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Relationship Between Overexpression of EML4-ALK Variant 1 and Inflammatory Moleculars and Immune Mediators Associated with Tumor Progression and Metastasis in BEAS-2B and H2126 Cells

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Abstract: The aim of this study was to investigate the relationship between overexpression of EML4-ALK and inflammatory factors about tumor progression and metastasis in a human bronchial epithelial cell (BEAS-2B) and a lung cancer cell (H2126). The recombinant plasmids with EML4-ALK variant 1 and EML4-ALK K589M (EML4-ALK variant 1 kinase inactive mutant) fusion gene were constructed and introduced into H2126 and BEAS-2B cells after transfection. The plasmid pcDNA3.1 was negative control. Subsequent, cell proliferation assay and scratch wound healing assay were used to examine the proliferation and invasion of BEAS-2B and H2126 cells after transfection. Finally, we analyzed 24 inflammatory moleculars and immune mediators associated with tumor progression and metastasis. Compared to the empty vector as control, the expression level of ALK was upregulated in BEAS-2B and H2126 cells after transfection the plasmid EML4-ALK variant 1 and plasmid EML4-ALK K589M. In vitro, EML4-ALK variant 1 promoted the proliferation and invasion ability of BEAS-2B and H2126 cells compared with EML4-ALK K589M and empty vector. The results of Q-PCR showed that factors more differentially expressed between both groups of BEAS-2B and H2126 cells were S100A8 and S100A9 after transfection EML4-ALK variant 1. In conclusion, an increased expression level in S100A8 and S100A9 by overexpression EML4-ALK variant 1 had a great biological interest because of their relation with tumor cell proliferation and migration.

Keywords: EML4-ALK Variant 1, Proliferation, Migration, S100A8, S100A9

1. Introduction

Lung cancer remains the number one cause of cancer-related deaths worldwide in 2016 [1]. It is divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common type of lung cancer and accounts for 80% of all lung cancers [2]. Most of the patients are diagnosed at advanced stage and survival rates for advanced disease remain low [3, 4]. With standard first-line chemotherapy with four to six cycles of platinum-based doublet chemotherapy, long-term survival for patients with advanced disease remains poor [5]. Therefore, a better understanding of cancer molecular mechanisms is expected to improve cancer prevention, diagnosis and treatment [6, 7].

The molecular targeted drugs of NSCLC have been introduced into routine clinical treatment since the presence of specific oncogenic alterations, including epidermal growth factor receptor (EGFR) gene mutations and anaplastic lymphoma kinase (ALK) rearrangements. EGFR inhibitors and echinoderm microtubule associated protein like 4-anaplastic lymphoma kinase (EML4-ALK) inhibitors can be useful for specific subgroups of patients [8, 9]. EML4-ALK, a novel transforming fusion gene, was

identified in NSCLC in 2007 [10]. But its pathogenetic mechanisms in NSCLC remain unclear [11, 12]. Tumor microenvironment is a crucial role in tumor pathophysiology, including cancer initiation, progression and metastasis [13, 14]. In tumor microenvironment, inflammatory cells and immune mediators influence tumor progression and metastasis in cancers [15]. Therefore, the aim of the present study was to evaluate 24 factors associated with inflammatory molecular and immune mediators influence tumor initiation, progression and metastasis in lung epithelial cells and lung cancer cells by overexpressing EML4-ALK.

2. Materials and Methods

2.1. Cell Lines

The cell lines used here included NSCLC cell line with a triple negative genotype (H2126, no EGFR mutation, Kras mutation and EML4-ALK fusion gene) and human bronchial epithelial cell (BEAS-2B). The cell lines were obtained from the American Type Culture Collection (ATCC, USA). BEAS-2B cell line was cultured in BEBM (Lonza, USA), at 37°C with 5% CO_2. H2126 cell line was cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C with 5% CO_2.

2.2. The Construction of Plasmids and Transfection in Vitro

EML4-ALK variant1 is the most common type of EML4-ALK fusion variants [10, 16]. Therefore, to analyse the function of EML4-ALK, we generated expression plasmid pcDNA3.1 for EML4-ALK variant 1 and EML4-ALK K589M (EML4–ALK variant 1 kinase inactive mutant, in which Lys 589 in the ATP-binding site of the kinase domain is replaced with Met) [10, 14]. The plasmid pcDNA3.1 was negative control. The plasmid pcDNA3.1, plasmid EML4-ALK variant 1 and plasmid EML4-ALK K589M were separately introduced into BEAS-2B and H2126 cells by Lipofectamine 2000 (Invitrogen, USA). Western blot analysis was used to identify the BEAS-2B and H2126 cells expressing EML4-ALK variant 1 and EML4-ALK K589M.

2.3. Western Blot Analysis

BEAS-2B and H2126 cells were seeded onto 6 well culture-plates before transfection. At 24h after transfection plasmid into BEAS-2B and H2126, the cells were collected and lysed on ice in a buffer containing 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40 and protease inhibitors. Proteins were quantitated by BCA assay (Thermo Scientific Pierce, USA). The proteins of the lysate were separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto the PVDF membranes. Then the membranes were blocked with 5% bovine serum albumin and incubated with a monoclonal rabbit anti-ALK antibody (1:1000 dilution, Cell Signaling Technology, USA) or a mouse anti-GAPDH monoclonal antibody (1:2000 dilution, abcam, USA) at 4°C overnight. After washed, the membranes were incubated with horseradish peroxidase-labelled secondary goat anti-rabbit antibody (dilution 1:2000; Dako, Denmark) or goat anti-mouse antibody (dilution 1:2000; Dako, Denmark) for 2 h at room temperature and enhanced chemoluminescence (Millipore Corporation, Billerica, USA) in substrate for 5 to 20 min.

2.4. Cell Proliferation Assay

BEAS-2B and H2126 cells were transfected with plasmid pcDNA3.1, plasmid EML4-ALK variant 1 and plasmid EML4-ALK K589M. At 24h following transfection, 3000 cell/well were seeded in 96-well plates. After 72h incubation, cell proliferation was determined by Cell Titer 96AQueous One Solution Cell Proliferation Assay (MTS assay) kit (Promega, USA).

2.5. Scratch Wound Healing Assay

The migration ability of the cells was assessed by scratch wound healing assay. BEAS-2B and H2126 cells were transfected with plasmid pcDNA3.1, plasmid EML4-ALK variant 1 and plasmid EML4-ALK K589M. A 3mm wound was introduced across the diameter of each plate until the cells were growth about 90% confluency after transfection. BEAS-2B cell migration was observed by microscopy at 72h later and analyzed objectively using Image J. H2126 cell migration was observed by microscopy at 24h later and analyzed objectively using Image J.

2.6. Quantitative Real-Time Reverse-Transcription PCR (Q-PCR)

BEAS-2B and H2126 cells were seeded onto 6 well culture-plates before transfection. At 24h after transfection plasmid into BEAS-2B and H2126, the cells were collected and lysed by Trizol (Invitrogen, USA). Total RNA was extracted with Trizol and quantified using the ND-1000 spectrophotometer (Nanodrop, USA). Complementary DNA was synthesized from 1μg total RNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions.

Q-PCR was performed to analyze the mRNA expression of 24 factors (Table 1). Q-PCR reactions were performed in triplicate and using the SYBR® *Premix Ex Taq*™ Perfect Real Time kit (Takara, Japan) in an Applied Biosystems 7500 Fluorescent Quantitative PCR System (Applied Bio-systems, USA). The reaction mixtures were incubated at 95°C for 30s, followed by 40 amplification cycles of 95°C for 5s and 60°C for 34s.

The relative expression levels of mRNAs between samples were calculated by the comparative delta CT (threshold cycle number) method ($2^{-\Delta\Delta CT}$) implemented in the 7500 Real-Time PCR System software. The representation of $2^{-\Delta\Delta CT}$ was fold changes in the relative gene expression than of target [17]. Primer sequences were presented in Table 2.

Table 1. *Factors analyzed by Q-PCR.*

Symbol	Official name
CXCL1	Chemokine (C-X-C motif) ligand1
CXCL3	Chemokine (C-X-C motif) ligand3
CXCL5	Chemokine (C-X-C motif) ligand5
CCL3	Chemokine (C-C motif) ligand3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IFNα	Interferon alpha
IFNβ	Interferon beta
MMP2	Matrix metalloprotease 2 (gelatinase A)
MMP9	Matrix metalloprotease 9 (gelatinase B)
M-CSF	Macrophage colony stimulating factor
S100A8	S100 calcium binding protein A8 (calgranulin-A)
S100A9	S100 calcium binding protein A9 (calgranulin-B)
TNF	Tumor necrosis factor
TGFβ1	Transforming growth factor beta 1
TGF-α	Transforming growth factor alpha
VEGFA	Vascular endothelial growth factor A

Table 2. *Primer sequence and product size used for qRT-PCR.*

Genes	Primer sequence	Product size (bp)
CXCL1	F-TCTTCCCTAGGAGCGTCCG	179
	R-GATGCAGGATTGAGGCAAGC	
CXCL3	F-AAAGCTTGTCTCAACCCCGC	131
	R-CACCCTGCAGGAAGTGTCAAT	
CXCL5	F-TGTGCAATTAACAAAGCTACTGC	128
	R-AGGCATCTAAAAAGCTCAGCA	
CCL3	F-GCTCTCTGCAACCAGTTCTCT	125
	R-CACTGGCTGCTCGTCTCAA	
G-CSF	F-AGAAGCTGGTGAGTGAGTGTG	168
	R-GGTAGAGGAAAAGGCCGCTA	
GM-CSF	F-CTCTGGGATCCCTCAGCTCT	148
	R-GTTCTGGGGAGGTAAACGGG	
IL-1	F-AACCTCTTCGAGGCACAAGG	107
	R-GGCGAGCTCAGGTACTTCTG	
IL-4	F-TCTTCCTGCTAGCATGTGCC	155
	R-TGTGTTCTTGGAGGCAGCAA	
IL-5	F-ACCCAGGAAGCGGGACAATA	135
	R-GCAGCTCCAAGAGCTAGCAA	
IL-6	F-CTCAATATTAGAGTCTCAACCCC CA	163
	R-GAGAAGGCAACTGGACCGAA	
IL-8	F-AGAGTGGACCACACTGCGC	251
	R-ACATCCCAACGGTCTACGTTA	
IL-10	F-CAACTGGCTCCCCTTACCTT	153
	R-GAGGCTGGATAGGAGGTCCC	
IL-12	F-ACTCTGGAAAGAATGCCACAAT	119
	R-ACCATGACTCACTTGGCTGC	
IL-13	F-TCTGCAATGGCAGCATGGTA	117
	R-GCATCCTCTGGGTCTTCTCG	
IFNα	F-TCGTATGCCAGCTCACCTTT	162
	R-CAGTCAGCATGGTCCTCTGT	
IFNβ	F-TGCTCTGGCACAACAGGTAG	192
	R-AGCCTCCCATTCAATTGCCA	
MMP2	F-GTCTGTGTTGTCCAGAGGCA	106
	R-CTAGGCCAGCTGGTTGGTTC	
MMP9	F-CCTGGGCAGATTCCAAACCT	172
	R-GTACACGCGAGTGAAGGTGA	
M-CSF	F-CCAAACTCACCAGGATGCTCA	140
	R-TCCACTCCCAATCATGTGGC	
S100A8	F-GGCCAAGCCTAACCGCTATAA	185
	R-CGGCATGGAAATTCCCCTTT	
S100A9	F-CGGCTTTGACAGAGTGCAAG	105
	R-GCCCCAGCTTCACAGAGTAT	
TNF	F-CACAGTGAAGTGCTGGCAAC	185
	R-AGGAAGGCCTAAGGTCCACT	
TGF-β1	F-ATGGAGAGAGGACTGCGGAT	167
	R-TAGTGTTCCCCACTGGTCCC	
TGF-α	F-CAGGGTGCGGAGATGGAAC	253
	R-CGAGGGCTCACGAGGAAGT	
VEGFA	F-CTGTCTAATGCCCTGGAGCC	124
	R-ACGCGAGTCTGTGTTTTTGC	

2.7. Statistical Analysis

All statistical analyses were performed by using SPSS version 18.0 (SPSS, Chicago, IL, USA). Differences between groups were analyzed using Student's t test, one-way ANOVA and Chi-square test. Datasets were described using descriptive statistics as means ± SD and differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Overexpression of the EML4-ALK Gene by Liposome-Mediated Transfection in BEAS-2B and H2126 Cells

Subsequent to 24 h of EML4-ALK transfection, the transfection of the ALK gene into BEAS-2B and H2126 cells was detected by western blotting. As shown in Figure 1, a successful transfer of EML4-ALK by the liposome complex was confirmed by western blotting of ALK protein. In the plasmid EML4-ALK variant 1-transfected cells and plasmid EML4-ALK K589M-transfected cells, the protein expression levels of ALK were upregulated. By contrast, no band was observed in the empty vector-plasmid pcDNA3.1-transfected cells.

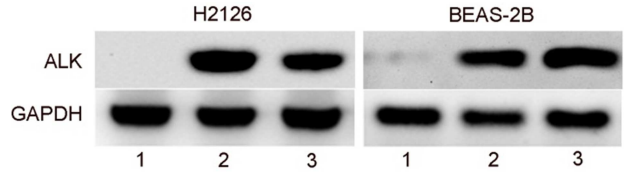

Figure 1. *Western blot analysis of ALK protein in BEAS-2B and H2126 cells by transfection plasmid EML4-ALK variant 1 and EML4-ALK K589M. Lane 1: plasmid pcDNA3.1-transfected cells; Lane 2: plasmid EML4-ALK variant 1-transfected cells; Lane 3: plasmid EML4-ALK K589M-transfected cells.*

3.2. Overexpression of the EML4-ALK Gene Promoted Cell Proliferation and Cell Migration Ability

To investigate whether EML4-ALK was involved in the cell proliferation, cell proliferation assay was performed after overexpression of EML4-ALK gene in H2126 and BEAS-2B cells. As shown in Figure 2, the growth of plasmid EML4-ALK variant 1-transfected cells was higher than plasmid EML4-ALK K589M-transfected cells and empty

vector-plasmid pcDNA3.1-transfected cells ($P<0.05$). The results demonstrated that EML4-ALK had an effect on cell proliferation.

The migration of BEAS-2B and H2126 cells was increased after transfection of plasmid EML4-ALK variant 1 compared to empty vector-plasmid pcDNA3.1 and plasmid EML4-ALK K589M (Figure 3). The results of quantitative analysis showed a significant increase of BEAS-2B and H2126 cells migration after transfection EML4-ALK variant 1 compared with empty vector-plasmid pcDNA3.1 and plasmid EML4-ALK K589M ($P<0.05$).

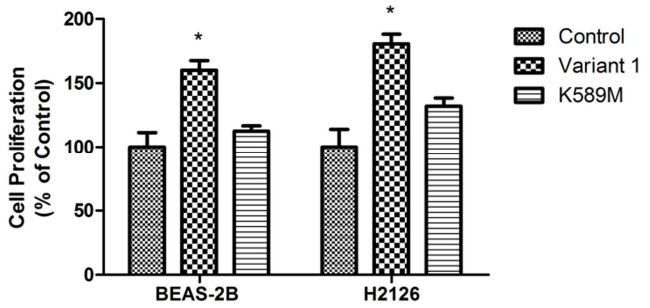

Figure 2. *Cell proliferation in BEAS-2B and H2126 cells: the growth of plasmid EML4-ALK variant 1-transfected cells was significantly accelerated compared with empty vector-plasmid pcDNA3.1-transfected cells and plasmid EML4-ALK K589M-transfected cells ($P<0.05$, the symbol * indicates statistically significant). Control: plasmid pcDNA3.1-transfected cells; Variant 1: plasmid EML4-ALK variant 1-transfected cells; K589M: plasmid EML4-ALK K589M-transfected cells.*

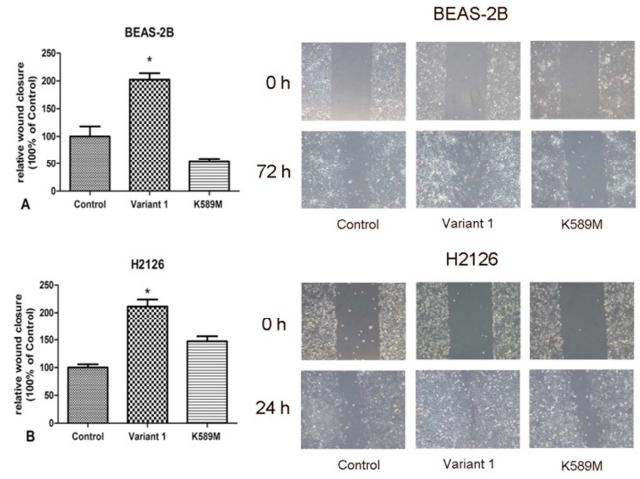

Figure 3. *Scratch wound healing assay in BEAS-2B and H2126 cells: The cell migration of plasmid EML4-ALK variant 1-transfected cells was increased compared with empty vector-plasmid pcDNA3.1-transfected cells and plasmid EML4-ALK K589M-transfected cells ($P<0.05$, the symbol * indicates statistically significant). Control: plasmid pcDNA3.1-transfected cells; Variant 1: plasmid EML4-ALK variant 1-transfected cells; K589M: plasmid EML4-ALK K589M-transfected cells.*

3.3. Preliminary Screening of Factors Related to Inflammatory Molecular and Immune Mediators Influence Tumor Progression and Metastasis After Overexpression of the EML4-ALK Gene

We analyzed the expression of 24 factors related with inflammatory molecular and immune mediators influence tumor progression and metastasis after transfection by the plasmid EML4-ALK variant 1 and plasmid EML4-ALK K589M through Q-PCR (Table 1). The empty vector-plasmid pcDNA3.1 was negative control. Though quantitative analysis, the results found differences in the RNA expression of 2 factors (S100A8 and S100A9) in BEAS-2B and H2126 cells after transfection (Figure 4). The plasmid EML4-ALK variant 1-transfected cells showed an increase in the mRNA level of S100A8 and S100A9 compared to plasmid EML4-ALK K589M-transfected cells and empty vector-plasmid pcDNA3.1-transfected cells.

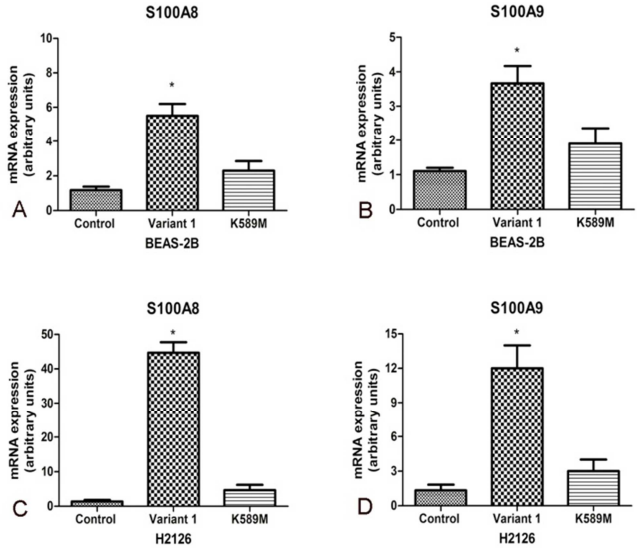

Figure 4. *Q-PCR analysis of S100A8 and S100A9 differentially expressed in BEAS-2B and H2126 cells by overexpression EML4-ALK: A: S100A8 mRNA expression in BEAS-2B cells; B: S100A9 mRNA expression in BEAS-2B cells; C: S100A8 mRNA expression in H2126 cells; D: S100A9 mRNA expression in H2126 cells ($P<0.05$, the symbol * indicates statistically significant). Control: plasmid pcDNA3.1-transfected cells; Variant 1: plasmid EML4-ALK variant 1-transfected cells; K589M: plasmid EML4-ALK K589M-transfected cells.*

4. Discussion

EML4-ALK, a novel fusion oncogene, was identified in NSCLC in 2007 [10]. EML4-ALK was formed as the result of a small inversion within the short arm of chromosome 2 because intron 13 of EML4 was disrupted and joined to intron 20 of ALK. Therefore, the fusion protein contained the amino-terminal half of EML4 and the intracellular catalytic domain of ALK [10, 18]. ALK rearrangements results in the activation of ALK and its downstream signaling pathways displaying tumorigenic activity [11].

At least 14 EML4-ALK variants have been reported in patients with ALK-positive NSCLC [19]. The most common variant is E13:A20 (the nomenclature refers to the exons in EML4 (E) that are fused to ALK (A)) which are referred to as variants 1(v1, 33%) [19, 20]. In this study, we constructed plasmid EML4-ALK variant 1 to overexpression EML4-ALK in NSCLC cell line with a triple negative genotype (H2126) and human bronchial epithelial cell (BEAS-2B). In order to eliminate other factors, we meanwhile constructed plasmid

EML4-ALK K589M which EML4–ALK kinase was inactive. Compared to the empty vector-plasmid pcDNA3.1, plasmid EML4-ALK variant 1 and EML4-ALK K589M were successfully transferred into BEAS-2B and H2126 cells by western blotting detection.

The results of the functional experiments *in vitro* showed that plasmid EML4-ALK variant 1 promoted tumor cell proliferation and migration in BEAS-2B and H2126 cells compared to EML4-ALK K589M. Then we performed a preliminary screening of inflammatory molecular and immune mediators influence tumor progression and metastasis in BEAS-2B and H2126 cells after overexpression EML4-ALK variant 1 in order to analyze the relationship between specific inflammatory factors and EML4-ALK. Our results demonstrated that factors more differentially expressed between BEAS-2B and H2126 cells after overexpression EML4-ALK variant 1 were S100A8 and S100A9 by Q-PCR.

In a word, our study showing an increased expression level in S100A8 and S100A9 by overexpression EML4-ALK variant 1 had a great biological interest because of their relation with tumor cell proliferation and migration. S100A8 and S100A9, two members of the S100 family of calcium-binding proteins, have been participated in numerous functions including protein phosphorylation, enzymatic activation, calcium homeostasis, and known to play roles in innate immune system [21, 22]. The overexpression of S100A8 and S100A9 have been reported in association with the tumor cell differentiation and tumor progression [23, 24, 25]. Therefore, the further work is to study the effects of S100A8 and S100A9 in EML4-ALK positive NSCLC in vivo and the mechanisms which cause the S100A8 and S100A9 overexpression.

5. Conclusion

Our study investigated the relationship between overexpression of EML4-ALK and inflammatory factors about tumor progression and metastasis in a human bronchial epithelial cell (BEAS-2B) and a lung cancer cell (H2126). The results of Q-PCR showed that factors more differentially expressed between both groups of BEAS-2B and H2126 cells were S100A8 and S100A9 after transfection EML4-ALK variant 1. And only EML4-ALK variant 1 exhibited higher proliferation rates and invasion ability compared to the controls indicating a role of EML4-ALK variant 1 of the pathogenetic mechanism in NSCLC. Therefore, an increased expression level in S100A8 and S100A9 by overexpression EML4-ALK variant 1 had a great biological interest because of their relation with tumor cell proliferation and migration. The data may provide the next research direction for molecular pathogenesis of EML4-ALK positive lung cancer.

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