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Review Article

NGAL and MMP9 can be used as early diagnosis and prognosis markers for patients with non-small cell lung cancer

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Abstract: Objective: To explore the value of NGAL and MMP9 in early diagnosis and prognosis of non-small cell lung cancer (NSCLC) patients. Method: 97 NSCLC patients admitted to Shanxi Bethune Hospital were selected as observation group (OG) (37 cases of lung squamous cell carcinoma, 60 cases of lung adenocarcinoma). 25 cases of non-lung cancer patients were selected as control group (CG). The peripheral blood of all subjects was collected. Serum NGAL and MMP9 mRNA expressions were detected by fluorescence quantitative PCR. The correlation between them was analyzed. ROC curve was used to analyze the clinical value of serum NGAL and MMP9 mRNA for predicting different types of NSCLC patients. The patients' death was counted. The predictive value of NGAL and MMP9 mRNA expression on death was analyzed. Result: NGAL and MMP9 mRNA expressions in OG were higher than those in CG. Spearman correlation results showed that NGAL and MMP9 expressions were positively correlated. ROC curve showed that area under ROC curve (AUC) of NGAL and MMP9 mRNA expression in diagnosing lung squamous cell carcinoma was 0.759 (95% CI: 0.640~0.878) and 0.796 (95% CI: 0.685~0.906), respectively. AUC of NGAL and MMP9 mRNA expression in diagnosing lung adenocarcinoma was 0.764 (95% CI: 0.662~0.867) and 0.894 (95% CI: 0.829~0.959). According to the best cut-off value, patients were divided into high and low expression groups of NGAL and MMP9. The 3-year survival rate in NGAL high expression group [25.00% (12/48)] was significantly lower than that in NGAL low expression group [42.86% (21/49)]. The 3-year survival rate in MMP9 high expression group [28.57% (14/49)] was significantly lower than that in MMP9 low expression group [39.58% (19/48)]. Conclusion: NGAL and MMP9 were highly expressed in NSCLC patients, which may have predictive significance for early clinical diagnosis, treatment and prognosis of lung adenocarcinoma and lung squamous cell carcinoma.

Keywords: NGAL, MMP9, non-small cell lung cancer, early diagnosis, prognosis

Introduction

According to the cancer data in the world in 2018, the incidence rate of lung cancer (11.6%) and the mortality rate (18.4%) are the first among malignant tumors [1]. NSCLC is one of the common types of lung cancer. Because of its low specificity of symptoms and the absence of symptoms in early stage, most NSCLC patients have reached the middle and late stage of tumor without indication of surgical treatment when they are informed of the disease progress. Moreover, the early stage of NSCLC is easy to metastasize and has strong ability of invasion and growth, which leads to a high degree of malignancy. In clinical treatment of NSCLC, surgery or systemic radiotherapy combined with chemotherapy are commonly used, but the prognosis is not ideal due to late treatment time, decrease of immunity and other factors [2, 3]. At present, the expansion of CT application scope can effectively improve the early diagnosis rate of lung cancer, but it is accompanied by high false positive rate, high economic cost, radiation damage and other technical defects [4]. However, the sensitivity and specificity of clinical biomarkers used in the early diagnosis of NSCLC are relatively low. Therefore, relevant researchers are urgently looking for new tumor markers with economical, efficient and high sensitivity to improve the early diagnosis rate of NSCLC and predict the level of tumor progression.

Neutrophil gelatinase-associated lipocalin (NGAL) is a protein coding product distributed on various epithelial surfaces of the body, which can mediate signal transduction and tumor cell adhesion functions [5]. Matrix metalloproteinase (MMP)-9 regulates angiogenesis, invasion and metastasis of malignant tumor cells by degrading basement membrane and extracellular matrix [6]. However, there is no relevant research on the expression level of NGAL and MP9 in NSCLC patients and whether it can reflect the heterogeneity of NSCLC and provide basis for its early diagnosis. This study aimed to explore the expression of NGAL and MMP-9 in tissues of different NSCLC patients and analyze the correlation between NGAL and MMP-9 in NSCLC and their diagnostic efficacy for early diagnosis and prognosis of disease, so as to achieve the purpose of clarifying their predictive value in early clinical diagnosis and treatment of NSCLC patients. The details are as follows.

Materials and methods

Baseline data

From January 2014 to December 2015, 97 NSCLC patients admitted to Shanxi Bethune Hospital were selected as OG (37 cases of lung squamous cell carcinoma and 60 cases of lung adenocarcinoma) and 25 patients with non-lung cancer were selected as CG. There were 64 males and 33 females in OG, 17 males and 8 females in CG.

Inclusion criteria: All patients in OG were diagnosed as NSCLC by pathological examination; The degree of pathological differentiation was in accordance with WHO criteria for pathological differentiation [7]; Clinical staging was in accordance with the international 7th edition TNM staging standard for lung cancer [8].

Exclusion criteria: In CG, pulmonary inflammatory pseudotumor and pulmonary tuberculosis patients were highly suspected to be pulmonary malignancies by pathological examination; Patients with pulmonary disease with airway inflammation were excluded; Patients with other types of tumors were excluded in the past. The experiment was designed, and all subjects knew the procedure and signed the informed consent. The research scheme was fully known and approved by the Shanxi Bethune Hospital Ethics Committee (Ethics No.: 2018-288).

Main reagents

RNA-TRIzol kit and cDNA synthesis kit were both purchased from Thermophilic Science and Technology (China) Co., Ltd. (item no. 10296010 and 00309721). Real-time fluorescence quantitative PCR instrument was purchased from Zhengzhou North-South Instrument Equipment (China) Co., Ltd. The fluorescence quantitative PCR kit was purchased from Applied Biosystems Company in the United States (item no. 1204003). All primers and sequences were designed and synthesized by Beijing Dingsheng Company.

Fluorescent quantitative PCR and primer design

Samples were taken from liquid nitrogen irrigation. 100 mg of tissue was taken with a centrifuge tube and loaded into a homogenizer treated with 0.1% DEPC. A little liquid nitrogen was added and ground into frozen powder samples. The integrity of RNA was detected by agarose gel electrophoresis. The absorbance of RNA was determined by ultraviolet spectrophotometer. According to the RNA extraction instructions, 8 μL of total RNA of the sample was extracted. Then 2 μL of total RNA was taken and reverse transcribed into cDNA by MLV inversion system for real-time fluorescence quantitative PCR. The rest was stored at -70°C. The reaction conditions were pre-denaturation at 94°C for 4 min, 94°C for 45 s, 58°C for 55 s and extension at 72°C for 50 s, with a total of 40 cycles. All samples were tested in 3 tubes in parallel. All PCR products were subjected to 2% agarose gel electrophoresis for DNA recovery and sequencing to detect their specificity. The ΔCt of all tested samples were compared and analyzed. NGAL upstream was 5'-GAAGACAAAGACCCGCAAAAG-3', downstream was 5'-CTGGCAACCTGGAACAAAAG-3' and target product was 135 bp. The upstream and downstream primers of MMP-9 were 5'-CGACGTCTTCCAGTACCGAG-3' and 5'-TTGTATCCGGCAAACTGGCT-3', respectively, and the target product was 82 bp. β-actin upstream was 5'-GAGCTACGAGCTGCCTGACG-3', downstream was 5'-GTAGTTTCGTGGATGCCACAG-3' and target product was 120 bp.

Table 1. Comparison of baseline data of patients in the three groups [n (%)]

Items	Lung squamous cell carcinoma group (n=37)	Lung adenocarcinoma group (n=60)	CG (n=25)	X^2	P
Gender				1.070	0.586
Male	23 (62.16)	41 (68.33)	17 (68.00)		
Female	14 (37.84)	19 (31.67)	8 (32.00)		
Age					
<60	16 (43.24)	26 (43.33)	11 (44.00)		
≥60	21 (56.76)	34 (56.67)	14 (56.00)		
Smoking history				0.080	0.961
Smoking	18 (48.65)	28 (46.67)	12 (48.00)		
No smoking	19 (51.35)	32 (53.33)	13 (52.00)		
Drinking history				1.587	0.452
Drinking	29 (78.38)	47 (78.33)	18 (72.00)		
No drinking	8 (21.62)	13 (21.67)	7 (28.00)		
Liver diseases				0.129	0.938
Yes	26 (70.27)	42 (70.00)	18 (72.00)		
No	11 (29.73)	18 (30.00)	7 (28.00)		
Cardiovascular diseases				0.384	0.825
Yes	12 (32.43)	21 (35.00)	9 (36.00)		
No	25 (67.57)	39 (65.00)	16 (64.00)		
Renal diseases				0.985	0.611
Yes	20 (54.05)	36 (60.00)	15 (60.00)		
No	17 (45.95)	24 (40.00)	10 (40.00)		
Infection				0.815	0.665
Yes	27 (72.97)	44 (73.33)	17 (68.00)		
No	10 (27.03)	16 (26.67)	8 (32.00)		

Observation indexes

(1) To compare the baseline data among all patients participating in the experiment; (2) To compare the expression levels of NGAL and MMP9 mRNA in all patient tissues and analyze the correlation between the two; (3) To observe and analyze the diagnostic value and prognostic value of NGAL and MMP9 expression for different NSCLC by ROC curve.

Statistical methods

SPSS 19.0 was used to carry out statistical analysis on the collected data. The measurement data conforming to normal distribution were expressed as mean number ± standard deviation. The counting data were all expressed as cases (%). The comparison between groups was conducted by χ^2 test. Spearman correlation was used to test the correlation between NGAL and MMP9 expression. Kaplan-Meier and Log-rank tests were used to calculate the survival curve of patients. ROC curve was used to analyze the diagnostic value and prognostic value of NGAL and MMP9 expression for different NSCLC. The difference was statistically significant with $P<0.05$.

Results

Comparison of baseline data

Compared with the CG, NSCLC patients with two different types of squamous cell carcinoma and adenocarcinoma had no significant difference in the past history of primary disease, age, gender and other baseline data ($P>0.05$), while there was no significant difference in tumor staging, vascular and nerve invasion between lung squamous cell carcinoma group and lung adenocarcinoma group ($P>0.05$), providing relatively stable data for comparison in the experiments. More details are shown in **Tables 1** and **2**.

Table 2. Comparison of baseline data of NSCLC patients [n (%)]

Items	Lung squamous cell carcinoma group (n=37)	Lung adenocarcinoma group (n=60)	X^2	P
Tumor stage			0.321	0.571
Stage I-II	18 (48.65)	27 (45.00)		
Stage III-IV	19 (51.35)	33 (55.00)		
Differentiation degree			0.570	0.450
Well differentiated	11 (29.73)	21 (35.00)		
Poorly and middle differentiated	26 (70.27)	39 (65.00)		
Vascular invasion			2.261	0.133
Yes	23 (62.16)	43 (71.67)		
No	14 (37.84)	17 (28.33)		
Perineuronal invasion			1.345	0.246
Yes	21 (56.76)	39 (65.00)		
No	16 (43.24)	21 (35.00)		
Pleural invasion			1.496	0.221
Yes	24 (64.86)	44 (73.33)		
No	13 (35.14)	16 (26.67)		

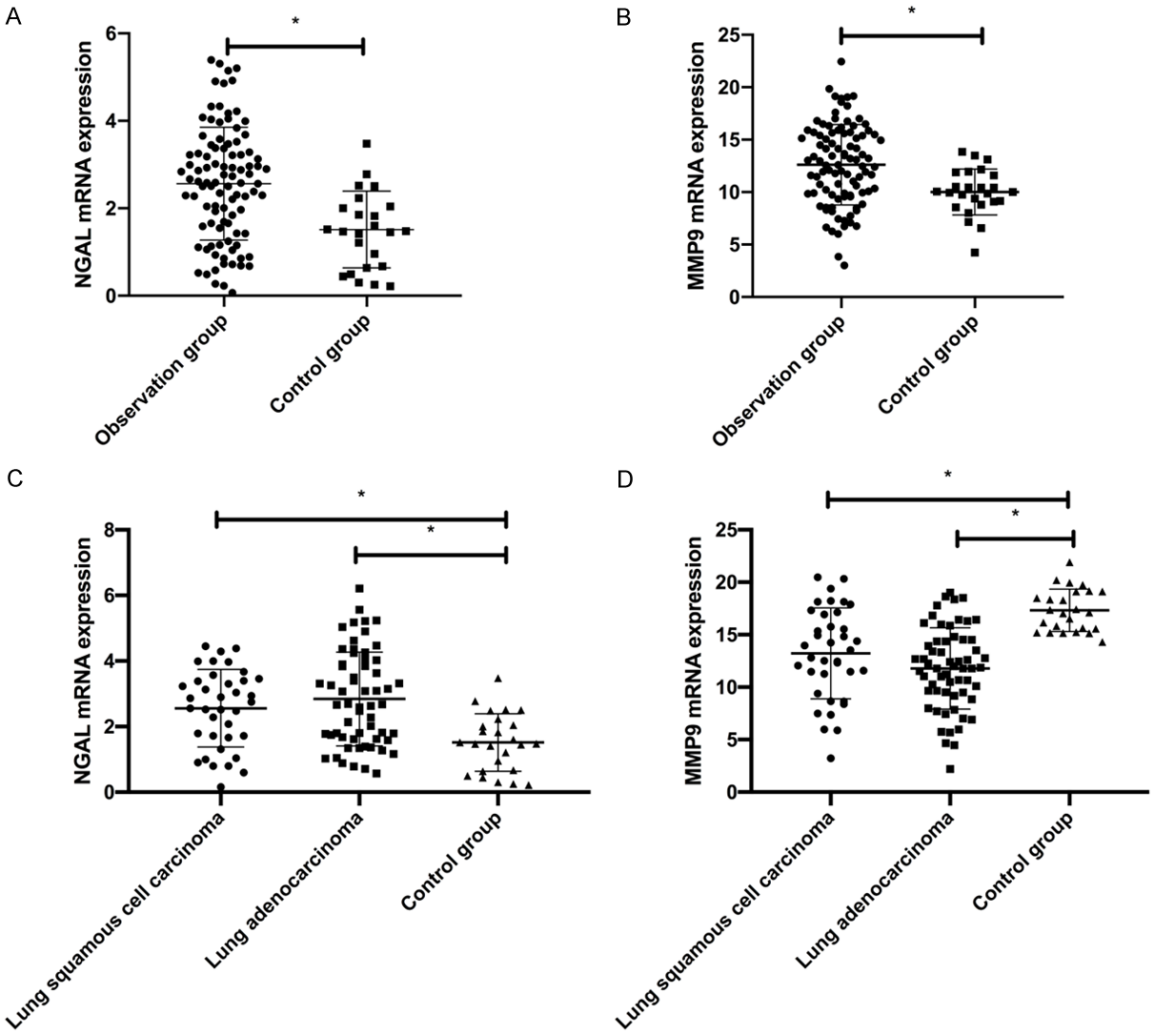

Figure 1. Comparison of NGAL and MMP9 mRNA expression levels of patients in each group. A. NGAL mRNA expression of all patients in OG (2.53±1.37) was significantly higher than that in CG (1.45±0.96). B. MMP9 mRNA expression of all patients in OG (12.62±3.78) was significantly higher than that in CG (9.38±2.14). C. NGAL mRNA expressions in lung squamous cell carcinoma and lung adenocarcinoma tissues were 2.33±1.23 and 2.86±1.47 respectively, which were significantly higher than those in CG. D. MMP9 mRNA expressions in lung squamous cell carcinoma and lung adenocarcinoma tissues were 13.42±4.42 and 11.65±3.52 respectively, which were significantly higher than those in CG. Note: *represents P<0.05.

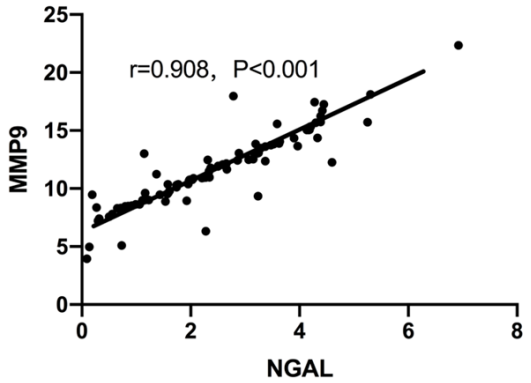

Figure 2. Detection of correlation between NGAL and MMP9 expression by Spearman correlation. Spearman correlation results showed that NGAL and MMP9 expressions were positively correlated (r=0.908, P<0.001).

Comparison of NGAL and MMP9 mRNA expression levels of patients in each group

The expressions of NGAL and MMMP9 mRNA were 2.33±1.23 and 13.42±4.42 respectively in lung squamous cell carcinoma tissues, and 2.86±1.47 and 11.65±3.52 respectively in lung adenocarcinoma tissues. However, the expressions of NGAL and MMP9 mRNA were 2.53±1.37 and 12.62±3.78 respectively in OG and 1.45±0.96 and 9.38±2.14 respectively in CG in all patients. The expressions of NGAL and MMP9 mRNA of patients in OG were higher than those in CG (P<0.05). More details are shown in **Figure 1**.

Detection of correlation between NGAL and MMP9 expression by Spearman correlation

Spearman correlation results showed that NGAL and MMP9 expressions were positively correlated (r=0.908, P<0.001). More details are shown in **Figure 2**.

Analysis of diagnostic efficacy of NGAL, MMP9 mRNA expression for different NSCLC

ROC curve showed that the area under ROC curve (AUC) of NGAL and MMP9 mRNA expression in diagnosing lung squamous cell carcinoma was 0.759 (95% CI: 0.640~0.878) and 0.796 (95% CI: 0.685~0.906), respectively. AUC of NGAL and MMP9 mRNA expression in diagnosing lung adenocarcinoma was 0.764 (95% CI: 0.662~0.867) and 0.894 (95% CI: 0.829~0.959), respectively. More details are shown in **Figure 3**.

Effects of high and low expression of NGAL and MMP9 on prognosis of NSCLC patients

The discharged patients were followed up for 3 years and there were no missed cases. According to the best cut-off value, the patients were divided into high and low expression groups of NGAL and MMP9. The 3-year survival rate in NGAL high expression group [25.00% (12/48)] was significantly lower than that in NGAL low expression group [42.86% (21/49)]. The 3-year survival rate in MMP9 high expression group [28.57% (14/49)] was significantly lower than that in MMP9 low expression group [39.58% (19/48)] (P<0.05). More details are shown in **Figure 4**.

Discussion

The number of people suffering from lung cancer has increased sharply due to the changes of environment and living habits such as worsening air pollution, increasing number of smokers and population aging. Lung cancer is a respiratory system disease that is prone to occur in bronchial mucosa and glandular tissue, accompanied by lymph node and blood metastasis [9, 10]. Early detection of lung cancer can improve the chemotherapy sensitivity and treatment effectiveness of patients. Staging judgment of lung cancer patients becomes one of the conditions for evaluating the survival rate of patients [11, 12]. However, due to the limited means of early prediction and diagnosis for lung cancer in clinic, the long-term survival improvement effect of patients including NSCLC is not significant. Tumor marker detection, as a new diagnostic technology with reusable, simple, efficient and obvious

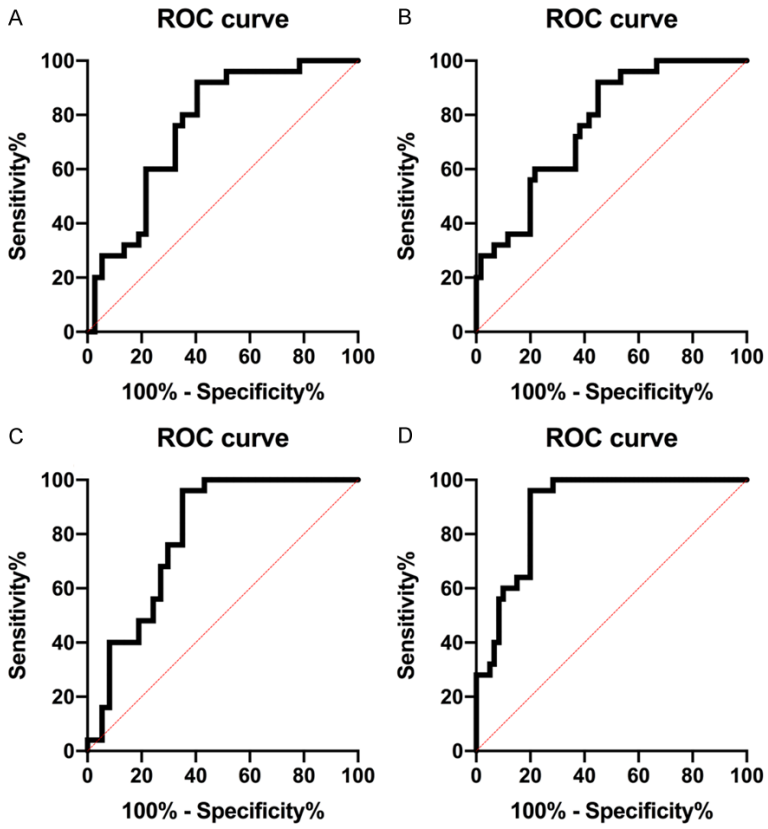

Figure 3. Analysis of diagnostic efficacy of NGAL, MMP9 mRNA expression for different NSCLC. A. ROC curve showed that the area under ROC curve (AUC) of NGAL mRNA expression in diagnosing lung squamous cell carcinoma was 0.759 (95% CI: 0.640~0.878). B. ROC curve showed that the AUC of NGAL mRNA expression in diagnosing lung adenocarcinoma was 0.764 (95% CI: 0.662~0.867). C. ROC curve showed that the area under ROC curve (AUC) of MMP9 mRNA expression in diagnosing lung squamous cell carcinoma was 0.796 (95% CI: 0.685~0.906). D. ROC curve showed that the AUC of MMP9 mRNA expression in diagnosing lung adenocarcinoma was 0.894 (95% CI: 0.829~0.959).

advantages, has a high frequency of clinical application. Through research and clinical practice, it has been found that there is extremely important value in the diagnosis, evaluation and prognosis of malignant tumors. However, at present, a tumor marker with specificity and sensitivity reaching appropriate standards that can effectively detect tumors early has not been found [13]. As one of the newly discovered apolipoprotein family members secreted into the blood circulation, NGAL is involved in and regulated in various processes such as apoptosis differentiation, lipid metabolism, verification of immune response and development and progression of tumors [14-18]. MMP9 originates from proenzyme formed by inflammatory cells and stromal cells invaded by tumor cells. After successful activation outside the cell through a series of protease cascades from the intracellular secretion to the extracellular, MMP9 initiates specific degradation of extracellular matrix and type IV collagen [19]. In this paper, the diagnostic efficacy of NGAL and MMP9 in NSCLC was explored. The correlation between them, the regulatory mechanism of NSCLC and how to affect the prognosis effect were analyzed.

Firstly, we recorded the expression records of NGAL and MP9 in patients with different types of NSCLC and non-lung cancer selected in this experiment and found that the expression of NGAL and MMP9 mRNA in all patients in OG was higher than that in CG. NGAL plays an important role in maintaining cellular homeostasis and immune regulation, but it is highly expressed in malignant tumors such as liver cancer, pancreatic cancer, ovarian cancer, and colorectal cancer under the state of infection, which may be related to its anti-infection effect [20-23]. MMP9 is over-expressed in many epithelial tumors or interstitial tumors including esophageal cancer and cervical cancer [24, 25]. It suggested that NGAL and MMP9 may play a role in regulating the progression of NSCLC, but there are few reports on them and NSCLC. We further found that the expression of the two in NSCLC was up-regulated and positively correlated. Previous studies [20, 26] have shown that NGAL is a protein with a molecular weight of 92 kD derived from the covalent bond between neutrophils and MMP-9. After NGAL/MMP-9 dimer formation and transformation, NGAL can produce protective reaction when MMP-9 is degraded by protease, which makes MMP-9 to play a strong gelatin decomposition function in cell matrix. Similarly, the enhancement of MMP-9

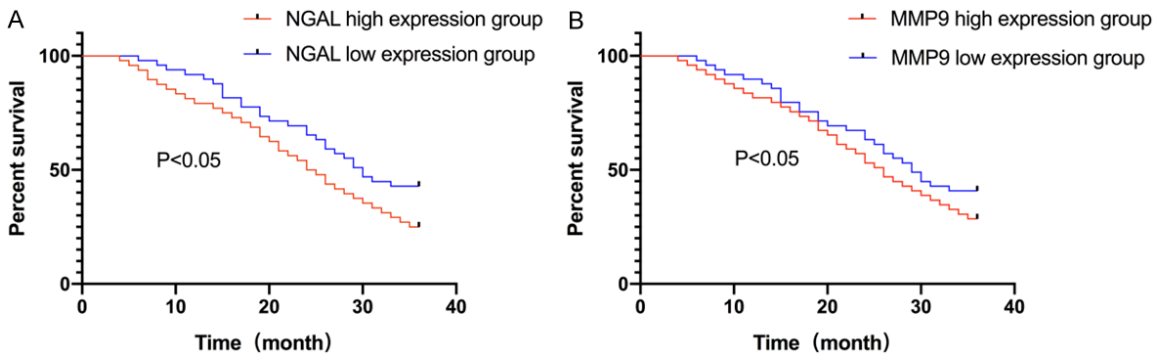

Figure 4. Effects of high and low expression of NGAL and MMP9 on prognosis of NSCLC patients. According to the best cut-off value, the patients were divided into high and low expression groups of NGAL and MMP9. A. The 3-year survival rate of NGAL high expression group [25.00% (12/48)] was significantly lower than that of NGAL low expression group [42.86% (21/49)] (P<0.05). B. The 3-year survival rate of MMP9 high expression group [28.57% (14/49)] was significantly lower than that of MMP9 low expression group [39.58% (19/48)] (P<0.05).

activity also promotes tumor invasion and metastasis. The possibility of NGAL and MMP9 in regulating the development and progression of NSCLC was further proved. In order to obtain evidence of the early diagnostic value of NGAL and MMP9 for NSCLC, we analyzed the diagnostic value of lung squamous cell carcinoma and lung adenocarcinoma and explored the ROC curve in this study. The results showed that in the diagnosis of NSCLC patients with different clinicopathological types, the area under ROC curve and youden index of NGAL and MMP9 were at the middle and higher levels, significantly reducing the occurrence of missed diagnosis and misdiagnosis. This suggested that the expression of NGAL and MMP9 mRNA may be non-invasive biomarkers for NSCLC, which has high clinical significance in the diagnosis of lung squamous cell carcinoma and lung adenocarcinoma. The observation of prognosis showed that the 3-year survival rate of NGAL and MMP9 high expression groups was significantly lower than that of all low expression groups. This indicated that high expression of NGAL and MMP9 was unfavorable to the prognosis of patients with NSCLC. Previous studies have shown that [27] NGAL relies on iron ion transport to regulate lung cancer cells in lung adenocarcinoma tissues and reduces the accumulation of intercellular E-cadherin, thus achieving the effect of promoting invasion and metastasis of new cancer cells. Studies have also shown [28] that MMPs has strong cell matrix dissolving ability and can stimulate angiogenesis through substances such as collagen in extracellular matrix to provide conditions for tumor metastasis. Combined with the literature, our research results were confirmed to explain the effect of over-expressed NGAL and MMP9 on the condition of patients with NSCLC and their diagnostic predictive value.

To sum up, NGAL and MMP9 were highly expressed in tissues of patients with NSCLC. The high level of NGAL and MMP9 expression indicated the malignant degree and poor prognosis of NSCLC, which can be used to predict the early clinical diagnosis of NSCLC, but has limited significance in predicting the malignant degree and poor prognosis of NSCLC. The limitation of this experiment is only testing abnormal expression of NGAL and MMP9 in NSCLC tissues, which is not enough to explain the correlation between NGAL and MMP9 and the development, progression, proliferation and metastasis of NSCLC. Compare the effect of a single test, we also does not explore the effect of the combined use of the two detection methods on the diagnosis and prognosis of NSCLC, so as to achieve a better and more efficient diagnosis level. In order to provide a better diagnosis and treatment idea and theoretical basis for NSCLC patients, it is necessary to carry out in-depth and improvement on the cell and gene research in the future.

Disclosure of conflict of interest

None.

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