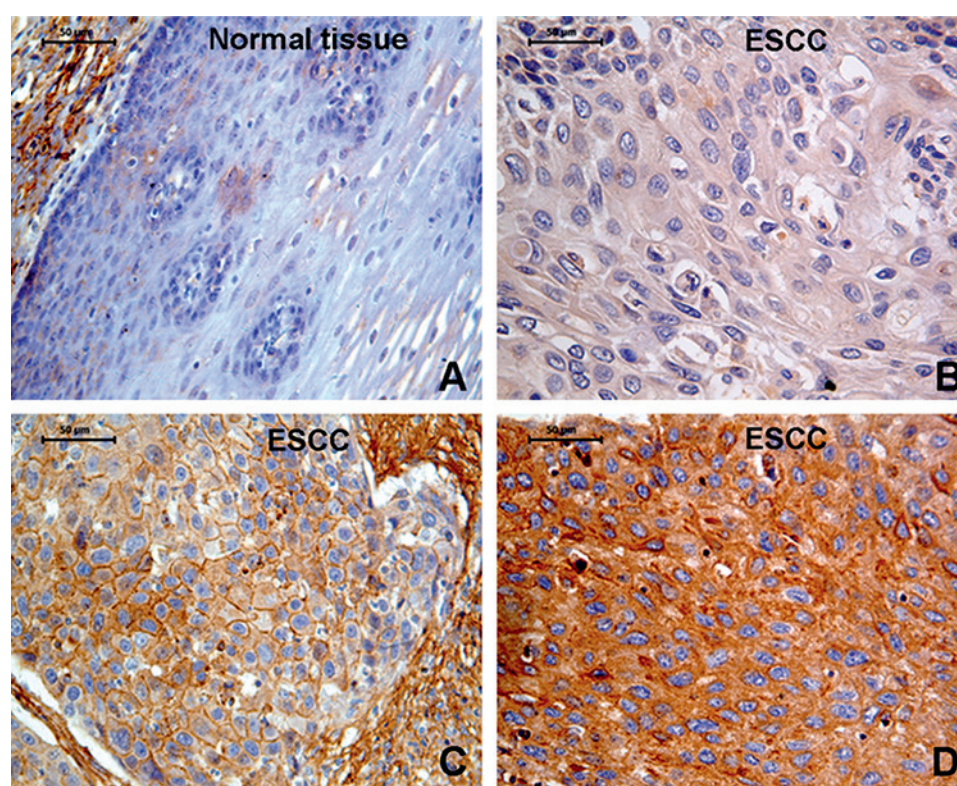
coexpression patterns of NGAL and NGALR into four categories: NGAL(-)/NGALR(-), NGAL(+)/NGALR(-), NGAL(-)/NGALR(+) and NGAL(+)/NGALR(+). Relationships between matched patient clinicopathological features and NGAL/NGALR status were analysed by χ^2 test. The data showed that there were no associations between NGAL/NGALR coexpression and clinicopathological features other

than histological differentiation grade ($p=0.033$) (online supplementary table S6).

NGAL expression correlated with the survival time of patients with ESCC

NGAL was overexpressed in a variety of tumours, which impelled us to analyse the relationship between its expression

Figure 2 Immunohistochemical staining of neutrophil gelatinase-associated lipocalin receptor (NGALR) in normal and cancerous oesophageal epithelial tissue. Weak expression of NGALR was detected in normal oesophageal epithelium (A). Representative staining of NGALR is shown in tumour tissues: negative (B), weak positive (C) and strong positive (D). Scale bar, 50 μ m.



level and the prognosis of patients with ESCC. The association between NGAL expression and the survival time of patients with ESCC was evaluated using Kaplan–Meier survival curves and log-rank test. The univariate analysis showed that positive expression of NGAL was associated with a decreased 5-year survival rate for patients with ESCC (figure 3).

Of the 202 cases of ESCC, the average survival time of 101 NGAL-negative patients was 74.7 months, with a 5-year survival rate of 49.8%; for 63 cases with weak positive staining of NGAL, the average survival time was 47.7 months and the 5-year survival rate was 33.2%; for the remaining 38 strongly positive cases, the average survival time was 38.1 months and the 5-year survival rate was 24.4% ($p=0.000$) (table 2). Using the Cox proportional hazards model, we performed multivariate analysis to assess the independent predictive value of NGAL expression for survival time of patients with ESCC. The following prognostic variables were also included: histological differentiation grade, tumour location and TNM (tumour, node, metastasis) stage. It showed that NGAL status ($p=0.000$), histological differentiation grade ($p=0.005$), tumour location ($p=0.017$) and TNM stage ($p=0.022$) were all independent prognostic factors (table 2).

NGALR expression correlated with the survival time of patients with ESCC

The association between NGALR expression and the survival time of patients with ESCC was also evaluated using Kaplan–Meier curves and log-rank test. This univariate analysis showed that positive expression of NGALR was also associated with a decreased 5-year survival rate for patients with ESCC (figure 4).

Of 205 cases of ESCC, for 154 with negative staining of NGALR, the average survival time was 67.5 months and the 5-year survival rate was 43.7%; for 32 cases with weak positive staining of NGALR, the average survival time was 41.0 months and the 5-year survival rate was 30.1%; for 19 cases of strong positive staining, the average survival time was 33.9 months and the 5-year survival rate was 26.3% ($p=0.002$) (table 3).

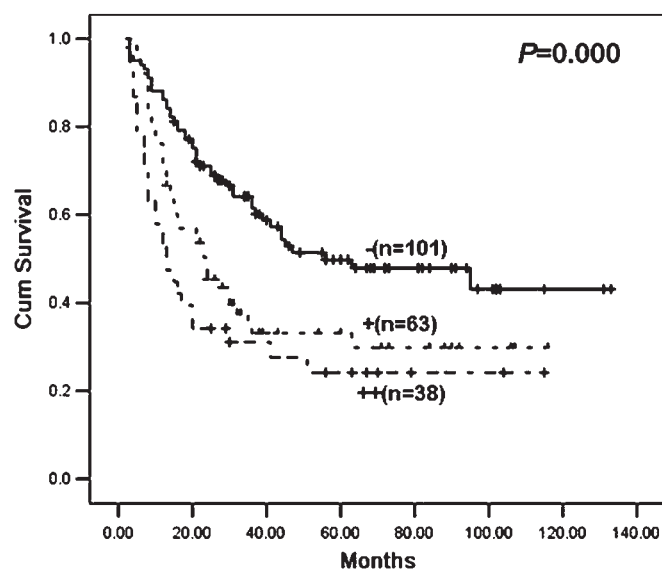


Figure 3 Kaplan–Meier analysis of survival by neutrophil gelatinase-associated lipocalin (NGAL) status in 202 cases of oesophageal squamous cell carcinoma (ESCC). The survival rate for patients with NGAL-positive expression was significantly lower than that for patients with NGAL-negative expression.

Table 2 Univariate and multivariate Cox regression analysis of neutrophil gelatinase-associated lipocalin (NGAL) status

NGAL status	Mean survival time (months)	Percentage of 5-year survival (95% CI)	p Value
Univariate			
Negative (—)	74.7	49.8% (3 to 115)	0.000**
Weak positive (+)	47.7	33.2% (5 to 107)	
Strong positive (++)	38.1	24.4% (4 to 104)	
Multivariate			
	HR	95% CI	p Value
Differentiation grade	1.607	1.2 to 2.2	0.005**
Location	0.643	0.4 to 0.9	0.017*
TNM stage	1.772	1.1 to 2.9	0.022*
NGAL status	1.893	1.5 to 2.4	0.000**

NGAL status: –, scores of 0–4; +, scores of 5–8; ++, scores of 9–12.

* $p<0.05$; ** $p<0.01$.

TNM, tumour, node, metastasis.

Furthermore, a multivariate Cox proportional hazards model revealed that NGAL status ($p=0.009$), histological differentiation grade ($p=0.023$), tumour location ($p=0.018$) and TNM stage ($p=0.004$) were all independent prognostic factors for overall survival (table 3).

NGAL/NGALR coexpression correlated with survival time of patients with ESCC

Considering the relationship of NGAL and NGALR as the ligand and the receptor, we also analysed the relationship between their coexpression and the survival time of matched patients with ESCC. The data showed that the expression pattern, NGAL (+)/NGALR(+), correlated with a shorter survival time for patients with ESCC. In contrast, the expression pattern, NGAL (–)/NGALR(–), favoured a longer survival time for patients with ESCC (figure 5).

Of the 131 ESCC cases, for 46 with the expression pattern of NGAL(–)/NGALR(–), the average survival time was 66.4 months and the 5-year survival rate was 52.2%; for 60 cases with NGAL

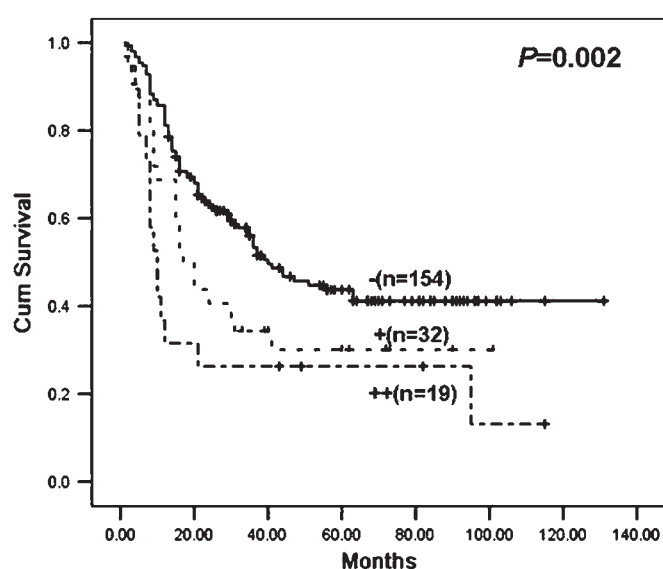


Figure 4 Kaplan–Meier curves showing a significantly different effect of neutrophil gelatinase-associated lipocalin receptor (NGALR) status on 205 patients with oesophageal squamous cell carcinoma (ESCC). NGALR-positive cases showed a trend towards shorter survival compared with NGALR-negative case.

Table 3 Univariate and multivariate Cox regression analysis of neutrophil gelatinase-associated lipocalin receptor (NGALR) status

NGALR status	Mean survival time (months)	Percentage of 5-year survival (95% CI)	p Value
Univariate			
Negative (—)	67.5	43.7% (4 to 102)	0.002**
Weak positive (+)	41.0	30.1% (3 to 101)	
Strong positive (++)	33.9	26.3% (4 to 115)	
Multivariate			
	HR	95% CI	p Value
Differentiation grade	1.482	1.1 to 2.1	0.023*
Location	0.637	0.4 to 0.9	0.018*
TNM stage	2.066	1.3 to 3.4	0.004**
NGALR status	1.421	1.1 to 1.8	0.009**

NGALR status: –, scores of 0–4; +, scores of 5–8; ++, scores of 9–12.

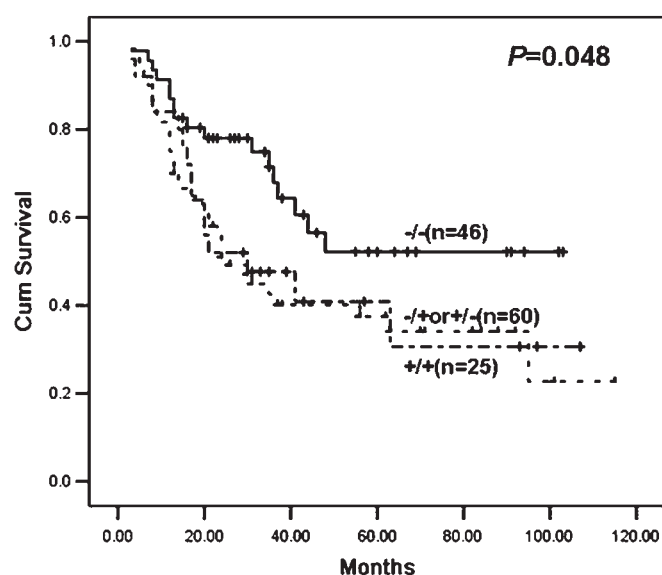
* $p < 0.05$; ** $p < 0.01$.

TNM, tumour, node, metastasis.

(–)/NGALR(+) and NGAL(+)/NGALR(–), the average survival time was 50.5 months and the 5-year survival rate was 37.5%; for the NGAL(+)/NGALR(+) coexpression group (25/131), the average survival time was 49.8 months and the 5-year survival rate was 40.9% ($p = 0.048$) (table 4). Multivariate Cox analysis to identify independent factors affecting patient outcomes showed that NGAL/NGALR status ($p = 0.026$), histological differentiation grade ($p = 0.009$), tumour location ($p = 0.018$) and TNM stage ($p = 0.000$) had significant prognostic value for the survival of patients with ESCC.

DISCUSSION

ESCC makes up more than 90% of oesophageal tumours in the Far East.¹⁷ In China, ESCC is the fourth most common malignancy with high mortality. The prognosis of patients with ESCC is still difficult to predict, and the emergence of effective new approaches for therapeutic and prognostic markers will depend on identifying the genes involved in the progression of this cancer.

**Figure 5** Kaplan–Meier estimates of the survival by neutrophil gelatinase-associated lipocalin (NGAL)/neutrophil gelatinase-associated lipocalin receptor (NGALR) status in 131 cases of oesophageal squamous cell carcinoma (ESCC). Positive expression of NGAL/NGALR was associated with a shorter survival time for patients with ESCC.**Table 4** Univariate and multivariate Cox regression analysis of neutrophil gelatinase-associated lipocalin (NGAL)/neutrophil gelatinase-associated lipocalin receptor (NGALR) status

NGAL/NGALR status	Mean survival time (months)	Percentage of 5-year survival (95% CI)	p Value
Univariate			
−/−	66.4	52.2% (7 to 102)	0.048*
−/+ or +/−	50.5	37.5% (4 to 101)	
+/+	49.8	40.9% (4 to 97)	
Multivariate			
	HR	95% CI	p Value
Differentiation grade	1.771	1.2 to 2.7	0.009**
Location	0.559	0.3 to 0.9	0.018*
TNM stage	3.150	1.9 to 5.2	0.000**
NGAL/NGALR status	1.432	1.0 to 1.9	0.026*

NGAL/NGALR status: –, scores of 0–4, +, scores of 5–12.

* $p < 0.05$; ** $p < 0.01$.

TNM, tumour, node, metastasis.

NGAL may be involved in diverse cellular processes, including transport of small hydrophobic molecules, protection against matrix metalloproteinase 9 proteolytic activity, and regulation of immune responses, in which NGAL functions as a carrier of signalling molecules.^{13 18 19} One of the most interesting and important biological functions of NGAL is to capture iron ions through the siderophore and then transport them into cells by binding to the specific membrane receptor, NGALR.¹⁴ Recently, we identified a CpG island within the NGALR promoter and showed that methylation of the island played a crucial role in the regulation of NGALR expression in ESCC.¹⁶ Moreover, raised NGAL was observed from mild to moderate to severe dysplasia of oesophageal epithelium, and both NGAL and NGALR were overexpressed in ESCC.^{13 16} These findings suggest that NGAL and NGALR may be involved in the progression of ESCC. Additionally, it has been reported that NGAL expression is highly suggestive of a poor prognosis or biomarker in other tumours, such as colon cancer, breast cancer and pancreatic cancer.^{7 20 21} These studies indicate that NGAL plays an increasingly important role and will probably become a diagnostic factor for a variety of tumours.

In the present study, we systematically investigated the patterns of NGAL and NGALR expression in ESCC and their effects on patient survival. High expression of NGAL and NGALR was observed in ESCC, but low expression in normal oesophageal tissue. The results show that expression of NGAL and NGALR did not correlate with any clinicopathological features. However, their coexpression was associated with histological differentiation grade. High expression of NGAL but low expression of NGALR has been reported in three independent chronic myelogenous leukaemia cells. In contrast, high expression of NGALR and low expression of NGAL was found in normal human peripheral blood lymphocytes.¹⁴ Our experimental results are not completely consistent with these previous reports, which indicated that NGAL may act synergistically with NGALR in the development of ESCC. However, it cannot be excluded that NGAL and NGALR may play different roles in solid tumours and non-solid tumours.

In this paper, we show that overexpression of NGAL was associated with poor prognosis of patients with ESCC. Similarly to NGAL, the higher expression of NGALR was associated with lower 5-year survival rate for patients with ESCC. These results obviously indicate that both NGAL and NGALR are independent prognostic factors of ESCC. To the best of our knowledge, this is

Take-home messages

- Tissue microarray technology and immunohistochemical study showed that neutrophil gelatinase-associated lipocalin (NGAL) and neutrophil gelatinase-associated lipocalin receptor (NGALR) are highly expressed in patients with oesophageal squamous cell carcinoma (ESCC). NGAL/NGALR coexpression correlated with histological differentiation grade.
- Kaplan–Meier survival curves and Cox proportional hazards regression model analysis indicated that positive expression of NGAL and/or NGALR was associated with a decreased 5-year survival rate for patients with ESCC.
- Multivariate analysis showed that both NGAL and NGALR are independent prognostic factors.

the first comprehensive immunohistochemical analysis to systematically reveal the coexpression of NGAL and NGALR and their relationship with prognosis for patients with ESCC, although the strong positive coexpression of NGAL/NGALR was found in a subset of ESCC. These results also imply that both NGAL and NGALR are involved in the development of ESCC. However, to confirm the hypothesis that NGAL/NGALR coexpression leads to formation of more aggressive tumours that require more aggressive treatment than others, it needs to be studied in a prospective way.

The interaction between ligands and their receptors involved in tumour progression has long been an important research area. It is generally believed that oestrogens regulate cellular responses through binding to two cognate receptors, ER α and ER β , which are ligand-regulated transcription factors with a broad range of physiological functions, especially in the pathological processes of hormone-dependent cancers, such as breast, endometrial, prostate, ovarian and thyroid.²² Notch–ligand interaction is a highly conserved mechanism which regulates specific cell fate decisions during cell development and maturation. Notch activation acts as a pro-oncogene in the onset and progression of many human malignancies, including lymphoid neoplasms, small cell lung cancer, neuroblastomas, skin cancer and cervical cancer.²³ It has been reported that forced overexpression of NGALR in HeLa cells induces binding and uptake of NGAL.¹⁴ However, the molecular mechanism by which this receptor mediates NGAL in vitro or in vivo remains to be determined in ESCC cells, especially when both are overexpressed.

In summary, we found that NGAL and NGALR are overexpressed in ESCC. When NGAL or NGALR was highly expressed, the prognosis of patients with ESCC was significantly worse, with shorter survival time, and their 5-year survival rate was significantly decreased. Both NGAL and NGALR are considered to be independent prognostic factors for ESCC. Measurement of NGAL and NGALR expression may help to provide novel prognostic factors and potentially therapeutic targets for patients with ESCC.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the ethics committee of the Central Hospital of Shantou City.

Contributors ZPD, ZL, BLW, EML and LYX conceived the study, participated in its design and coordination and the drafting of the manuscript. ZYW, JHS and QH provided clinical samples and background. ZPD, ZL, JS and XEX performed the immunohistochemical evaluation. ZPD, ZL, BLW, JYW, HBC and LYX contributed to the statistical analysis.

Provenance and peer review Not commissioned; externally peer reviewed.

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Correction

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