CCL18 Induces EMT via CEP192 Down-Regulation in Breast Cancer

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Abstract

Background: Chemokine (C-C motif) ligand 18 (CCL18) secreted by TAMs induces epithelial-mesenchymal transformation (EMT) in breast cancer cells. Centrosome scaffold protein KDA192 (CEP192), as the important pericentriolar material (PCM), plays a critical role in microtubule nucleation and centrosome replication. Decreased expression of CEP192 will cause tumor cell polarization and elevated motility. Whether CEP192 is involved in the process of breast cancer EMT induced by CCL18 has not been reported.

Methods: CEP192 mRNA level in lymph node metastasis and non-lymph node metastasis breast cancer was evaluated by bioinformatics analysis. CCL18 and CEP192 expressions in breast tissues were examined using immunochemistry, CEP192 expression in breast cancer cells was examined using qPCR and Western blot. CEP192 was silenced using siRNA. EMT phenotype proteins were examined using Western blot. Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc).

Results: CEP192 expression was decreased in lymph node metastasis breast cancer tissues compared with non-lymph node metastasis. CEP192 expression was down-regulated by CCL18 in breast cancer cells and silencing CEP192 promoted the migration and invasion of cancer cells. Based on the promotion of breast cancer EMT by CCL18, silencing CEP192 in breast cancer further increased E-cadherin and decreased KRT-8 which are both EMT phenotype proteins in vitro.

Conclusions: Our study elucidates that CCL18 promotes breast cancer EMT by down-regulating CEP192 expression. Down-regulated CEP192 plays a key role in invasion and migration of breast cancer cells. CEP192 is a potential new marker for tumor metastasis induced by CCL18.

Background

Centrosome is the key microtubule assembly centers (MTOC) in vertebrate cells, consists of two perpendicular centrioles, which regulates the tumor cells cytoskeleton and motility. Centrosome is wrapped around pericentriolar material (PCM) [1–4]. Aurora-A kinase (Aurora-A) and polo-like kinase-1 (PLK-1) as PCM proteins regulate the replication and division of centrosomes. Increased expressions of Aurora-A and PLK-1 promote the development of tumor which cause tumor cells epithelial-mesenchymal transformation (EMT) have been reported in gastric cancer, nasopharyngeal cancer, laryngeal cancer, non-small cell lung cancer [5–14]. Centrosome protein 192 KDa (CEP192) is the key protein of the PCM, which is responsible for microtubule nucleation and centrosome replication mediated by γ -TuRC (γ -tubulin ring complex) [3]. The absence of CEP192 leads to cell morphological polarization, promotion of tumor cells migration [15, 16]. In this paper, we find that CEP192 mRNA level in lymph node metastasis of breast cancer was significantly decreased compared with lymph node metastasis through bioinformatics analysis by TCGA database in Oncomine website. Besides, CEP192 cooperate with Aurora-A and PLK-1 to regulate microtubule nucleation [16]. Although the PCM closely associated with CEP192 promotes EMT, the mechanism of CEP192 affects the motility of cancer cells has not been reported.

Current studies have found that tumor cells grown in the tumor microenvironment, which plays a significant role in promoting the motility of tumor cells [17, 18]. Tumor-associated macrophages infiltrate in the microenvironment, secreting CCL18 which can promote the migration and invasion of breast cancer and other solid tumors [19–22], through the PITPNM3 receptor [23–25]. It has been reported that CCL18 promotes epithelial-mesenchymal transformation (EMT) through PI3K/AKT and NF-kb signaling pathways [26–27]. Although both CCL18 and CEP192 correlates with cancer cells migration, whether CEP192 participates in tumor cells EMT induced by CCL18 remains unknown.

Here, our data indicated that CCL18 down-regulated CEP192 expression, and the reduction of CEP192 promoted EMT in breast cancer cells. This finding provides a new biomarker for tumor migration induced by CCL18.

Materials And Method

Tissue samples

30 cases of tissue samples were collected from the breast cancer patients in the Affiliated People's Hospital of Guangzhou Medical University, including 8 cases of tissue adjacent to carcinoma, 10 cases of non-lymph node metastasis carcinoma tissue, 12 cases of lymph node metastasis carcinoma tissue, and informed consent was obtained from all patients. The pathological diagnosis was made independently by two pathologists. None of the patients had undergone chemotherapy or radiotherapy. The study was approved by the Human Research Ethics Committee of the Affiliated People's Hospital of Guangzhou Medical University.

Cell lines and cell culture

Human breast cancer cell lines (MCF-7, MDA-MB-231) were obtained from the American Type Culture Collection (ATCC, USA), and were cultured in DMEM F12 culture medium containing 20% foetal bovine serum (FBS, SIGMA-ALDRICH) for MCF-7 and 10% for MDA-MB-231 supplemented with 100 U/ml penicillin sodium and 100 µg/ml streptomycin sulphate (Sigma-Aldrich). These cell lines were incubated in the humidified incubator with the atmosphere of 37 °C containing 5% CO₂.

Quantitative RT-PCR

Cells were collected, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT) was conducted using Prime Script RT Master Mix (Takara, Dalian, China) to synthesize cDNA. Q-RT-PCR was performed with SYBR Green PCR Master Mix (Takara, Dalian, China) on an ABI 7900HT (PE Applied Biosystems) qPCR machine. For relative quantification, target gene mRNA expression was normalized to β-actin expression. The 2^{-ΔΔCt} method was applied to analyse the data, and each experiment was performed in triplicate. The primers sequence used were CEP192 Forward (5'-TCCCTCGACTCACACTCTTCT-3'), CEP192 Reverse (5'-

TTTGGTGAGGACACTCTGCC-3'), GAPDH Forward (5'-CATCATCCCTGCCTCTACTG-3'), GAPDH Reverse (5'-GCCTGCTTCACCACCTTC-3'), β -actin Forward (5'-AGGCCAACCGCGAGAAGATG-3'), Reverse (5'-CACACGGAGTACTTGCGCTCAG-3')

RNA interference

CEP192 siRNA sequences and the si-GFP sequence were designed by RIBOBIO (Guangzhou, China) and used to transfect cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The target sequences of CEP192 siRNA used were siCEP192(1) (5'-AAGGAAGACATTTTCATCTCT-3'), siCEP192(2) (5'-CCAGGAGCCTATAGATGAA-3').

Western blot

RIPA buffer (Beyotime, China) was used to extract the protein following the appropriate steps. BCA Protein Assay Kit (Beyotime, China) was used to measure the concentration of extracted protein. Cells were collected and lysed with RIPA buffer. Proteins were quantified using a bicinchoninic acid assay (Thermo Scientific), resolved by SDS-PAGE, and transferred onto PVDF membranes (Millipore, Billerica, USA). Antibody detection was conducted using an enhanced chemiluminescent substrate kit (Yeasten). Antibodies against CEP192 (Bioss, 1:1000, China), E-cadherin (Abcam, 1:500, Cambridgeshire, UK), KRT-8 (Abcam, 1:1000, Cambridgeshire, UK) were used to the related protein level. β -actin (1:1000, Abcam, UK) and GAPDH (1:2500, Abcam, UK) were used for normalization.

Cell invasion and migration assays

Boyden chamber invasion assay was used to detect the invasiveness of MDA-MB-231 cell. Transfected MDA-MB-231 cells were digested and resuspended in serum-free DMEM, and were placed at the top of the Matrigel-coated chambers (BD Biosciences, USA). The culture medium with 10% fetal bovine serum was used as the chemical attractant and added to the lower chamber. After 24 h, the fixed invasive cells were stained with crystal violet, counted and photographed. Boyden chamber migration experiment repeated the invasion procedure, but the Matrigel was not applied.

Immunohistochemistry (IHC)

IHC was performed using 4- μ m-thick sections of representative formalin-fixed tissue blocks. Briefly, the slides were dewaxed in xylene, passed through graded alcohols, and placed into 0.01 mol/L phosphate-buffered saline (PBS; pH = 7.4). The slides were then pretreated with 1.0 mM citrate, pH 6.0 (Invitrogen), in a steam pressure cooker for epitope retrieval and were washed in PBS. Next, they were incubated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and were subsequently incubated with a monoclonal rabbit anti-human CCL18 antibody (Biodragon, 1:125, China), CEP192 (BIOSS 1: 250, China) at 4 °C overnight. On the following day, the slides were washed with PBS and incubated with an anti-rabbit secondary antibody (Dako) for 60 min at room temperature. After being washed in PBS, the

slides were stained with DAB+ (Dako) and then counterstained for 1 min with Harris hematoxylin (BASO), differentiated in 1% hydrochloric acid in alcohol, dehydrated, and mounted. All PT and LNM specimens were stained using the same protocol.

Results

In breast cancer tissues with lymph node metastasis, CCL18 expression is increased, while CEP192 expression is decreased

Based on the analysis of the TCGA database on the Oncomine website (<https://www.oncomine.org/resource/main.html>), CEP192 expression showed a 37.50% reduction in mRNA levels in breast cancer tissues with lymph node metastasis (n = 203) compared with non-lymph node metastasis (n = 178) (Fig. 1a, $P < 0.001$). A cohort of 30 breast cancer cases (patients information Table S1) included 8 cases of para-tumor tissues, 10 cases of non-lymph node metastasis tissues, and 12 cases of lymph node metastasis tissues. Compared with para-tumor tissues, IHC proved that CEP192 expression in lymph node metastasis breast cancer tissues decreased by 35.90% (Fig. 1b, c, $P < 0.01$), in non-lymph node metastasis obviously decreased by 63.37% (Fig. 1b, c, $P < 0.001$). Compared with para-tumor tissues, the expression level of CCL18 in lymph node metastasis breast cancer tissues increased by 1.26-fold, and in non-lymph node metastasis obviously increased by 1.97-fold (Fig. 1b, d, $*P < 0.05$, $**P < 0.001$). Our data indicated that increased CCL18 and decreased CEP192 is associated with breast cancer metastasis.

CCL18 down-regulates CEP192 expression in breast cancer cells

To study the correlation between CCL18 and CEP192 in breast cancer cells, cell experiments were performed. After MCF-7 and MDA-MB-231 cells were treated with CCL18 (20 ng/ml) for 48 h, Q-RT-PCR showed that CEP192 mRNA levels in MCF-7 and MDA-MB-231 decreased by 42.89% (Fig. 2a, $**P < 0.01$) and 50.86% (Fig. 2b, $**P < 0.01$), respectively, in breast cancer cells compared with the untreated group. Western blot demonstrated that CEP192 expression in cells was decreased compared with the untreated group (Fig. 2c-d). Together this data suggested that CCL18 down-regulates CEP192 expression in breast cancer cells.

CEP192 down-regulation in breast cancer cells promotes tumor migration and invasion

To prove the function of CEP192 down-regulation in breast cancer metastasis, CEP192 was silenced in breast cancer cells MDA-MB-231 to perform Boyden chamber assays. The numbers of migration cancer cells in siCEP1 and siCEP2 group were increased by nearly 3-fold compared with the PBS group (Fig. 3a and 3c, $P < 0.01$). Boyden chamber invasion assay showed that cell numbers of siCEP1 and siCEP2 group

also increased by closely 3-fold (Fig. 3b and 3d, $P < 0.01$). Collectively, our data suggested that silence of CEP192 in breast cancer cells promotes tumor migration and invasion.

CCL18 promotes EMT in breast cancer cells by down-regulating the expression of CEP192

It has been reported that CCL18 promotes tumor invasion and migration by promoting EMT in breast cancer cells, so we further discussed whether CEP192 is involved in CCL18 promoting EMT in breast cancer cells. CEP192 was silenced in breast cancer MCF-7 cells without CCL18 treatment to detect EMT-related phenotype proteins E-cadherin and KRT8 by Western blot. Compared with PBS group, expressions of CEP192, E-cadherin and KRT8 in mock and siGFP transfection were stable. Transfection with CEP192-siRNAs showed decreased expression of E-cadherin and increased expression of KRT8 (Fig. 4a). Compared with PBS group, the expression of CEP192 in cancer cells with CCL18 treatment (non-transfection, mock transfection and siGFP transfection) was decreased, while the expression of E-cadherin was decreased and the expression of KRT8 was increased. In CCL18-treated MCF-7 cells, compared with the non-transfected group, transfection with CEP192-siRNAs revealed a further decrease in CEP192 expression, while the E-cadherin expression was further reduced and the KRT8 expression was further increased (Fig. 4b). These data suggested that CCL18 promotes tumor cells EMT by down-regulating CEP192 expression in breast cancer cells.

Discussion

In the tumor microenvironment, CCL18 secreted by tumor-associated macrophages promotes the invasion and migration of breast cancer and angiogenesis. CCL18 induces EMT in tumor cells by activating the NF- κ B signaling pathway. The change of cytoskeleton regulates the motility of tumor cells [28]. Vimentin as a critical cytoskeleton protein promotes tumors migration. Whereas the relationship between CCL18 and cytoskeleton is unclear. Previous studies have confirmed that AURORA-A and PLK1 cause tumor cells EMT in vivo and in vitro. Additionally, CEP192 as a critical centrosome protein, plays an important role in the motility of tumor and the regulation of cytoskeleton. CEP192 combines with PLK1 and AURORA-A to provide attachment points for NEDD1 as γ -TURC to assist microtubule nucleation [16]. Although both CCL18 and CEP192 cause changes in the morphology and movement of tumor cells, it has not been reported whether CEP192 participates in EMT induced by CCL18.

Our study found that CCL18 derived from TAMs causes down-regulation of the CEP192 expression to promote tumor cells EMT in breast cancer. This finding suggests that CCL18 regulating the protein correlated cytoskeleton management to promote migration of breast cancer. Other studies have showed that CCL18 is found in various tumors, including lung cancer, liver cancer and ovarian cancer. Therefore, whether this phenomenon is universal in tumors can be further studied.

In EMT, E-cadherin, N-cadherin, Vimentin, Fibronectin and KRT8 are important phenotypic proteins. The previous study has reported KRT8 overexpression in gastric cancer cells in vitro can promote EMT

through TGF- β 1 / SMAD2 / SMAD3 and integrin-1/FAK signaling pathways activation [29]. However, whether EMT induced by CCL18 is promoted by KRT8 in breast cancer has not been reported yet. We found that the decrease of CEP192 in breast cancer cells can promote the obvious over-expression level of KRT8. Therefore, whether KRT8 can promote the next potential regulatory molecule of EMT after CEP192 down-regulation in breast cancer induced by CCL18 is also worthy of further investigation.

Conclusion

Our study confirmed that CCL18 as the TAMs-derived chemokine promotes tumor EMT and the invasion and migration of tumor cells by down-regulating CEP192 in breast cancer from clinical tissues and cell experiments. This study provides a new biomarker for the invasion and migration of breast cancer induced by CCL18.

Abbreviation

CCL18 Chemokine C-C motif Ligand 18

TAMs Tumor-associated macrophages

CEP192 Centrosome protein of 192 KDa

MTOC microtubule organizing center

PCM pericentriolar material

PVDF Polyvinylidene fluoride

Q-RTPCR Quantitative Real-time polymerase chain reaction

PBS Phosphate Buffered Saline

SDS-PAGE Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

TBS Tris buffered saline

PMSF Phenylmethyl sulfonylfluoride

DAPI 4',6-diamidino-2-phenylindole

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Jingqi Chen designed this research and provided guidance for the research ideas and experiments and checked the writing format. Zichao Wu performed the majority of experiments and all statistics and wrote the paper. Xinrong Ke, Yanli Liu, Yihong Dong participated in parts of Western blot and Q-RT-PCR experiments.

Ethics approval and consent to participate

All samples were collected with informed consent according to the Internal Review and the Ethics Boards of the Second Affiliated Hospital of Guangzhou Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Figures

Figure 1

CCL18 promotes breast cancer cells EMT via CEP192. a. Western blot detected the expression of EMT-related phenotypic proteins E-cadherin and KRT8 in breast cancer MCF-7 cells untransfected (Un), mock transfected (mock), transfected with GFP-siRNA (si-GFP), and CEP192-siRNAs (si-CEP-1 and 2) (n=3). b. Western blot detected the expression of EMT-related phenotypic proteins E-cadherin and KRT8 in breast cancer MCF-7 cells treated with PBS or CCL18 and untransfected (Un), mock transfected (mock), transfected with GFP-siRNA (si-GFP), and COL4A1-siRNAs (si-CO-1 and 2) (n=3).

Figure 2

Silencing CEP192 promotes invasion and migration of breast cancer. a. Boyden chamber migration assays of breast cancer cells MDA-MB-231 untransfected (Un), mock transfected (mock), transfected with GFP-siRNA (si-GFP), and CEP192-siRNAs (si-CEP1 and 2) (n=3). b. Boyden chamber invasion assays of breast cancer cells MDA-MB-231 transfected as described in (a) (n=3). c. Cell counts of migration breast cancer cells MDA-MB-231 transfected as described in (a). The experimental data represented three independent repeated experiments. Bars equal to mean $\pm$ SD. ** P<0.01 one-way ANOVA with post analysis compared with GFP-siRNA transfected cells (siGFP). d. Cell counts of invasion breast cancer cells MDA-MB-231 transfected as described in (a). The experimental data represented three independent

repeated experiments. Bars equal to mean $\pm$ SD. ** $P < 0.01$ one-way ANOVA with post analysis compared with GFP-siRNA transfected cells (siGFP).

Figure 3

The expression of CEP192 in breast cancer cells. a. In MCF-7, mRNA levels of CEP192 in CCL18 untreated group and CCL18 treated group (the experimental data represent mean $\pm$ SD of three independent replicates, ** $P < 0.01$, analyzed by student's t-test). b. In MDA-MB-231, mRNA levels of CEP192 in CCL18 untreated group and CCL18 treated group (the experimental data represent mean $\pm$ SD of three independent repeated experiments, ** $P < 0.01$, analyzed by student's t-test). c. Western blot detected the expression of CEP192 in CCL18 untreated group and CCL18 treated group in MCF-7 (n=3). d. Western blot detected the expression of CEP192 in the CCL18 untreated group and the CCL18 treated group in MDA-MB-231 (n=3).

Figure 4

Expressions of CCL18 and CEP192 in breast cancer tissues. a. The expression of CEP192-mRNA in non-lymph node metastatic breast cancer (n=178) and lymph node metastatic breast cancer (n=203) in the Oncomine TCGA database (*** $P < 0.001$). b. The expression of CEP192 and CCL18 in adjacent breast cancer tissues (n= 8), non-lymph node breast cancer tissues (n=10), lymph node breast cancer tissues (n=12) was determined by immunohistochemistry ($\times 400$). c. Immunohistochemical grayscale analysis of

CEP192 expression in human breast cancer adjacent tissues, non-lymph node metastatic breast cancer tissues and lymph node metastatic breast cancer tissues (mean $\pm$ SD, * $p < 0.05$, ** $P < 0.01$, *** $p < 0.001$, one-way ANOVA with post analysis compared with “breast cancer adjacent tissues” or “non-lymph node metastatic breast cancer tissues”). d. Immunohistochemical grayscale analysis of CCL18 expression in human breast cancer adjacent tissues, non-lymph node metastatic breast cancer tissues and lymph node metastatic breast cancer tissues (mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with post analysis compared with “breast cancer adjacent tissues” or “non-lymph node metastatic breast cancer tissues”).