

Clinicopathological and prognostic significance of metastasis-associated protein 1 expression and its correlation with angiogenesis in lung invasive adenocarcinomas, based on the 2011 IASLC/ATS/ERS classification

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Abstract. Based on previous findings regarding the angiogenic activities and prognostic roles of metastasis-associated protein 1 (MTA1) in early-stage non-small cell lung cancer, the clinicopathological and prognostic significance of MTA1 protein expression, and its correlation with angiogenesis in lung invasive adenocarcinoma, were further assessed in the present study, according to the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. High protein expression levels of MTA1 were commonly observed in patients with lung invasive adenocarcinoma, and were significantly correlated with tumor size ($P=0.030$), lymph node metastasis ($P=0.021$) and microvessel density ($P=0.015$). Survival analysis demonstrated that patients with high protein expression levels of MTA1 exhibited significantly shorter five-year disease-free and overall survival than those patients whose protein expression levels of MTA1 were low (24.5% vs. 48.7%, $P=0.001$, and 34.7% vs. 59.2%, $P=0.005$, respectively). In addition, Cox regression multivariate analysis demonstrated that high protein expression levels of MTA1 significantly correlated with unfavorable five-year disease-free survival ($P=0.024$). These findings indicate that MTA1 protein

expression may possess clinical potential as an indicator of progressive phenotype. Therefore, MTA1 is a promising prognostic predictor to identify subgroups of patients with high risk of relapse, and a potentially novel therapeutic target for anti-angiogenesis in patients with lung invasive adenocarcinoma.

Introduction

Lung cancer is the most common malignancy and the leading cause of cancer-associated mortality worldwide (1-3). The incidence of adenocarcinoma has gradually increased in the past decades, and has become the most prevalent histological subtype of primary lung cancer worldwide (4-6). Lung adenocarcinoma is characterized by its diverse clinical, radiological, pathological, histological and molecular heterogeneity. This histological heterogeneity has led to modifications in the classification of lung adenocarcinoma (7,8). According to the novel architectural classification of lung adenocarcinoma proposed by the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society (IASLC/ATS/ERS) in 2011, the previous morphological classification of the different histological subtypes of lung adenocarcinoma has been substituted by a comprehensive multidisciplinary classification (9). According to this novel classification, patients with adenocarcinoma *in situ* (AIS) and minimally invasive adenocarcinoma (MIA) are expected to present favorable five-year survival, whereas the prognoses of patients with lung invasive adenocarcinomas, including those with pathological stage IA, are relatively poor (10-12). Clearly, a further understanding of the mechanisms underlying the pathogenesis and progression of lung invasive adenocarcinoma would promote the development of novel prognostic markers and therapeutic targets that may improve the treatments and clinical outcomes of patients with lung cancer (13,14).

Metastasis-associated protein 1 (MTA1) has been identified as a critical regulator of the carcinogenesis and aggressiveness of a wide variety of human malignancies (15-19). Previous studies by Li *et al* (20) demonstrated that high protein expression levels of MTA1 are involved in tumor angiogenesis and unfavorable prognosis in patients

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Abbreviations: MTA1, metastasis-associated protein 1; IASLC/ATS/ERS, International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society; AIS, adenocarcinoma *in situ*; MIA, minimally invasive adenocarcinoma; NSCLC, non-small cell lung cancer; MVD, microvessel density

Key words: metastasis-associated protein 1, angiogenesis, microvessel density, prognosis, lung invasive adenocarcinoma

with early-stage non-small cell lung cancer (NSCLC), and MTA1 acts as a proangiogenic factor by promoting the migration, invasion and angiogenesis of NSCLC cells *in vitro* (21), thus contributing to the aggressive biological behavior and metastatic propensity of this type of cancer. However, to the best of our knowledge, the clinicopathological and prognostic roles of MTA1 protein expression, and its correlation with angiogenesis in lung invasive adenocarcinoma, have not been investigated thus far.

To address these questions, the protein expression levels of MTA1 were analyzed in the present study, and its clinicopathological and prognostic significance, in addition to its angiogenic activity in lung invasive adenocarcinoma, were evaluated based on the 2011 IASLC/ATS/ERS classification of lung adenocarcinoma (9).

Materials and methods

Patients. Medical records were reviewed to identify patients with primary lung invasive adenocarcinoma who had undergone complete lobectomy and systematic mediastinal lymph node dissection consecutively between January 2006 and December 2008 at the Department of Thoracic Surgery of Qilu Hospital, Shandong University (Jinan, China). Patients who received preoperative adjuvant chemotherapy and/or radiotherapy, succumbed to perioperative complications or were not subjected to follow-up examinations were excluded from the study. A total of 125 patients were selected for the study. The histology slides of each patient enrolled in the study were reviewed independently by two pathologists, and the histological subtypes were classified according to the criteria proposed by the 2011 IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma (9). The pathological staging was determined based on the 7th edition of the Union for International Cancer Control Tumor Node Metastasis classification of malignant tumors (22). Informed consent was obtained from all the individual participants included in the study. The present study was approved by the institutional review board of Qilu Hospital, Shandong University. The general clinicopathological characteristics of the patients are presented in Table I.

Follow-up. All patients were regularly followed up subsequently to surgery. The patients were recommended to attend follow-up visits every three months for the first two years following surgery, and every six months thereafter. The follow-up protocol consisted of physical examination, blood tests, sonography, chest radiography, computed tomography (CT), magnetic resonance imaging, whole body bone scans and positron emission tomography-CT scans, if necessary. Recurrent disease was confirmed by fine-needle aspiration or cytopathological diagnosis when clinically feasible. All patients were followed up until mortality or last day of follow-up. The deadline of follow-up was December 2013, and the median clinical follow-up time was 58 months (range, 16-90 months).

Evaluation of MTA1 protein expression and microvessel density (MVD). Immunohistochemical staining for MTA1 and cluster of differentiation (CD)105 was performed using

an immunohistochemical detection kit (cat no. SP-9000; ZSGB-BIO, Beijing, China) according to the procedure previously described (20,23), using a goat anti-human polyclonal antibody against MTA1 (sc-9446; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and a rabbit anti-human polyclonal antibody against CD105 (sc-20632; Santa Cruz Biotechnology, Inc.), respectively. The slides were incubated at 60°C for 30 min, and deparaffinized in xylene, followed by rehydration with graded alcohol. Antigen retrieval was performed in citrate buffer for 15 min using a microwave oven. Once cooled down to room temperature, the slides were immersed in 3% hydrogen peroxide for 10 min, and incubated in blocking serum for 30 min to reduce nonspecific binding. Upon discarding any excess of blocking solution, primary goat anti-MTA1 polyclonal antibody (1:100) and rabbit anti-CD105 polyclonal antibody (1:100) were applied to the slides, and incubated overnight at 4°C. Next, the slides were incubated at 37°C for 30 min with biotinylated antibodies and streptavidin-peroxidase complex, followed by the addition of 3,3'-diaminobenzidine solution to visualize the staining corresponding to antibody-specific binding. Subsequently, the slides were counterstained with hematoxylin (ZLI-9609; ZSGB-BIO), and mounted with neutral balsam.

Semiquantitative determination of MTA1 protein expression was performed based on the staining intensity, as follows: i) A value of 0 was assigned to negative staining; ii) a score of 1 was assigned to weak staining; iii) 2 indicated moderate staining; and 3, intense staining. The proportion of positively stained cancer cells was 0, 0~5%; 1, 6~25%; 2, 26~50%; 3, 51~75%; and 4, ≥76%. The sum of the scores corresponding to the staining intensity and the percentage of positively stained cells was used to classify the different cases, and those tumors that displayed a final staining score of ≥4 were defined as exhibiting high protein expression levels of MTA1.

CD105 was observed to be expressed in the cytoplasm and membrane of endothelial cells, and the MVD count was performed as previously described (23,24). Briefly, the number of CD105⁺ microvessels was counted in a x200 microscopic field, and the mean value of the microvessels counted in five different vascular 'hot spots' was regarded as the final value for each case. All the immunostained slides were independently evaluated by two pathologists blinded to the clinicopathological and prognostic information of the patients. If disagreement emerged on the same slide, the reviewers would together use a multihead microscope, and discussed until a consensus score was achieved.

Statistical analysis. All data was statistically analyzed with SPSS statistical software version 18.0 (SPSS Inc., Chicago, IL, USA). The χ^2 test was used to examine the association between the protein expression levels of MTA1 and the clinicopathological characteristics of the patients. The correlation between the protein expression levels of MTA1 and intratumoral MVD was analyzed by the nonparametric Mann-Whitney U test. Survival curves were plotted by the Kaplan-Meier method, and survival differences were compared by the log-rank test. Multivariate analysis was performed to identify significantly independent prognostic factors. $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. Correlation between the protein expression levels of MTA1 and the clinicopathological factors of the patients.

Variables	No. of patients	MTA1 protein expression levels		P-value ^a
		Low	High	
Gender				0.758
Man	54	32	22	
Woman	71	44	27	
Age (years)				0.204
<60	55	30	25	
≥60	70	46	24	
Histological subtypes				0.351
Lepidic	13	10	3	
Acinar	15	11	4	
Papillary	51	28	23	
Solid	35	19	16	
Micropapillary	11	8	3	
Differentiation				0.978
Well	37	23	14	
Moderate	55	33	22	
Poor	33	20	13	
Pleural invasion				0.819
Absent	52	31	21	
Present	73	45	28	
Tumor size (cm)				0.030
≤3	61	43	18	
>3	64	33	31	
Lymph node metastasis				0.021
Absent	67	47	20	
Present	58	29	29	
Pathological stage				0.054
I	64	45	19	
II	40	22	18	
III	21	9	12	

^a, χ^2 test; MTA1, metastasis-associated protein 1.

Results

Correlation between MTA1 protein expression and clinicopathological factors. Immunohistochemical analysis revealed positive immunostaining for MTA1 in the nuclei of the cancer cells, and different immunoreactivity for MTA1 protein was detected in regards to the percentage of positive cells stained and the intensity of the nuclear staining (Fig. 1A and B). Of the 125 primary lung invasive adenocarcinoma specimens analyzed, high protein expression levels of MTA1 were detected in 49 cases, and the association between the protein expression levels of MTA1 and the clinicopathological characteristics of the patients was analyzed by the χ^2 test. As indicated in Table I, high protein expression levels of MTA1 were significantly correlated with the size of the tumor (P=0.030) and lymph node metastasis (P=0.021), whereas no significant differences were detected among the protein expression levels of MTA1

on the basis of the patients' gender (P=0.758), age (P=0.204), histological subtypes (P=0.351), differentiation (P=0.978), pleural invasion (P=0.819) and pathological stage (P=0.054, which may be considered borderline significance).

Correlation between MTA1 protein expression and tumor angiogenesis. Intratumoral MVD was quantified by counting the number of CD105⁺ endothelial cells present in the cancer tissues (Fig. 1C). The staining intensity of MVD varied from 6.6 to 49.6, with a median of 32.2/high power field (HPF). Double-staining of MTA1 and CD105 in the same serial sections of cancer tissues revealed that low protein expression levels of MTA1 were generally associated with few microvessels (6.6-49.4; median, 29.3/HPF), whereas high protein expression levels of MTA1 were generally associated with abundant microvessels (9.0-49.6; median, 37.0/HPF). Statistical analysis further demonstrated a significantly higher

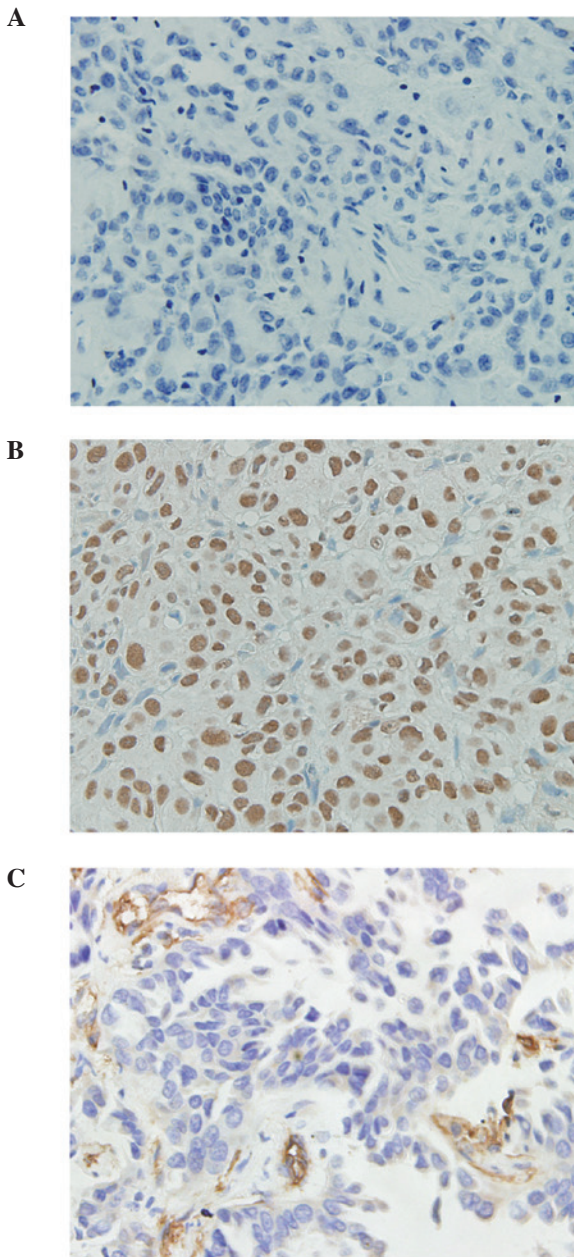


Figure 1. Immunohistochemical staining of lung invasive adenocarcinoma specimens, displaying (A) low and (B) high protein expression levels of metastasis-associated protein 1, respectively. (C) Intratumoral microvessels were immunopositive for cluster of differentiation 105 (magnification, x200).

MVD in tumors with high protein expression levels of MTA1 than in those with low protein expression levels of MTA1 ($P=0.015$, Mann-Whitney U test; Fig. 2).

Follow-up results and analysis of prognostic factors. During the follow-up period, tumor relapse developed in 76 patients (60.8%), and 63 patients (50.4%) succumbed to the disease. The median survival time of the subgroup of patients exhibiting high protein expression levels of MTA1 was 48.0 months, while the median survival time of the subgroup of patients displaying low protein expression levels of MTA1 was 61.5 months. As indicated in Table II, the results of the log-rank test demonstrated that the protein expression levels of MTA1 were significantly associated with the five-year disease-free survival

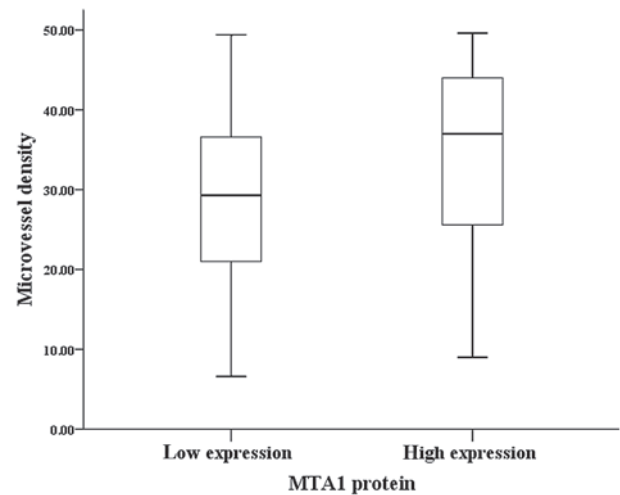


Figure 2. Mann-Whitney U test demonstrated that tumors with high protein expression levels of MTA1 exhibited significantly higher intratumoral microvessel density than tumors with low protein expression levels of MTA1 ($P=0.015$). MTA1, metastasis-associated protein 1.

($P=0.001$; Fig. 3A). Other parameters such as tumor size, lymph node metastasis and pathological stage were also observed to be significantly associated with five-year disease-free survival ($P<0.001$). Furthermore, the protein expression levels of MTA1 were significantly associated with the five-year overall survival ($P=0.005$; Fig. 3B), in addition to tumor size, lymph node metastasis and pathological stage ($P<0.001$). The significantly independent prognostic factors were further analyzed by a multivariate Cox regression model, and the results indicated that MTA1 protein expression, tumor size, lymph node metastasis and pathological stage were independent prognostic factors for five-year disease-free survival ($P=0.024$, 0.044, 0.029 and 0.001, respectively), whereas only the pathological stage ($P=0.028$) was identified as a significantly independent prognostic factor for five-year overall survival.

Discussion

Major advances in the treatment of lung adenocarcinoma are the result of the combined therapy that is currently administered to patients with lung cancer. This combined therapy consists of surgical resection, chemotherapy, radiotherapy and molecular targeting agents based on epidermal growth factor receptor (EGFR) (8). However, due to the high heterogeneity of lung adenocarcinoma, the overall five-year survival of patients affected by this disease is $<30\%$ (25-28). This heterogeneity has led to modifications in the histological categories employed to classify the different types of lung adenocarcinoma, as described in the 2011 IASLC/ATS/ERS classification of lung adenocarcinoma (9). According to this novel classification, patients with AIS and MIA present favorable five-year disease-free survival, whereas the prognoses of patients with lung invasive adenocarcinoma, including those with pathological stage IA, are relatively poor (11,12,29). The aim of the present study was to further investigate the underlying mechanisms involved in the invasive ability and metastatic properties of lung invasive adenocarcinoma, and to identify possible targets for novel treatments, including

Table II. Results of univariate and multivariate survival analyses.

Variables	Disease-free survival			Overall survival		
	Univariate analysis P-value	Multivariate analysis		Univariate analysis P-value	Multivariate analysis	
		95% CI	P-value		95% CI	P-value
Gender	0.893	0.549-1.494	0.699	0.956	0.528-1.545	0.710
Age	0.664	0.745-2.060	0.409	0.304	0.676-2.023	0.575
Histological subtypes	0.172	0.973-1.517	0.086	0.221	0.956-1.525	0.114
Differentiation	0.087	0.975-1.819	0.072	0.075	0.961-1.900	0.083
Pleural invasion	0.374	0.622-2.047	0.690	0.279	0.602-2.227	0.660
Tumor size	<0.001	1.017-3.809	0.044	<0.001	0.883-3.893	0.103
Lymph node metastasis	<0.001	0.112-0.888	0.029	<0.001	0.268-2.350	0.676
Pathological stage	<0.001	1.682-6.686	0.001	<0.001	1.088-4.368	0.028
MTA1 protein levels	0.001	1.076-2.899	0.024	0.005	0.773-2.256	0.309

CI, confidence interval; MTA1, metastasis-associated protein 1.

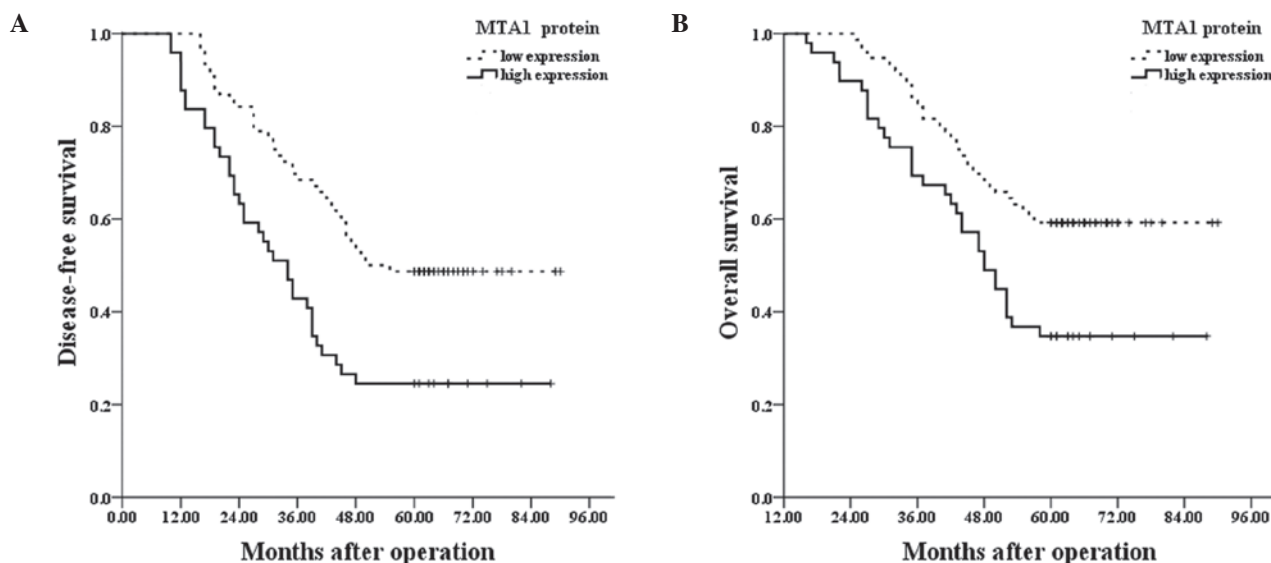


Figure 3. Kaplan-Meier curves of (A) disease-free and (B) overall survival, stratified according to the protein expression levels of MTA1. Log-rank test demonstrated that patients with high protein expression levels of MTA1 exhibited significantly shorter five-year disease-free and overall survival than those with low protein expression levels of MTA1 (24.5% vs. 48.7%, $P=0.001$, and 34.7% vs. 59.2%, $P=0.005$, respectively). MTA1, metastasis-associated protein 1.

individualized therapy, which may improve the clinical outcomes of patients with this type of cancer.

MTA1 is a component of the nucleosome remodeling and histone deacetylation complex, which functions in histone deacetylation, alteration of chromatin structure and control of transcription (30,31). As a vital regulator, MTA1 has been observed to be aberrantly expressed in various human malignant tumors, and its expression levels are positively correlated with aggressive phenotypes characterized by their invasiveness and metastatic potential (15). Thus, MTA1 may be a target for overcoming tumor progression (15,17). The association between MTA1 expression, angiogenesis and unfavorable prognosis in patients with early-stage NSCLC has been previously reported (20). Thus, it is clinically required to further assess the protein expression levels of MTA1 and analyze its

clinicopathological and prognostic significance in human lung invasive adenocarcinoma. A total of 125 patients with primary lung invasive adenocarcinoma were enrolled in the present study, which aimed to elucidate the angiogenic activities and the clinicopathological and prognostic significance of MTA1 protein expression. The results revealed that high protein expression levels of MTA1 were significantly associated with tumor size and lymph node metastasis, and markedly associated with pathological stage, all factors known to contribute to aggressive phenotypes.

Antiangiogenesis is a pivotal strategy for treating malignancies (32), and MTA1 has been defined as a proangiogenic factor (15,21,33,34). A previous study demonstrated that high intratumoral protein expression levels of MTA1 were significantly associated with angiogenesis in NSCLC (20).

In addition, RNA interference-mediated downregulation of MTA1 protein expression in the lung adenocarcinoma cell line 95D was able to substantially inhibit the formation of capillary tube-like structures *in vitro* (21). However, the correlation of MTA1 protein expression with tumor angiogenesis in lung invasive adenocarcinoma has not been investigated thus far. In the present study, the expression of CD105, a homodimeric cell membrane glycoprotein, was evaluated in order to quantify tumor angiogenesis, since this marker is able to discriminate immature neovascularization from mature and established blood vessels (35,36), thus indicating the presence of active angiogenesis in the tumor (37-39). The results demonstrated that high protein expression levels of MTA1 were significantly associated with increased angiogenic activity, as measured by the number of CD105-associated intratumoral microvessels, suggesting that MTA1 may be involved in tumor progression by participating in the process of angiogenesis in lung invasive adenocarcinoma. Tumor angiogenesis is a complex process, and the mechanism by which MTA1 modulates angiogenesis remains unknown (22,30-34). Therefore, further studies are required in order to elucidate the mechanisms by which MTA1 induces angiogenesis.

With regard to prognosis, the results of the univariate survival analysis conducted in the present study demonstrated that patients with high protein expression levels of MTA1 presented a significantly shorter five-year disease-free and overall survival than those patients with low protein expression levels of MTA1. Subsequent multivariate analysis demonstrated that high protein expression levels of MTA1 were an independent prognostic factor for unfavorable disease-free survival, but not for overall survival. Clinically, the long-term survival of patients with cancer may be influenced by multiple factors, including postoperative chemotherapy with different regimens and cycles, radiotherapy and EGFR-tyrosine kinase inhibitor therapy, which may cause statistical bias, thus resulting in the inability to objectively and adequately evaluate the prognostic significance of MTA1. Nevertheless, the subgroup of patients with high protein expression levels of MTA1 may require further oncologic evaluation and clinical attention following surgical resection than those patients with low protein expression levels of MTA1, due to their higher risk of relapse.

Taken together, the results of the present study demonstrated for the first time that aberrantly high protein expression levels of MTA1 are involved in the malignant phenotype and unfavorable prognoses of patients with lung invasive adenocarcinoma, possibly due to its potent angiogenic activity. However, the stepwise progression and facilitated neoangiogenesis is too complicated to be clearly elucidated by a simple clinical study like the present one. Therefore, future studies will contribute to a further understanding of the underlying mechanisms targeting MTA1, and provide a greater insight into the aggressive phenotype of lung invasive adenocarcinoma, which may aid the design of novel drugs targeting MTA1 and the development of more efficient antiangiogenic therapies aimed to improve the survival of certain subgroups of patients affected by lung cancer.

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