Aberrant Maspin mRNA Expression is Associated with Clinical Outcome in Patients with Pulmonary Adenocarcinoma

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to investigate the expression level of maspin mRNA in pulmonary adenocarcinoma and to clarify its clinical significance in prediction of prognosis.

Material/Methods: RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks of 30 pairs of pulmonary adenocarcinoma (AC) tissues and adjacent noncancerous tissues (ANT) and in another 81 AC tissues. Expression of maspin mRNA was tested by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and the potential relationship between maspin mRNA expression and clinic pathological features of AC patients was analyzed.

Results: The expression of maspin mRNA was upregulated in AC samples compared with the ANT ($p<0.001$). Patients at advanced clinical stage (III) and patients with lymphatic metastasis showed higher maspin mRNA expression level than those in early-stage patients (I and II) ($p=0.038$) or with non-lymphatic metastasis ($p=0.034$). The Kaplan-Meier survival curves indicated that disease-free survival (DFS) was significantly worse in high maspin mRNA expression AC patients ($p=0.007$). Furthermore, multivariate analysis revealed that the expression of maspin mRNA was an independent prognostic marker for AC ($p=0.040$).

Conclusions: Our study reveals that maspin mRNA was significantly up-regulated in tissues of AC patients. Maspin mRNA may be useful as a new marker of prognosis in AC.

MeSH Keywords: **Adenocarcinoma, Bronchiolo-Alveolar • Gene Expression Profiling • Prognosis • Serine Proteinase Inhibitors**

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Background

Lung cancer is one of the most common malignancies worldwide, and its incidence is significantly increasing, especially in China [1,2]. Non-small-cell lung cancer (NSCLC) is the most frequent type of lung cancer, accounting for 80–85% of all lung cancer cases. Patients with NSCLC are often diagnosed at advanced stage. Invasion and metastasis of neoplasms are the major cause of cancer mortality [3]. Overall survival of this disease is less than 10%, and the 5-year survival rate is only about 20–30% after surgery [4]. Therefore, more efforts, such as detecting the gene expression profiling of NSCLC by bioinformatics methods and investigation of the underlying mechanisms, are needed to block the tumor progression and improve the survival of patients [5]. NSCLC can be further divided into several histological subtypes, including squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma. Pulmonary adenocarcinoma (AC) is the most frequently diagnosed NSCLC, representing 35–40% of all lung cancers and originates more commonly from epithelial cells at the terminal bronchioles/alveoli in the periphery of the lung.

The serine protease inhibitors (Serpins) superfamily was first identified as a set of proteins which can inhibit proteases [6]. The clade B serpins (SERPINB family) is the largest human serpin superfamily, containing 13 genes located on chromosome 6p25 (SerpinB1, SerpinB6 and SerpinB9) and 18q21 (the remaining members of the family) [7], all of which were identified as important regulators of malignant transformation [8]. SerpinB5, also known as maspin (mammary serine protease inhibitor), is an epithelial-specific member of the serpin superfamily, with tumor-suppressing activity and is expressed predominantly in human mammary epithelial cells [9,10]. The differential expression of maspin protein has been reported to predict a better prognosis for several types of carcinomas, including breast, prostate, colon, and oral squamous cell carcinoma [11]. In lung cancer, nuclear localization, rather than a combined nuclear and cytoplasmic localization, of maspin protein has been reported to segregate with increased overall survival in early-stage pulmonary adenocarcinoma, and maspin protein shows distinct patterns of expression in different histological subtypes of NSCLC [12]. However, the expression of maspin mRNA in AC, especially in early-stage adenocarcinoma (without distant metastasis), remains unclear, as does the diagnostic and prognostic potential of maspin mRNA.

In this pilot study we verified the endogenous expression levels of maspin mRNA from primary tumor specimens of early-stage AC patients. Then we studied the clinical significance of maspin mRNA by analyzing the potential relationship between maspin mRNA levels and clinicopathological features of early-stage AC patients, and also evaluated its correlation with overall survival of patients.

Material and Methods

Study samples

All 111 human early-stage AC tissue specimens were provided by the First Affiliated Hospital of Nanjing Medical University. Patients were histopathologically diagnosed as having pulmonary adenocarcinoma at our hospital from 2003 to 2009. The exclusion criteria included previous other disease of the respiratory system (e.g., bronchial asthma, chronic bronchitis, chronic obstructive pulmonary disease, chronic pulmonary heart disease, and pulmonary tuberculosis), metastasized cases, and previous radiotherapy or chemotherapy patients. All patients were staged based on the International Association for the Study of Lung Cancer (IASLC) Tumor-Node-Metastasis (TNM) classification, 7th edition [13]. Written informed consent was obtained from all patients involved in the study. The study was performed with the approval of the hospital ethics committee.

Tumor selection

The histopathological slides with hematoxylin-eosin (H&E) staining were microscopically reviewed to select the tumor blocks with preserved, viable tumor tissue comprising over 50% of tumors in the paraffin blocks. The tumor area was marked and cut. Slides with large areas of necrosis were excluded from the study. Two pieces of 10-μm-thick sections were cut from each selected block marked for tumor areas and avoiding normal tissues [14].

RNA extraction and real-time PCR

RNA was extracted from the FFPE tumor samples using Qiagen kits according to the manufacturers' instructions. The maspin mRNA expression level relative to GAPDH mRNA level was measured by RT-qPCR using TaqMan probes, as described previously [14].

Statistical analysis

All statistical analyses were done with SPSS 19.0 statistical software. Differences between groups were evaluated by the t test for continuous variables and χ^2 test for categorical variables. The associations between maspin expression and prognosis of patients were analyzed using the Kaplan-Meier method, and differences in survival were estimated by using the log-rank test. Disease-free survival was defined as the time from surgery to the time of first evidence of radiographic metastatic disease or AC-specific death. Prognostic factors were examined by univariate and multivariate analyses (Cox proportional hazards regression model). P value <0.05 indicates a statistically significantly difference.

Table 1. The relationship between Maspin expression and clinicopathologic parameters.

Characteristics	No. of patients	High expression		Low expression		*P* value
Age (years)						0.527
≤60	74	39	(52.7%)	35	(47.3%)	
>60	37	20	(54.1%)	17	(45.9%)	
Gender						0.512
Male	50	27	(54.0%)	23	(46.0%)	
Female	61	32	(52.5%)	29	(47.5%)	
Tumor size (cm)						0.106
≤3	76	45	(59.2%)	31	(40.8%)	
>3	35	21	(60.0%)	14	(40.0%)	
T stage						0.179
I	49	29	(59.2%)	20	(11.8%)	
II	52	26	(50.0%)	26	(42.3%)	
III	6	1	(16.7%)	5	(16.7%)	
IV	4	3	(75.0%)	1	(25.0%)	
N stage						0.045*
N0	60	36	(60.0%)	24	(40.0%)	
N1	17	9	(52.9%)	8	(47.1%)	
N2	33	14	(42.4%)	19	(57.6%)	
N3	1	1	(100%)	0	(0.0%)	
TNM stage						0.309
I	52	31	(59.6%)	21	(40.4%)	
II	22	12	(54.5%)	10	(45.5%)	
III	37	16	(43.2%)	21	(56.8%)	

* *P*<0.05.

Results

Patients' characteristics

The characteristics of the 111 AC patients enrolled are summarized in Table 1, including 50 male and 61 female, with a median age of 58 (range: 36–72 years) years. Follow-up lasted until 30 March, 2014, with a median period of 40.4 months for living patients (range: 4.0–76.2 months). During the follow-up time, 29 deaths from AC were observed. For all 111 cases, there were 52 (46.9%), 22 (19.8%), and 37 (33.3%) patients of clinical stage I, II, and III, respectively.

Maspin is upregulated in human pulmonary AC tissues and correlated with progression of AC

To analyze the expression of maspin in pulmonary AC, we firstly assessed 30 pairs of cancer tissues and adjacent normal tissues using the RT-qPCR. As shown in Figure 1A, Maspin expression was significantly increased in AC tissues than that of ANT (p<0.001) and was observed in 20 (66.7%) cases (Figure 1B). Further analysis demonstrated that the expression of maspin in patients with lymphatic metastases and advanced TNM stage (III) was higher than those with non-lymphatic metastases (p=0.034, Figure 2A) and early TNM stage (I and II) (p=0.038, Figure 2B).

Maspin expression and clinicopathologic features in AC

To assess the association of maspin expression with clinicopathologic data, the cases were divided into a low-maspin group and a high-maspin group according to the median value. As shown in Table 1, the maspin level was associated with N stage (i.e., lymph node metastasis) (P=0.045). However, there was no significant correlation between maspin expression and other clinicopathological features, such as age, sex, tumor size, depth of invasion (i.e., T stage), or TNM stage (P>0.05).
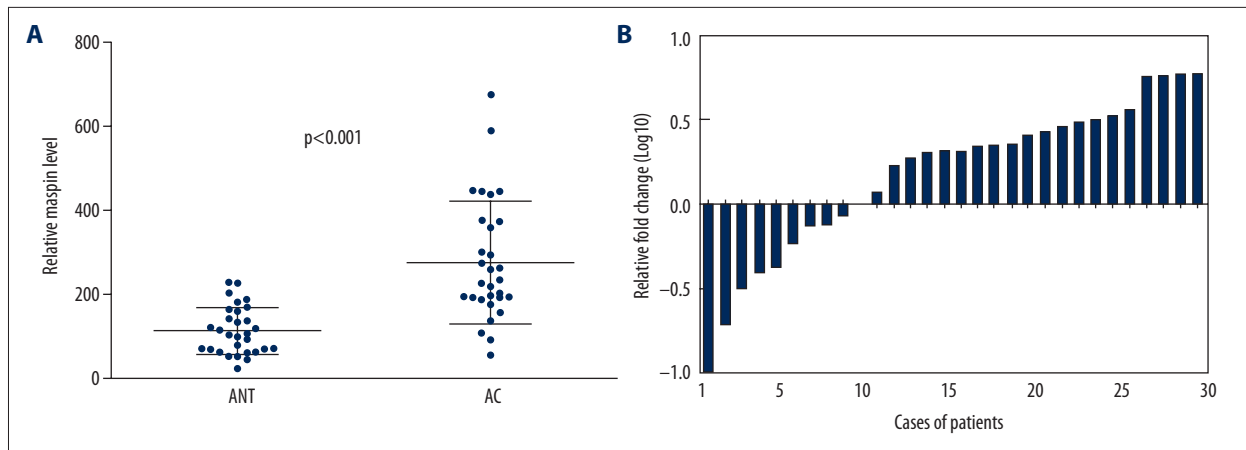
Figure 1. Detection of relative maspin mRNA expression in human AC tissues. (**A**) qRT-PCR was performed to detect the relative maspin expression in 30 pairs of AC tissues (AC) and corresponding noncancerous lung tissues (ANT). The mean expression level of maspin in AC tissues was significantly higher than that in ANT (P<0.001) (**B**). High maspin expression levels were observed in AC tissues in 20 (66.7%) cases. GAPDH was used as an internal control.
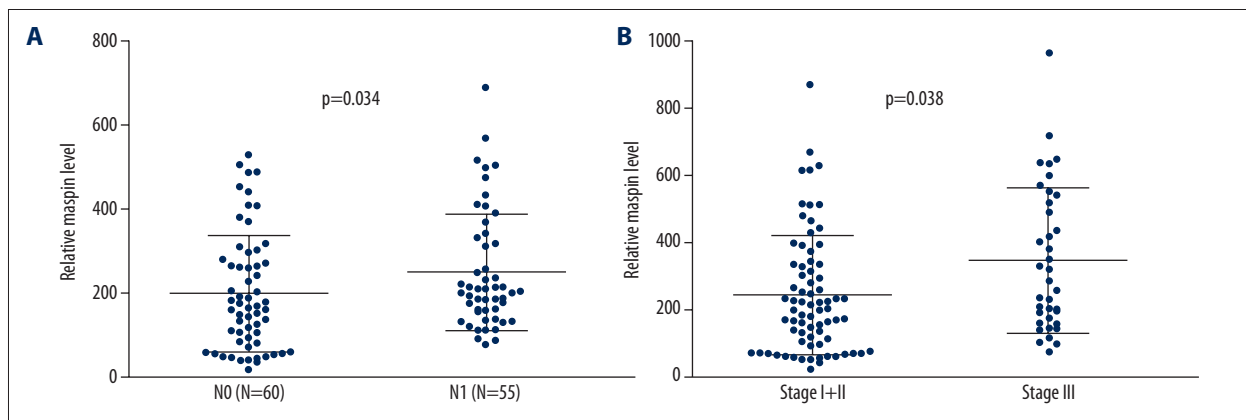

Figure 2. High levels of maspin mRNA are correlated with progression in AC. (**A**) AC patients with lymphatic metastasis displayed significantly higher maspin expression levels (P=0.034). (**B**) AC patients with advanced TNM stage (III) displayed significantly higher maspin expression levels (P=0.038).

Maspin expression is a prognostic factor for disease-free survival of AC patients

To study the correlation between maspin and prognosis, we used the long-rank test by performing Kaplan-Meier survival analysis. The maspin-high group showed obvious shorter DFS compared with the low-maspin group, with the DFS of 37.3 months and 59.6 months, respectively (log-rank chi-square=4.416, P=0.007; Figure 3). Further studies were performed by univariate and multivariate analysis for the prognostic factors of disease-free survival in AC patients. Data are summarized in Table 2. In Cox univariate analysis, tumor size, T stage, N stage, TNM stage, and maspin expression were significantly correlated with disease-free survival (P=0.010, P=0.000, P=0.000, P=0.000, and P=0.007, respectively.). Cox multivariate analysis defined T stage, TNM stage, and maspin expression as independent prognostic factors for disease-free survival (P=0.006, P=0.000, and P=0.040, respectively).

Discussion

Maspin is an epithelial-specific tumor suppressor that participates in the regulation of various physiological and pathological processes, including cell adhesion, apoptosis, and the inhibition of cell invasion, motility, and angiogenesis [15]. Consistent with its antitumor properties, maspin protein downregulation has been found in human prostate cancer [16], gastric cancer [17], and breast [10] cancer. However, it was also found to be overexpressed in pancreatic [18], thyroid [19], gallbladder [20], colorectal [21], and ovarian [22] cancers. These contradictory results suggest that maspin protein may play a complex role in the tumorigenesis of different cancers through different molecular mechanisms. However, to the best of our knowledge, until now no primary study has focused on the expression level and clinical significance of maspin mRNA in human pulmonary adenocarcinoma (AC).
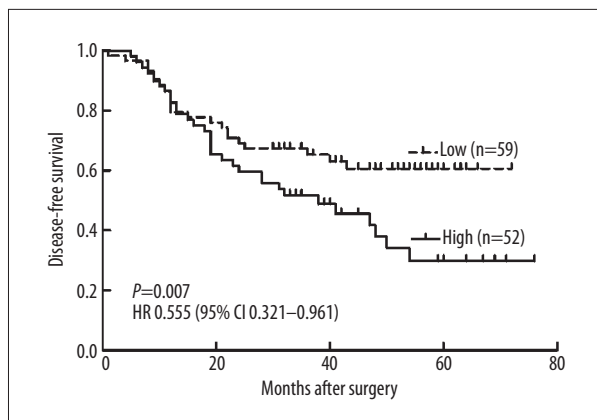
Figure 3. Prognostic value of maspin mRNA in AC patients. Kaplan-Meier analysis shows that the DFS rates in the maspin low-expression group (n=59) were significantly higher than those of patients in the maspin high-expression group (n=52) (P=0.007).

In this study, through detecting the mRNA expression of FFPE tumor samples using qRT-PCR assay, we provide the first signature of maspin mRNA in AC, which was significantly upregulated in most early-stage AC tissues. Meanwhile, we analyzed the correlation of maspin mRNA expression with different clinicopathologic parameters (including depth of tumor invasion, lymphatic metastasis, and TNM stage). We found that maspin mRNA expression was higher in patients with lymphatic metastasis and with advanced clinical stage (III). Taken together, these results suggest that maspin mRNA might be involved in the progression of pulmonary AC.

Nakashima et al. [23] reported on the prognostic role of maspin protein in 78 cases of AC. They revealed that increased maspin expression was a significant factor in predicting a favorable prognosis in AC patients. Frey et al. [24] reported that in stage I AC, high nuclear maspin protein expression predicted favorable

overall survival. Other studies also strongly suggested that cytoplasmic pattern predicts good survival for AC cases [25,26]. These results are not consistent with our findings. One reason for the differences between these results might be the different level assessed, since we analyzed maspin mRNA expression using qRT-PCR, and the previous study focused on the expression of maspin protein and used immunohistochemistry. In addition, previous studies analyzed the prognostic implications of maspin protein based on independent intracellular localization, whereas we focused on the total mRNA expression of maspin in AC tissues.

This study had some limitations. Firstly, nuclear and cytoplasmic RNA should be extracted to determine maspin mRNA localization and expression, and prognostic implications in AC tissues. Secondly, the significant association observed in our study should be further verified in larger-scale studies including distant-metastatic cancers.

Conclusions

Our results demonstrated that the expression of maspin mRNA was significantly upregulated in early-stage pulmonary AC tissues. Patients with positive-lymphatic metastasis or advanced clinical stage (III) showed higher maspin mRNA expression than those with non-lymphatic metastasis or early-stage patients (I and II). Furthermore, the upregulation of maspin mRNA was an unfavorable prognostic factor for DFS in addition to T stage and TNM stage. These findings suggested that maspin mRNA might be a novel prognostic indicator in pulmonary AC.

Competing interests

The authors declare that they have no competing interests.

Table 2. Univariate and multivariate analysis of different prognostic factors for disease-free survival in 111 patients with AC.

Prognostic factors	HR	95% CI	P value	HR	95% CI	P value
Age (≤60/>60)	0.995	0.959–1.034	0.814			
Gender (male/female)	1.010	0.221–1.476	0.489			
Smoking status (yes/no)	1.211	0.936–1.933	0.673			
Tumor size (≤3 cm/>3 cm)	0.489	0.284–0.844	0.010*			
T stage	2.110	1.537–2.898	0.000*	1.602	1.148–2.234	0.006
N stage	1.839	1.379–2.453	0.000*			
TNM stage (I–II/III)	2.096	1.535–2.862	0.000*	1.836	1.312–2.570	0.000*
Maspin (low/high)	1.391	1.094–1.768	0.007*	1.563	1.325–1.975	0.040*

HR – hazard ratio; CI – confidence interval. * $P<0.05$.

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