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Correlation between metastasis-associated gene 1 expression and tumor-associated macrophages in non-small cell lung cancer

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The role of metastasis-associated gene 1 (MTA1) in the metastasis of non-small cell lung cancer (NSCLC) has been proved, but its role in the tumor microenvironment is still insufficient. The study was performed to explore the correlation between MTA1 and tumor-associated macrophages (TAMs) in NSCLC. The expression profile data of lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) were downloaded from TCGA database. The tumor-infiltrating immune cells in each LUAD and LUSC patient were estimated using the CIBERSORT method. Then, the online TIMER database containing multiple algorithms was used to analyze the relationship between MTA1 and TAMs. Besides, correlations between MTA1 and TAMs markers were also explored. Additionally, the immunohistochemistry staining of MTA1 protein and CD206 was performed in 75 NSCLC tissue specimens. Associations of MTA1 and CD206 with the clinicopathological characteristics were analyzed, as well as the correlation between MTA1 and CD206. Based on different algorithms, MTA1 expression was correlated with the distribution of infiltrating immune cells in the tumor microenvironment and negatively correlated with tumor immune-stromal score. MTA1 was associated with TAMs markers according to TCGA database. In 75 NSCLC tissue specimens, the positive rate of MTA1 was 60.00% (45/75), which of CD206 was 42.67% (32/75). The MTA1 expression was significantly correlated with T stage, lymph node metastasis, and TNM stage. The CD206 expression was significantly correlated with T stage, lymph node metastasis, TNM stage, and tumor type. Additionally, we found that MTA1 was positively correlated with CD206 in NSCLC and LUSC. In NSCLC, MTA1 expression was correlated with the infiltrations of different types of macrophages and the expression of TAMs markers, as well as the M2-TAMs marker CD206, suggesting that MTA1-promoting tumor metastasis may mediate the infiltration of different types of macrophages in the tumor microenvironment.

Key words: metastasis-associated gene 1, tumor-infiltrating immune cells, tumor-associated macrophages, CD206

Lung cancer remained the leading cause of cancer-related mortality worldwide, with an estimated 1.8 million deaths (18%) in 2020, seriously threatening the lives and health of people, including those in China [1]. Non-small cell lung cancer (NSCLC) accounts for about 85% of lung cancers. Although great achievements had been made in the treatment of NSCLC, especially the rapid development of precision molecular targeted therapy and immunotherapy, the overall five-year survival rate in China was less than 20% [2]. Metastasis was the main cause of treatment failure and death in patients. Metastasis-associated gene 1 (MTA1) was an oncogene that had been discovered in recent years and was closely related to tumor metastasis [3]. A meta-analysis confirmed that MTA1 was highly expressed in a variety of tumors including lung cancer, and was associated with poor prognosis of tumors [4]. Studies found that MTA1 was involved with the vascular and lymphatic formation, epithelial-mesenchymal transition (EMT), inflammatory cytokines secretion, and the release of immune reaction-related cytokines are closely correlated [5], suggesting MTA1 may promote tumor metastasis due to its mediated tumor microenvironment.

Tumor-associated macrophages (TAMs) are the macrophages that infiltrate the interior of the tumor. TAMs can provide a unique microenvironment for tumor cells through a variety of ways to promote tumor growth, invasion, and metastasis [6]. Targeting TAMs had become a research hotspot in anti-tumor microenvironmental therapy and a large number of preclinical and clinical studies were in full swing [7].

In NSCLC, the infiltration ratio of M1 and M2 macrophages was closely related to the survival time of patients [8].

Interestingly, MTA1 was associated with both M1/M2 type TAMs. For example, in the process of LPS activating macrophages (type M1), MTA1 can assist in activating NF-κB signaling to secrete inflammatory cytokines, such as IL-1β, TNF-α, and macrophage inflammatory protein-2 (MIP2) [9]. In breast cancer, MTA1 can promote the transcription of STAT3 [10], and the activation of STAT3 can enhance the polarization effect of M2-type macrophages and promote the release of inflammatory factors, such as IL-8 and IL-23. These inflammatory factors can not only promote the occurrence and development of tumors but also promote the differentiation of Th17 cells, exacerbating tumor-associated inflammation [11]. Besides, IL-17 secreted by Th17 cells can upregulate MTA1 expression [12]. A previous study had shown that AKT inhibitors can downregulate MTA1 expression [13]. Loss of PTEN (a negative regulator of AKT) enhanced CCL2 secretion and promoted brain metastases of NSCLC [14]. The binding of CCL2 to the monocyte surface receptor CCR2 can promote the aggregation and polarization of macrophages to form M2-TAMs and was associated with a poor prognosis of esophageal carcinogenesis [15].

Based on the above studies, we speculated that MTA1 may be involved in the regulation of TAMs-mediated tumor microenvironment. Therefore, we performed the study with the aim to explore the associations of MTA1 with different types of macrophages and their markers using a variety of online tools and 75 tissue specimens.

Patients and methods

Databases. The mRNA-seq data in FPKM format of the TCGA-LUAD cohort and TCGA-LUSC cohort were obtained from TCGA dataset (https://portal.gdc.cancer.gov/). Then the mRNA-seq data were transformed into TPM format for further analysis. And patients with LUAD and LUSC were divided into the high-MTA1 expression group and the low-MTA1 expression group according to the median value of MTA1 expression.

ESTIMATE score. Immune cells and stromal cells were the two main types in the tumor microenvironment and the ESTIMATE algorithm had been used to calculate the immune score and stromal score [16]. So, we firstly used the R software package to estimate the immune score and stromal score of each tumor sample, and then to observe the relationship between MTA1 expression and immune-stromal score in LUAD and LUSC. ESTIMATE score was calculated by combing the stromal score and immune score.

CIBERSORT algorithm. CIBERSORT is a bioinformatics approach to evaluate immune cell composition using standardized gene expression data [17]. With the aim to understand the profiles of tumor-infiltrating immune cells (TIICs) between low- and high-MTA1 groups in LUAD and LUSC patients, we ran the "CIBERSORT" package with 1000 permutations. After the samples were filtrated through $p>0.05$, the LUAD cohort and LUAC cohort each had 448 samples (each including 233 low-MTA1 group and 215 high-MTA1 group) were selected to perform the immune cells analysis. The differential infiltrating density between low- and high-MTA1 groups was analyzed by Wilcoxon rank-sum test and was generated as a violin plot using the ggplot2 R package.

TIMER database. TIMER (https://cistrome.shinyapps.io/timer/) is a comprehensive resource for systematic analysis of immune infiltrates, including TIMER, XCELL, EPIC, quanTIseq, and TIDE algorithms [18]. Based on the public resources, we analyzed the correlations between MTA1 and the infiltrating level of different types of macrophages, and Spearman's correlation coefficient and the estimated p-values were calculated.

Correlation analysis of macrophage markers. We further analyzed the relationship between MTA1 and common macrophage markers (macrophage, CD11b and CD68; M1- macrophage, CD80 and CD86; M2- macrophage, CD163 and CD206) based on TCGA database by using the "Psych" package. The Pearson's correlation coefficient and the estimated p-values were calculated.

Clinical tissue specimen. 75 surgical tissue samples of NSCLC patients (including 26 adenocarcinomas cases and 49 squamous cell carcinoma cases) diagnosed in 2021 at the First Affiliated Hospital of Zhengzhou University were collected to evaluate the associations between MTA1 expression and CD206 expression. Patients included 62 males and 13 females. The age ranged from 31 to 80, with an average age of 58.4. TNM staging of all patients was based on the 8th edition AJCC.

The study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the patients selected for our study were fully informed about our experiment protocols and signed informed consent.

Immunohistochemical staining. MTA1 rabbit anti-human polyclonal antibody (working concentration 1:100, AB71153) and CD206 rabbit anti-human polyclonal antibody (working concentration 1:500, AB64693) were purchased from Abcam Company. EnVision Two-step Immunohistochemistry Kit (REAL EnVision K5007) was purchased from Agilent (Dako). All specimen tissues were formalin-fixed, embedded in paraffin, and sectioned into 4 μm serial sections. The sections were stained according to the Envision two-step working procedure specification after antigen retrieval. PBS was used as a negative control instead of the primary antibody. The staining score criteria were as previously described [13]. Tissue sections with a final staining score ≥4 were considered to be positive.

Statistical analysis. SPSS21.0 statistical software was used for tissue microarray data analysis. Chi-square test was used to analyze differences between clinicopathological variables. Correlation analysis of CD206 and MTA1 was performed using Pearson Correlation Coefficient. Data from TCGA database was performed in RStudio (Version 3.6.3).

All statistical analyses were two-sided probability tests, and $p<0.05$ was considered statistically significant.

Results

MTA1 negatively correlated with tumor immune-stromal score. Immune cells and stromal cells are two main types in the tumor microenvironment, which play important roles in the occurrence and development of tumors. So, we firstly used the ESTIMATE algorithm to calculate the immune score and stromal score. The results showed that both in LUAD and LUSC, MTA1 was negatively associated with the immune score (Figures 1A, 1D) and stromal score (Figures 1B, 1E), as well as the ESTIMATE score (Figures 1C, 1F). Especially in LUSC, the relationship between MTA1 and ESTIMATE score (the sum of the immune score and stromal score) was more obvious (R=–0.482).

Associations of MTA1 with tumor-infiltrating immune cells (TIICs). In LUAD, the infiltrating levels of T cells follicular helper, T cells regulatory, T cells gamma delta, and M0-type macrophages were significantly increased in the high-MTA1 expression group, while the infiltrating levels of neutrophils, eosinophils, mast cells resting, dendritic cells resting, and plasma cells were significantly decreased in the high-MTA1 expression group (Figure 2A). In LUSC, the infiltrating levels of B cells naive, T cells follicular helper, T cells gamma delta, and M0-type macrophages were significantly increased in the high-MTA1 expression group, while the infiltrating levels of T cells CD4 memory activated, dendritic cells resting, and neutrophils were significantly decreased in the high-MTA1 expression group (Figure 2B).

Associations of MTA1 with the infiltration levels of different types of macrophages. Based on the TIMER database, we found that the expression of MTA1 and the infiltration levels of different types of macrophages were different with different calculation methods. Different from the CIBERSORT algorithm, MTA1 was negatively correlated with the infiltration level of macrophages (Figures 3E, 3J, 3M). In addition, MTA1 was also negatively correlated with M1-macrophages (Figures 3K, 3N) in LUSC. Only according to the TIDE algorithms in LUSC, MTA1 was positively associated with the infiltrating levels of M2-type macrophages (Figure 3P), while based on several other algorithms, MTA and M2-type macrophages were negatively correlated (Figures 3D, 3G, 3H).

Associations of MTA1 with different types of macrophage markers. Studies have found the clinical significance of the expression of different macrophage cell marker molecules [19]. So, we further explored the correlations between MTA1 and macrophage markers. The results showed that MTA1 was negatively correlated with different types of macrophage markers (Figure 4).

Associations of MTA1 expression with patient clinicopathological characteristics. Immunostaining of MTA1 was observed in the nucleus of cancer cells (Figures 5B–5D) and CD206 was observed in the cytoplasm and/or membrane (Figures 5F–5H).

In the explored 75 NSCLC tissue specimens, the positive expression rate of MTA1 was 60.00% (45/75), and the positive rate of CD206 was 42.67% (32/75).

Associations between MTA1 or CD206 expression and various patient clinicopathological characteristics are shown in Table 1.

Correlation between MTA1 expression and CD206 expression Correlations between MTA1 and CD206 expres-

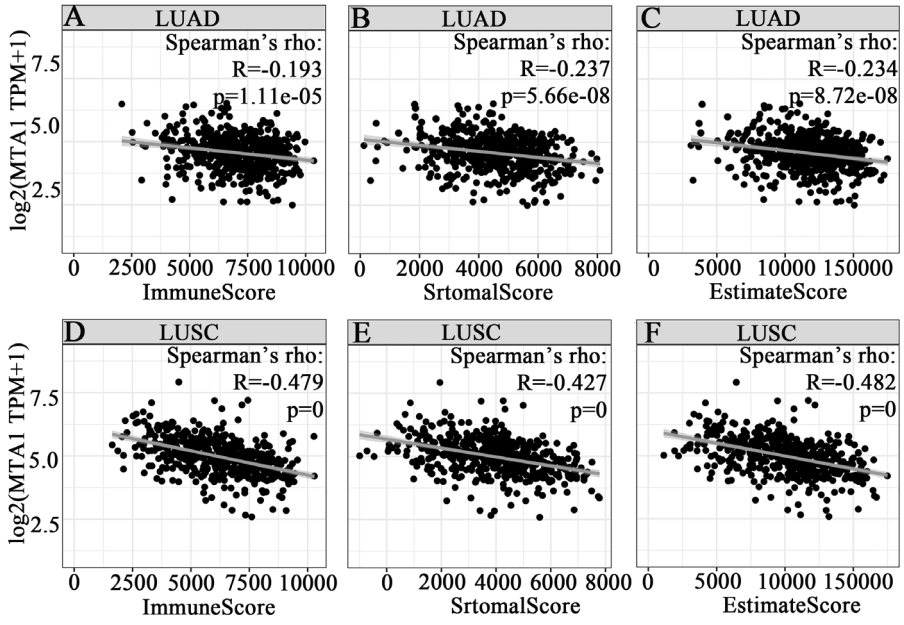

Figure 1. MTA1 was negatively related to the immune score (A, D), stromal score (B, E), and estimate score (C, F) in both LUAD and LUSC. Abbreviations: MTA1-metastasis-associated gene 1; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma

Table 1. Relationships of MTA1 or CD206 expression and the clinicopathological characteristics of NSCLC.

Variable	No.	MTA1		χ^2	p-value	CD206		χ^2	p-value
		-	+			-	+		
Gender								0.803	0.370
Male	62	25	37	0.016	0.901	37	25		
Female	13	5	8			6	7		
Age					0.119			3.828	0.05
≤60 years	47	22	25	2.432		31	16		
>60 years	28	8	20			12	16		
T stage								3.271	0.071
T1–2	59	28	30	4.057	0.044	376	2210		
T3–4	16	2	10						
Lymph node metastasis				6.490	0.011			6.946	0.008
Yes	36	9	27			1528	21		
No	39	21	18				11		
AJCC stage				4.501[a]	0.034			6.105	0.013
I/II	63	29	3411			40	23		
III	12	1				3	9		
Tumor type					0.766			11.480	0.001
AC	26	11	15	0.88		8	18		
SC	49	19	30			35	14		

Notes: [a] Continuously corrected chi-square test was used; -: Negative; +: Positive. Abbreviations: SC-squamous carcinoma; MTA1-metastasis-associated gene 1; AC-adenocarcinoma; NSCLC-non-small cell lung cancer

Table 2. Correlation between MTA1 expression and CD206 expression in NSCLC.

	CD206				
NSCLC					
MTA1	Negative	Positive	Total	Pearson	p-value
Negative	24	6	30	0.326	0.004
Positive	19	26	45		
Total	43	32	75		
LUAD					
MTA1	Negative	Positive	Total	Pearson	p-value
Negative	6	5	11	0.314	0.119
Positive	2	13	15		
Total	8	18	26		
LUSC					
MTA1	Negative	Positive	Total	Pearson	p-value
Negative	18	1	19	0.371	0.009
Positive	17	13	30		
Total	35	14	49		

Abbreviations: MTA1-metastasis-associated gene 1; NSCLC-non-small cell lung cancer; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma

sion is shown in Table 2. High MTA1 expression was positively associated with high CD206 expression in both NSCLC and grouped LUSC, but not in LUAD.

Discussion

MTA1 is an oncogene closely related to tumor metastasis. Our previous studies had confirmed that high expression of MTA1 was a poor prognostic factor of NSCLC, which could increase the invasive and metastatic potential of NSCLC cell lines by inducing epithelial-mesenchymal transformation [13]. In the present study, we explored the correlation between MTA1 and macrophages with different phenotypes in the tumor microenvironment.

Based on TCGA database analysis, there was a different distribution of various infiltrating immune cells in the tumor microenvironment between the MTA1 high expression group and the low expression group, suggesting MTA1 may be involved in the different distribution of immune cells and resulted in the specific tumor microenvironment. Targeting MTA1 may help to improve the tumor microenvironment and antitumor effect. In fact, the latest research by Zhou et

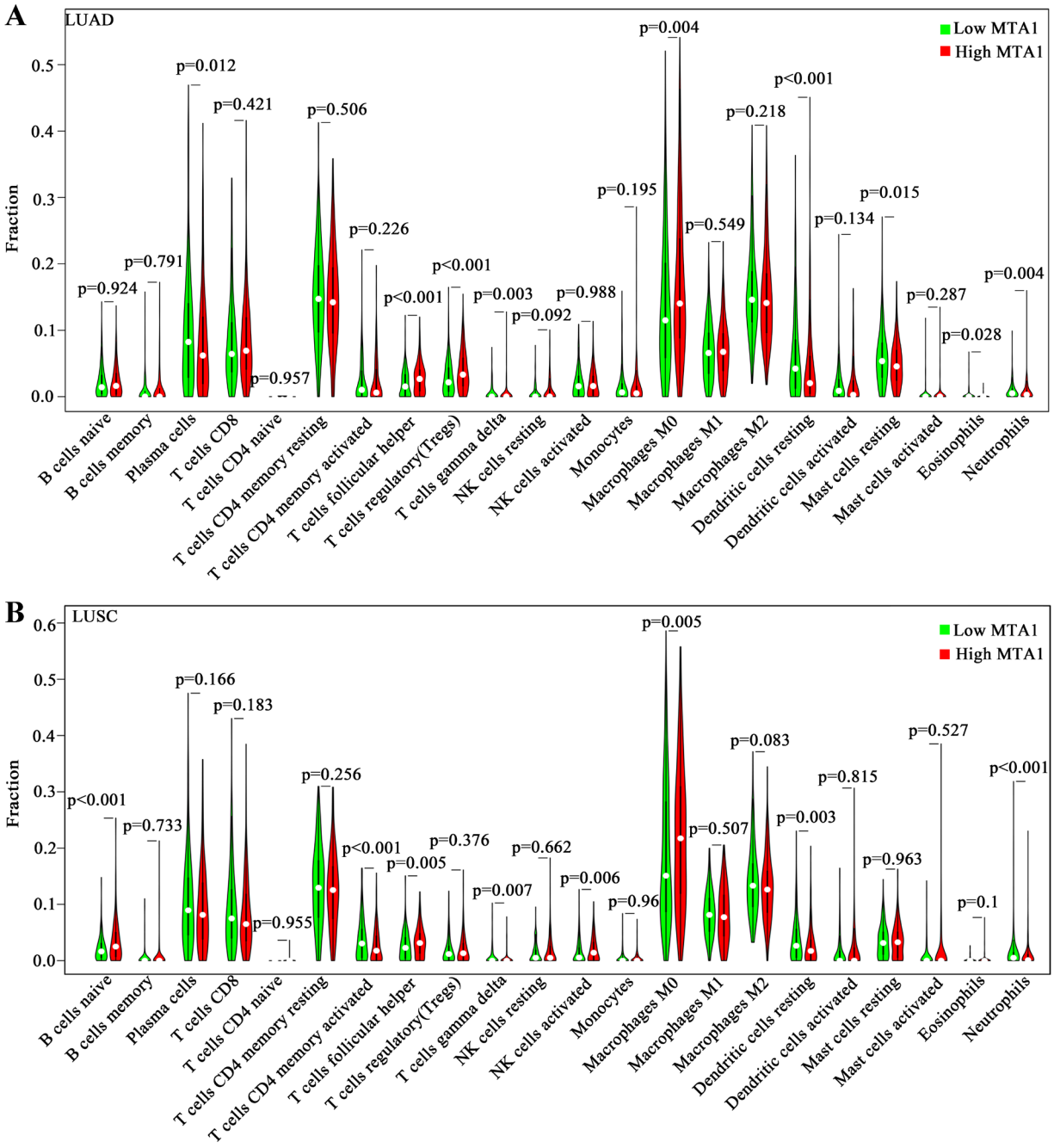

Figure 2. Evaluation of tumor-infiltrating immune cells using the CIBERSORT algorithm. A) The distribution of 22 tumor-infiltrating immune cells in the high- and low-MTA1 expression group in LUAD. B) The distribution of 22 tumor-infiltrating immune cells in the high- and low-MTA1 expression group in LUSC. Abbreviations: MTA1-metastasis-associated gene 1; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma

al. revealed that upregulation of MTA1 in colorectal cancer drives an immunosuppressive tumor microenvironment by decreasing the microphages from the tumor and inducing the residual macrophages into tumor-associated microphage phenotypes to block the activation of the killing cytotoxic T lymphocytes (CTLs), which contributes to cancer progression [20]. Some scholars had constructed the recombinant MTA1 antigenic peptide, which can enhance CD4+ CTLs immune response, promote IFN-γ secretion, and play an anti-tumor effect as a tumor vaccine [21]. Besides, MTA1 was negatively associated with the immune score and stromal score, which was related to the efficacy of tumor immunotherapy [22].

Immunotherapy, especially the immune checkpoint inhibitors (ICIs) had made great achievements in NSCLC,

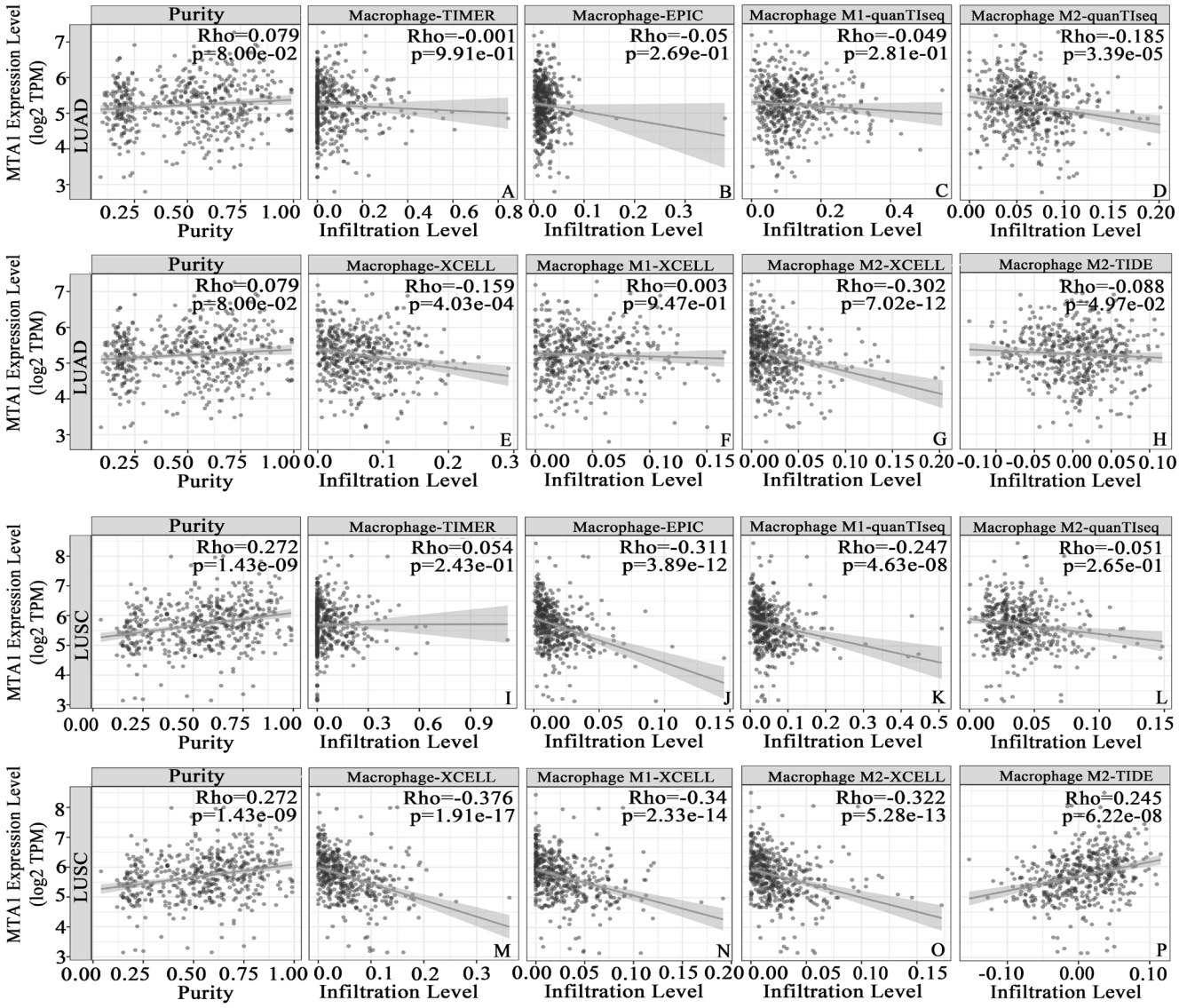

Figure 3. Associations of MTA1 with the infiltration levels of different types of macrophages based on online TIMER (https://cistrome.shinyapps.io/timer/). Abbreviations: MTA1-metastasis-associated gene 1; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma

but the sobering fact remains that a large group of patients does not respond to PD-(L)1 inhibition at all (primary resistance) [23]. The response rate of ICIs monotherapy in the overall population was less than 20% [24]. How to improve immunotherapy response is a hot topic in current research. The formation of the MTA1/HDAC complex with HDAC is an important regulatory mode of MTA1's function [3, 25, 26]. Booth et al. found that HDACs inhibitors AR42 and Sodium Valproate can enhance the efficacy of PD-1 immunotherapy. A combination of HDACs inhibitors with anti-PD-1 can increase activated T cells and increase infiltration of M1-type macrophages, neutrophils, and natural killer cells [27]. A paper published in Cell in 2017 showed that inhibition of HDACs can reverse tumor immune escape by inhibiting angiogenesis, HIF-α expression, and TAMs cell number in the tumor microenvironment, and improve the efficacy of immunotherapy for NSCLC, especially for myC-deficient lung cancer [28]. At present, a large number of clinical trials of HDAC inhibitors combined with ICIs are being carried out in various cancers [29]. Based on this, we speculated that MTA1 expression may be related to the effect of anti-tumor immune therapy, but further experiments were needed to verify.

Then we used database analysis to find that MTA1 was associated with different types of macrophages, although not exactly. But the immunohistochemical staining analysis

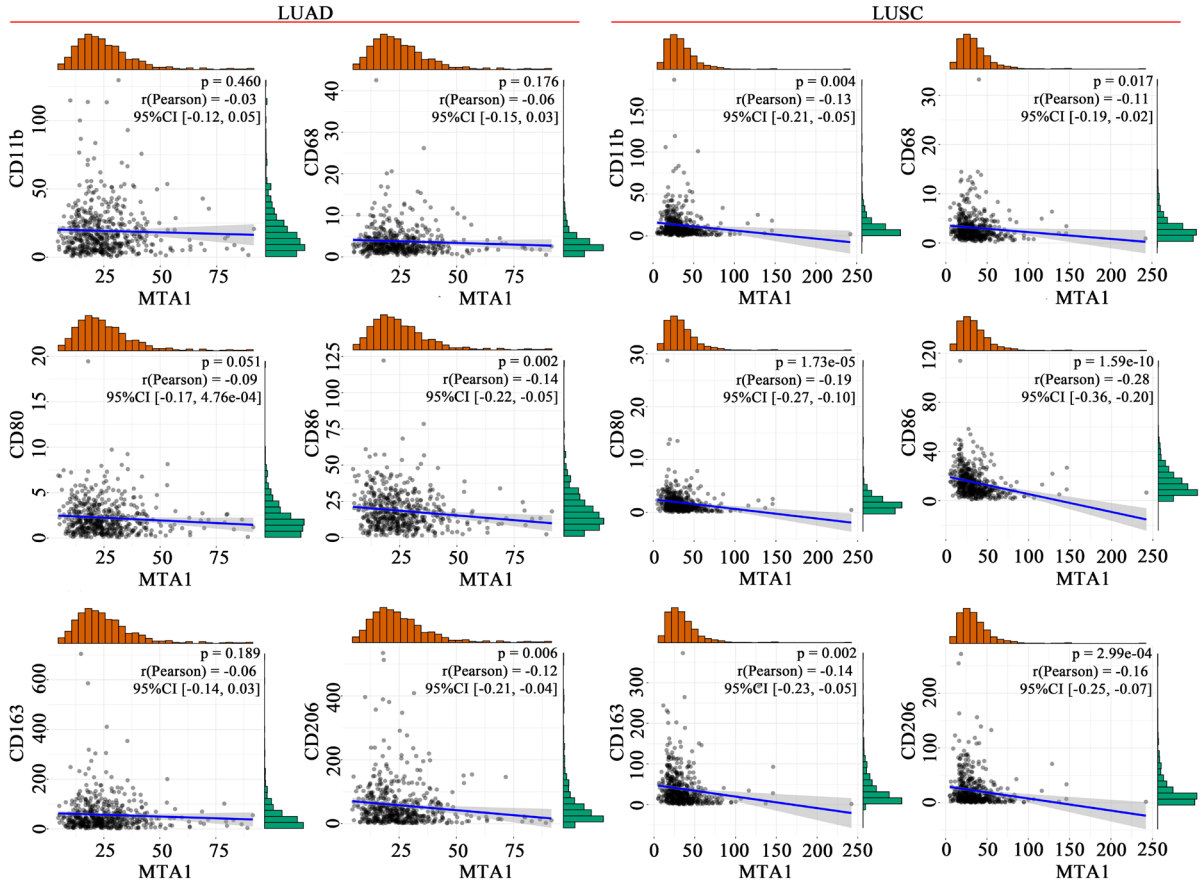

Figure 4. Associations of MTA1 with different types of macrophage markers based on TCGA database. Macrophage markers: CD11b and CD68; M1-macrophage markers: CD80 and CD86; M2- macrophage markers: CD163 and CD206. Abbreviations: MTA1-metastasis-associated gene 1; LUAD-lung adenocarcinoma; LUSC-lung squamous cell carcinoma

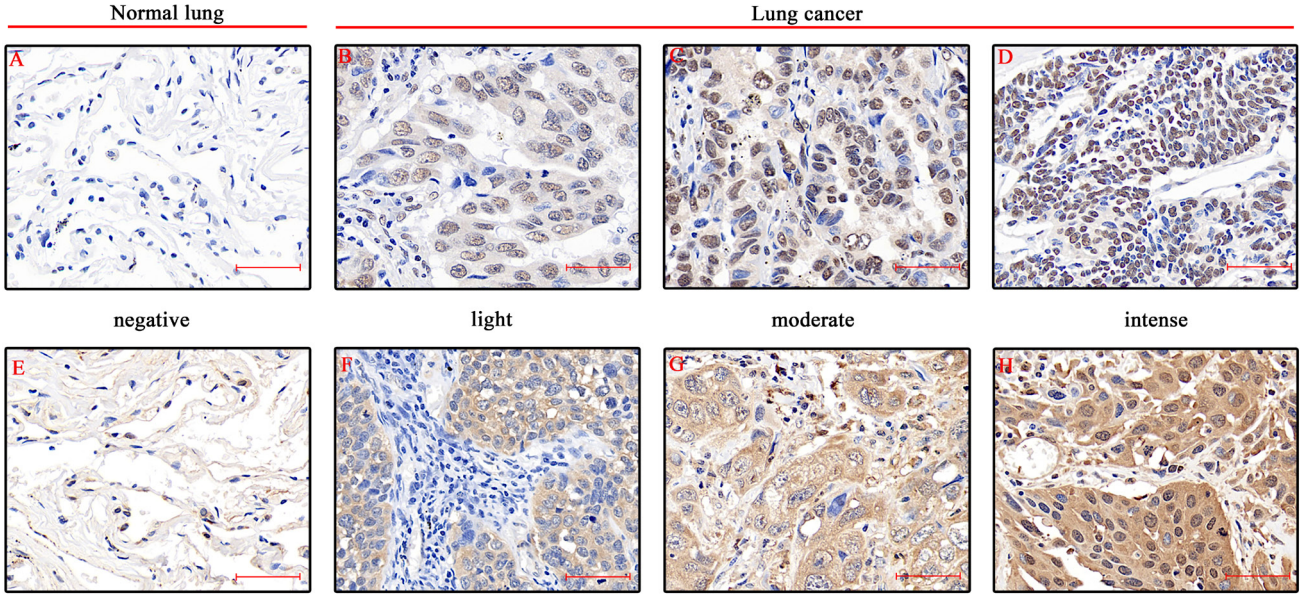

Figure 5. The expression of MTA1 and CD206 in NSCLC. A) No staining of MTA1 in normal lung tissue. B) Light staining of MTA1 in NSCLC. C) Moderate staining of MTA1 in NSCLC. D) Intense staining of MTA1 in NSCLC. E) Light staining of CD206 in normal lung tissue. F) Light staining of CD206 in NSCLC. G) Moderate staining of CD206 in NSCLC. H) Intense staining of CD206 in NSCLC. Scale bars: 50 μm Abbreviations: MTA1-metastasis-associated gene 1; NSCLC-non-small cell lung cancer

of the tissue specimens further confirmed that MTA was associated with M2-TAMs marker CD206. M2 TAMs were closely related to the immunosuppressive microenvironment for tumor development [30] and M2 markers are associated with worse survival across epithelial tumors [19]. CD206 as a marker of M2-TAMs is associated with a poor prognosis in liver cancer, leukemia, and other tumors [31, 32]. In the present study, CD206 is not only related to tumor stage and lymph node metastasis, but also related to the tumor type, suggesting that the level of M2 macrophage infiltration is different in different lung cancer tissue types, and whether this difference is related to the efficacy of tumor immunotherapy is a good research direction in the field of immunotherapy. But in LUSC, there was no significant correlation between MTA1 and CD206, indicating the correlation between MTA1 and TAMs was also related to the histological types of tumors. What deserved mentioning was that the correlation coefficient between MTA1 and CD206 is not strong enough, suggesting that MTA1 may not be directly correlated with CD206. And in the present study, the correlations between MTA1 and M0- or M1- macrophages markers were also observed.

It has been reported that the infiltration ratio of M1 and M2 macrophages in the NSCLC microenvironment is closely related to patient survival [8]. Therefore, MTA1 may be more closely related to the proportion of M1/M2 macrophages, which deserved further exploration.

In conclusion, in the present study, we explored the association of MTA1 and TAMs using a variety of databases. In the immunostaining analysis of tissue sections, MTA1 was found to be positively correlated with M2-TAMs and associated with tumor malignant phenotype, suggesting that MTA1-promoting tumor metastasis may mediate the infiltration of different types of macrophages in the tumor microenvironment. Combined targeting MTA1 has the potential to improve the efficacy of immunotherapy. This study preliminarily explored another mechanism of action of MTA1 in the tumor microenvironment of NSCLC, but further experimental data are needed to confirm.

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