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CARMA3 regulates the invasion, migration, and apoptosis of non-small cell lung cancer cells by activating NF-κB and suppressing the P38 MAPK signaling pathway

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ABSTRACT

In our previous study, CARMA3 overexpression in lung cancer cells promoted cell proliferation and invasion; however, the mechanism underlying the role of CARMA3 in cancer cell invasion remained unclear. In the present study, knockdown of CARMA3 in A549 and H1299 cells suppressed cell invasion and migration, and downregulated matrix metalloprotease 9 expression at the protein and mRNA levels, as shown by Western blotting and real-time PCR. CARMA3 knockdown increased cell apoptosis, as shown by flow cytometry, increased the mRNA and protein expression levels of Bax and Caspase3, and downregulated Bcl-2 in A549 and H1299 cells. Phosphorylated P38 levels increased and NF-κB activation decreased following knockdown of CARMA3. SB203580, a P38 MAPK inhibitor, activated NF-κB, increased cell migration, and inhibited cell apoptosis after knockdown of CARMA3 compared to knockdown of CARMA3 without SB203580. These findings indicate that CARMA3 may suppress the activation of the P38 MAPK signaling pathway to regulate invasion, migration and apoptosis of lung cancer cells by activating NF-κB (P65) in the nucleus.

1. Introduction

The human CARMA3 gene located on chromosome 22 q13. 1, also known as caspase recruitment domain (CARD) and membrane-associated guanylate kinase-like (GUK) domain gene, belongs to the CARMA family. The proteins in the family are scaffold proteins, and there are three members: CARMA1 (CARD11), CARMA2 (CARD14), and CARMA3 (CARD10). These three proteins share similar structural motifs, with an N-terminal CARD domain, followed by a coiled-coil domain, a PDZ domain, an SH3 domain, and a C-terminal GUK domain. However, they have different expression patterns, as CARMA1 is expressed in hematopoietic cells, CARMA2 is expressed in the placenta and several mucosae, and CARMA3 is expressed in non-hematopoietic cells (Bertin et al., 2001; Gaide et al., 2001; Wang et al., 2001; McAllister-Lucas et al., 2001).

Numerous studies have confirmed that CARMA3 is overexpressed in breast cancer, ovarian cancer, colorectal cancer and kidney cancer tissues (Wu et al., 2013; Zhao et al., 2013; Miao et al., 2012; Mahanivong et al., 2008), and that CARMA3 affects the proliferation, invasion and migration of tumor cells in association with the activation of nuclear factor-κB (NF-κB) (Jiang et al., 2011). Recent studies showed that guanosine-binding protein coupled receptor (GPCR), epidermal growth factor receptor (EGFR) and protein kinase C (PKC) induced the activation of NF-κB by CARMA3 (Jiang et al., 2011; Grabiner et al., 2007; McAllister-Lucas et al., 2007), and several other studies indicated that GPCR and PKC require CARMA3 as well as its downstream signal components, MALT-1 and BCL10, to activate NF-κB (Jiang et al., 2011; McAllister-Lucas et al., 2007; Wang et al., 2007; Klemm et al., 2007; Pan and Lin, 2013). Protease activated receptor 1 signaling to NF-κB depends on initial PKC activation, and a complex of proteins containing CARMA3, Bcl10 and MALT-1 links PAR-1 activation to stimulation of the IκB kinase complex. IκB kinase in turn phosphorylates IκB, leading to its degradation and the release of active NF-κB in vascular endothelial cells (Delekta et al., 2010). Activation of NF-κB confers resistance to apoptosis and enhances the proliferation and migration ability of cancer cells (Hanahan and Weinberg, 2000).

Early research in our laboratory showed that CARMA3 is overexpressed in non-small cell lung cancer tissues, and its expression is associated with Tumor Node Metastasis (TNM) staging, lymph node metastasis, and the expression of EGFR. CARMA3 promoted the proliferation, invasion, and migration of lung cancer cells, and may promote the proliferation of lung cancer cells by upregulating cyclin D1 (Li et al., 2012). However, the mechanism by which CARMA3 promotes the

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invasion and migration of lung cancer cells remains unclear. The present study focused on the mechanism underlying the promotion of cancer cell invasion and migration, and the inhibition of lung cancer cell apoptosis by CARMA3.

2. Materials and methods

2.1. Quantitative real-time PCR (SYBR Green Method)

For cDNA synthesis, a Prime Script ® RT reagent Kit with gDNA Eraser (TakaRa Dalian, China) was used. The reaction system consisted of 10 μl reaction mix (total RNA, 5 μl, 300 ng); the gene amplification (G-Storm, UK) complete reverse transcription experiment process was as follows: 37 °C, 15 min; 85 °C, 5 s. Amplification products were prepared for real time PCR the next day, after −20 °C storage. Quantitative real-time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 ml on a 7900HT Fast Real-Time PCR System (Applied Biosystems) as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 30 s. A dissociation step was performed to generate a melting curve to confirm the specificity of the amplification. GAPDH was used as the reference gene. The relative levels of gene expression were expressed as $\Delta Ct = Ct\ gene - Ct\ reference$, and the fold change of gene expression was calculated by the $2-\Delta\Delta Ct$ method. The primer sequences are provided in Table 1. Experiments were performed in triplicate.

2.2. Western blot analysis

Total proteins from primary tissues and cell lines were extracted in lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) and quantified using the Bradford method. Sixty micrograms of protein was separated by SDS-PAGE (12%). After transferring, the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were incubated overnight at 4 °C with the following antibodies (as shown in Table 2). After incubation with peroxidase-coupled anti-mouse or rabbit or goat IgG (Santa Cruz Biotechnology) at 37 °C for 2 h, bound proteins were visualized using ECL (Thermo Fisher Scientific) and detected using a BioImaging System (UVP Inc., Upland, CA, USA). The relative protein levels were calculated based on GAPDH as the loading control.

2.3. Cell lines and small interfering RNA treatment

A549 and H1299 lung cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), and 100 mg/ml streptomycin (Sigma). Cells were grown on sterile tissue culture dishes and passaged every 2 days using 0.25% trypsin (Invitrogen). SiRNA against CARMA3 and non-targeting siRNA were purchased from RiBoBio (Guangzhou, China). For transfections, cells were seeded in a six-well plate 24 h before the experiment. The cells were transfected with siRNA (10 μl/well; RiBoBio) according to the manufacturer's protocol. Following transfection, the mRNA and protein levels were assessed 48 h later.

Table 1
Real time PCR primer sequences.

Primer name	Primer sequences
CARMA3 forward	5′-TCTTCCACCGTTGCCAATCT-3′
CARMA3 reverse	5′-TTCGCCTGCCAGGAACATC-3′
GAPDH forward	5′-AGAAGGCTGGGGCTCATTTG-3′
GAPDH reverse	5′-AGGGGCCATCCACAGTCTTC-3′
Bax forward	5′-TGTGGTCTATAATGCGTTTTCC-3′
Bax reverse	5′-GGCACTAATCAAGTCAAGGTCA-3′
BCL-2 forward	5′-ATTTCTCCTGGCTGTCTCTGAA-3′
BCL-2 reverse	5′-CAGGCATGTTGACTTCACTTGT-3′
Caspase3 forward	5′-AGTAGATGGTTTGAGCCTGAGC-3′
Caspase3 reverse	5′-GTATGGAGAAATGGGCTGTAGG-3′
MMP2 forward	5′-CCATTTTGATGACGATGAGCTA-3′
MMP2 reverse	5′-ACAAGAAGGGGAACTTGCAGTA-3′
MMP9 forward	5′-ACTTTGACAGCGACAAGAAGTG-3′
MMP9 reverse	5′-GGCACTGAGGAATGATCTAAGC-3′
TIMP-1 forward	5′-GCACATCACTACCTGCAGTC-3′
TIMP-1 reverse	5′-GAAACAAGCCCACGATTTAG-3′

Table 2
Dilution ratio of antibodies.

Name of the antibodies	Manufacture	Dilution ratio
Anti-CARMA3	Sigma	1:100
Anti-MMP9	Proteintech	1:500
Anti-MMP2	Proteintech	1:500
Anti-MMP7	Proteintech	1:500
Anti-BCL-2	Proteintech	1:500
Anti-Bax	Proteintech	1:500
Anti-Caspase3	Proteintech	1:500
Anti-Caspase9	Proteintech	1:500
Anti-P65	Santa Cruz Biotechnology	1:100
Anti-P38	Cell Signaling	1:500
Anti-P-P38	Cell Signaling	1:500
Anti-P21	Cell Signaling	1:500
Anti-P73	Santa Cruz Biotechnology	1:100
Anti-N-Cadherin	BD Biosciences	1:500
Anti-E-Cadherin	BD Biosciences	1:500
Anti-AKT1/2	Santa Cruz Biotechnology	1:100
Anti-P-AKT	Cell Signaling	1:500
Anti-GAPDH	KANGCHEN	1:5000
Anti-LaminB	Cell Signaling	1:200

2.4. Flow cytometry

After knocking down CARMA3 for 48 h, cells were harvested and washed twice with cold PBS by gentle shaking. Resuspended cells were added to binding buffer (1×) and cell density was adjusted to 200,000–500,000/ml. In the dark, 5 μl of Annexin V-FITC (50 mM TRIS, 100 mM NaCl, 1% BSA, 0.02% sodium azide, pH 7.4) was added to the cell suspension in a mix of 195 μl and incubated for 10 min at room temperature before adding 190 μl binding buffer (1×) and 10 μl propidium iodide (PI). Ten thousand events per sample were acquired using a FACS-scan flow cytometer (Becton-Dickinson, San Jose, CA, USA) and the percentage of apoptotic cells was analyzed using CellQuest analysis software (Becton-Dickinson).

2.5. Cell migration and invasion assays

Cell invasion was assessed using a 24-well Transwell chamber with a pore size of 8 mm (Costar, Cambridge, MA, USA). The inserts were coated with 20 ml Matrigel (1:3 dilution, BD Biosciences, San Jose, CA, USA). Forty-eight hours after the transfection, cells were trypsinized and 40,000 cells in 100 μl of serum-free medium were transferred to the upper Matrigel chamber and incubated for 24 h. The cell migration assay was performed using a 24-well Transwell chamber with a pore size of 8 mm (Costar). Forty-eight hours after the transfection, cells were trypsinized and 30,000 cells in 100 μl of serum-free medium were transferred to the upper Matrigel and incubated for 16 h. After incubation, the non-invaded or non-migrated cells on the upper membrane surface were removed with a cotton tip, and the cells that passed through the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. The number of invaded or migrated cells was counted under microscope (OLYMPUS, Japan) in five randomly selected high power fields. This experiment was performed in triplicate.