Original Article Natriuretic peptide receptor a promotes breast cancer development by upregulating MMP9

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Received May 24, 2019; Accepted June 14, 2019; Epub July 1, 2019; Published July 15, 2019

Abstract: Natriuretic peptide receptor A (NPRA), one of the natriuretic peptide receptors, plays important roles in circulatory system. Recently some studies showed that NPRA was involved in tumorigenesis, however, its role in the development of breast cancer remains unclear. In this study, we observed that NPRA expression was upregulated in breast cancer tissues and NPRA high expression was associated with poor clinicopathological features. In addition, we found that patients with high NPRA expression had a worse 5-year survival and NPRA was an independent factor for predicting the prognosis of breast cancer cells. Nocking down NPRA expression reduced the proliferation, migration and invasion of breast cancer cells. Overexpressing NPRA was able to enhance the malignant behaviors of breast cancer cells. Furthermore, NPRA promoted the invasive phenotype through upregulating matrix metalloproteinase-9 (MMP9). Mechanistically, NPRA increased MMP9 expression through activating STAT3. We identified that NPRA might serve as a prognostic marker and p-STAT3 and MMP9 could be a potential target of NPRA in breast cancer patients.

Keywords: NPRA, breast cancer, MMP9, STAT3, prognosis

Introduction

Breast cancer, which mainly derives from mammary ducts or lobules of women, is the top killer with highest incidence and second mortality in all kinds of female cancer [1]. With lifestyle changing, the incidence rate of breast cancer increased year by year, especially in Chinese women [2]. Despite the average survival rate of breast cancer patients increased significantly by advanced diagnostic and therapeutic techniques in recent years, once the metastasis occurred, the prognosis of breast cancer patients was still very poor [3]. The validation of biomarkers, which could help doctors predict regional tumor or distant metastasis, is still a challenge. Thus, it is critical to investigate more molecular mechanisms of breast cancer metastasis and find some potential molecular biomarkers that could predict prognosis of patients.

Natriuretic peptide receptor A (NPRA) is one of the natriuretic peptide receptors, it is a kind of

membrane-bound guanylate cyclase which serves as the receptor for both atrial and brain natriuretic peptides (ANP and BNP, respectively) [4]. Its canonical function is very important for cardiovascular system, including regulating blood pressure, salt and water balance and body fluid homeostasis [5]. Recently, several studies also showed that NPRA involved in modulating cell proliferation [6] and tumor development [7-9] in different organs. These studies suggested that overexpression of NPRA was related to the malignant phenotypes, such as promoting proliferation and metastasis in some kinds of cancer cells [7-10]. However, the expression level of NPRA and whether NPRA modulates malignant behaviors in breast cancer remain unknown.

Invasion and metastasis are major characteristics of cancer with poor prognosis. During this process, tumor cells can break through several collagen-enriched tissue barriers, which form the basement membrane with vascular endothelial cells to prevent metastasis. And this pro-

cess is largely regulated by matrix metalloproteinases (MMPs), which are a family of Ca²⁺ and Zn²⁺ ions dependent endopeptidases, well known for their ability to degrade extracellular matrix (ECM) components [11]. Breast cancer cells with high level of MMP9 could have enhanced migration and invasion ability [12, 13]. Some studies reported that MMP9 was highly related to STAT3 activation, which was involved in tumorigenesis, cellular proliferation, apoptosis, invasion, angiogenesis and evasion of host immune response [14-17]. Furthermore, inhibition of STAT3 suppressed metastasis and MMP9 expression of breast cancer cells [18]. It's reported that NPRA could bind with STAT3 [19], suggesting that there might be a link between these two molecules. However, this link remains unknown in breast cancer.

In current study, we investigated the role of NPRA in breast cancer metastasis and its downstream signaling in regulating p-STAT3 and MMP9 expression. Besides, we explored the potential of NPRA to be a prognostic biomarker in breast cancer. These findings provided insights into the possible mechanisms by which NPRA modulates p-STAT3 and MMP9, and showed some potential applications of NPRA in breast cancer diagnosis and treatment.

Materials and methods

Patients and specimens

Cancer tissues and adjacent normal (>2 cm distance to the resection margin) breast tissues were obtained from 98 breast cancer patients who underwent modified radical mastectomy from 2009 to 2012 in the First Affliated Hospital of Xi'an Jiaotong university and No. 3201 Hospital of Xi'an Jiaotong University. These patients did not have any other malignancies and were not treated by chemotherapy or radiotherapy before surgery. Fresh tissues were stored at -80°C for protein and RNA analysis. Clinical data were collected from the medical records. All patients had signed informed consent before the study began. The Investigation and Ethics Committee in our hospital approved all protocols.

Immunohistochemical (IHC) staining

We use our previous protocol [20]. The intensity was assessed by four grades: 0 for none; 2 for

weak; 4 for moderate; 6 for strong. The percentage of positive cells was divided into four degrees: 0 for <5%; 2 for 5-25%; 4 for 26-50%; 6 for >50%. The total scores \geq 4 were defined as positive. The overall intensity and percentage were evaluated at 10 independent fields (×400).

Cell culture

The human breast cancer cell lines MCF-7, SKBR-3, MDA-MB-231, MDA-MB-435, MDA-MB-436 and non-transformed breast cell line MCF-10A were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China. All cancer cells were maintained in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1% penicillin-streptomycin under humidified conditions with 5% CO₂ at 37°C. MCF-10A was supplemented with 5% donor horse serum, 20 ng/ml EGF, 10 mg/ml insulin, 1 ng/ml cholera toxin, 100 mg/ml hydrocortisone, 50 U/ml penicillin and 50 mg/ml streptomycin.

Quantitative real-time PCR (qRT-PCR)

We use our previous protocol [20]. Primers: NPRA forward, GGG ATA CAG TCA ACA CAG CCT CAA; reverse, CGA AGC TCC AGC TCG AAA CC; MMP9 forward, GAT GCG TGG AGA GTC GAA AT, reverse, CAC CAA ACT GGA TGA CGA TG; β -actin forward, TGG CAC CCA GCA CAA TGA A, reverse, CTA AGT CAT AGT CCG CCT AGA AGC A.

Western blot

Cells and Tissues were collected with RIPA lysis buffer, and were detected as previous report [20]. Antibody: NPRA (Abcam ab70848), MMP9 (CST 13667), STAT3 (CST 9139), Phospho-Stat3 (Tyr705) (CST 9145).

Cell transfection and clone selection

To establish stably knock down NPRA in MDA-MB-231 and MDA-MB-436 cells, we used 2 small hairpin RNA (shRNA) constructs, targeting the NPRA 3' untranslated region, with puromycin (2 µg/ml) as selection marker. Nonsilencing shRNA was used as control for both above mentioned shRNA knockdown experiments. shNPRA#1: Target Sequence: CGC CTG ACG TTG CGC AAA TTT Hairpin Sequence: 5' CCG GCG CCT GAC GTT GCG CAA ATT TCT CGA GAA ATT TGC GCA ACG TCA GGC GTT TTT TG 3'; shNPRA#2: Target Sequence: GCC TCA AGA ATG GAG TCT AAT Hairpin Sequence: 5' CCGG GCC TCA AGA ATG GAG TCT AAT CTC GAG ATT AGA CTC CAT TCT TGA GGC TTT TT 3'. To overexpress NPRA, lentivirus vector pCDH containing the NPRA open reading frame under the control of CMV promotor with puromycin (2 µg/ml) as selection marker was used. Lentiviral vectors (with ORFs or shRNA) were transfected into the packaging cell line 293T, together with a packaging DNA plasmid (psPAX2) and an envelope DNA plasmid (pMD2G), through Lipofectamine transfection. After 48 h, viruses were collected, filtered, and incubated with target cells in the presence of 10 µg/mL Polybrene for 24 h. The infected cells were selected with suitable selection markers, as mentioned above, to generate the stable clone.

RNA interference

The human STAT3 specific siRNA: forward, GGA UAA CGU CAU UAG CAG AdT dT and reverse, UCU GCU AAU GAC GUU AUC CdT dT. siRNA was synthesized from Sigma (USA) and was transfected according to the protocol of Lipofectamine[®] RNAiMAX transfection reagents (Invitogen[™]).

Wound healing assay

The cancer cells were seeded into 6-well plates and cultured to >90% confluency in monolayer. Scratch wounds were made by a 200- μ l pipette tip. Wounds Imaging was taken with a phase contrast microscope at 0, 18 or 24 h. The distance of the gap was measured through Image Pro-Plus 6.0 software. 6 visual fields and 2 repeated wells were measured with three replications.

MTT assay

Cancer cells were plated at 10000-20000 cells/well in triplicate in 24-well cell culture plates. Two hours before each time point (0, 2, 4, and 6 day), 100 μ l of 5 mg/ml MTT in PBS were added into 1000 μ l of culture medium and incubated at 37°C in the dark. The medium with MTT was removed, and 500 μ l of DMSO was added into each well after incubation. After the wells were mixed, the absorbance was determined at 570 nm and 650 nm with a microtiter plate reader.

Migration assay and invasion assay

Transwell chambers were used for both migration and invasion assays. We use our previous protocol [20].

In vivo experiments

Ten BALB/c female nude mice (Shanghai Institute of Materia Medical, Chinese Academy of Science), 8-week-old, were kept in laminarflow cabinets under specific pathogen-free conditions, and handled according to the National Institutes of Health guidelines for care and use of laboratory animals. For the tumor Xenograft model, 1×10^7 shCtrl or shNPRA transfected MDA-MB-436 cells in 100 µl of 50% Matrigel were conducted subcutaneous injection around the fourth mammary fat pad for each mouse, tumor growth was monitored each 5 days. There were five mice per group. The mice were sacrificed on 25 days after injection. Tumor tissues were prepared for IHC staining.

Result

NPRA expression is significantly increased in breast cancer patients and cell lines

NPRA protein expression was detected in 98 samples of human breast cancer tissues and corresponding adjacent noncancerous breast tissues by Western Blot, low-level NPRA expression was found in some noncancerous breast tissues (Figure 1A). Moreover, the average NPRA/β-actin level in breast cancer tissues was significantly higher than that in non-cancer tissues (P<0.05) (Figure 1B). Consistent with its protein expression, NPRA mRNA expression was also dramatically higher in breast cancer tissues than that in non-cancer tissues (P<0.05) (Figure 1C). Immunohistochemical staining showed that NPRA was detectable in both cancer and adjacent non-cancer tissues and mainly expressed in cytomembrane and cytoplasm (Figure 1D). IHC scores showed About 65% cancer tissues showed positive NPRA staining (64/98), however, only 34% noncancer tissues (33/98) showed positive NPRA staining (Chi-squared Test, χ^2 =19.61, P<0.01). IHC scores using Mann-Whitney test showed that NPRA protein expression was dramatically enhanced in cancer tissues compared with the corresponding adjacent noncancerous tissues (P<0.05) (Figure 1F). Meanwhile, we also



Figure 1. NPRA expression was significantly increased in breast cancer, which is associated with the poor prognosis. (A) NPRA protein expression of breast cancer patients' cancer tissues and corresponding adjacent noncancerous tissues was analyzed by Western Blot. (B) The average NPRA/β-actin level quantification of (A). (C) NPRA mRNA expression of breast cancer tissues and corresponding adjacent noncancerous tissues was analyzed by qRT-PCR. (D) Patients immunohistochemistry using anti-NPRA antibodies. Representative IHC staining of NPRA in adjacent normal tissues (a) and in cancer tissues (c and d); (a) low NPRA expression ×200 magnification (b) negative NPRA expression control in cancer tissues; (c) high NPRA expression ×200 magnification; (d) high NPRA expression ×400 magnification. (E) overall survival curves of patients with breast cancer. (F) Quantification of IHC staining in (D). (G) Distance metastasis free survival curves of patients with breast cancer. (H) NPRA protein expression in five breast cancer cell lines and one non-transform breast cell line was analyzed by Western Blot. (*P<0.05; **P<0.01).

		NF	PRA	_	
Clinical Parameter	Ν	Positive	Negative	X ²	P value ^a
		(n=64)	(n=34)		
Age (years)				1.923	0.166
<60	60	36	24		
≥60	38	28	10		
lymph node mets				10.148	0.001ª
Yes	56	44	12		
No	42	20	22		
Grade				2.806	0.246
G1	31	17	14		
G2	45	33	12		
G3	22	14	8		
TNM				9.224	0.002ª
+	35	16	19		
+ V	63	48	15		
Position				0.735	0.865
Up outside	65	44	21		
Down outside	15	9	6		
Up inside	9	5	4		
Down inside	9	6	3		
Tumor size (cm)				4.471	0.034ª
≤2 cm	30	15	15		
>2 cm	68	49	19		

 Table 1. Correlations between NPRA expression and clinicopathological characteristics

°P<0.05.

detected NPRA protein expression level in breast cancer cell lines (MDA-MB-436, MDA-MB-231, MDA-MB-435, SKBR3 and MCF-7) and non-transformed cell line (MCF-10A), finding that NPRA protein expression was higher than that in non-transformed cell line (**Figure 1**H). These data suggested that NPRA expression was increased in breast cancer.

NPRA expression is a prognosis predictor of breast cancer patients

To investigate the potential role of NPRA in breast cancer, we assessed the correlation between NPRA expression levels and histopathological parameters in breast cancer patients. Clinical association analysis by Pearson χ^2 test revealed that the increased NPRA expression in breast cancer tissues was associated with lymph node metastasis (P=0.001), advanced tumor stage (TNM stage III + IV; P=0.002) and large tumor size (>2 cm; P=0.034) (**Table 1**). And NPRA expression level was not related to age, histological differentiation grade and tumor position. These results indicated that high NPRA expression might relate to breast cancer aggressive phenotypes.

As NPRA expression level was significantly increased in breast cancer, we further evaluated whether NPRA expression in tumor tissues could predict the prognosis of breast cancer patients. The 5 years prognostic information of 85 breast cancer cases (86.7%) was available for analysis. The median IHC score of NPRA in breast cancer tissues was used as cut-off value, the Kaplan-Meier survival curve showed that patients with high NPRA expression had worse overall survival (OS) (Figure 1E) and distance metastasis free survival (DMFS) (Figure 1G). Multivariate analysis that enrolled all of the significant clinical parameters for OS and DMFS indicated that NPRA expression (Table 2) was an independent prognostic factor for breast cancer patients. These data suggested that NPRA might be an

effective marker to predict prognosis of breast cancer patients.

NPRA is required for breast cancer cell proliferation, migration and invasion

Basing on the result of different NPRA expression in breast cancer cell lines (Figure 1H), we found that NPRA expressed higher in metastatic breast cancer cell lines (MDA-MB-436, MDA-MB-231, MDA-MB-435) than that in nonmetastasis cancer cell lines (MCF-7, SKBR3). Thus, to investigate whether NPRA was required for the invasive phenotypes of breast cancer cells in vitro, we used two shRNA, shNPRA#1 and shNPRA#2, to knock down NPRA expression in MDA-MB-231 and MDA-MB-436 cell lines. Western blot and qRT-PCR results showed that compared with control shRNA transfected cells, NPRA protein expression and mRNA level were dramatically decreased in both NPRAshRNA#1 and NPRA-shRNA#2 transfected cells, respectively (Figure 2A and 2B). Then we

Variables		Overall survival		Distant Metastasis-Free Survival			
	HR	95% CI	P-value	HR	95% CI	P-value	
NPRA	2.384	1.223-5.132	0.012ª	1.925	1.051-4.525	0.026ª	
TNM	3.513	1.356-8.012	0.009ª	6.514	2.124-22.432	0.010ª	
lymph node mets	3.154	1.034-4.932	0.006ª	7.542	2.588-25.314	0.002ª	

Table 2. Multivariate Cox regression analysis of 5-year overall survival and Distant Metastasis-FreeSurvival of breast cancer patients

HR, hazard ratio; CI, confidence interval. ^aP<0.05.

tested whether NPRA was required for tumor cell growth. Using MTT assay, we compared the proliferation curves between control shRNA cells and NPRA knocking down cells in MDA-MB-231 and MDA-MB-436 cell lines. We observed that in both MDA-MB-231 and MDA-MB-436 cell lines, knocking down NPRA could decrease cell proliferation dramatically compared with the control shRNA cells (Figure 2C). Furthermore, we explored whether NPRA regulated migration and invasion of breast cancer cells. We firstly tested the role of NPRA in tumor cell migration by wound scratch assay. We scratched a line in tumor cells and then observed the percentage of healed wounds 18 to 24 hours later. We found that knocking down NPRA expression significantly inhibited wound closure in MDA-MB-231 (Figure 2D) and MDA-MB-436 (Figure 2E) compared with the control shRNA cells. To further validate this phenotype, we evaluated the effects of NPRA on breast cancer cells migration by transwell migration assay. We plated tumor cells in the upper chamber without Matrigel and observed that tumor cells transfected with shNPRA showed attenuated ability of migration compared with those transfected with shCtrl cells (Figure 2F). Then, we assessed the role of NPRA in invasive capacity of tumor cells. We conducted these experiments using transwell chamber with Matrigel. It showed clearly that compared with the control shRNA cells, downregulating NPRA significantly decreased the number of tumor cells which invaded through Matrigel (Figure 2G). These results indicated that NPRA was required for breast cancer cell proliferation, migration and invasion.

Overexpressing NPRA enhanced breast cancer cell proliferation, migration and invasion

To further validate NPRA functions, we repeated experiments mentioned above by transfecting NPRA plasmid to overexpress NPRA in MCF-7 and SKBR3 cells with low endogenous NPRA. Western Blot and qRT-PCR results confirmed that NPRA expression was significantly upregulated after NPRA overexpression plasmid transfection compared with the cells transfected vector plasmid in both MCF-7 and SKBR3 cells (Figure 3A and 3B). Then, we investigated whether NPRA upregulation affected tumor cell proliferation. Using MTT assay, we assessed proliferation curve between tumor cells transfected vector plasmid and NPRA overexpression plasmid. Results showed in both MCF-7 and SKBR3 cells with NPRA overexpression grew faster than control cells (Figure **3C**). Then, we tested whether overexpressing NPRA could enhance tumor cell migration ability. Using wound scratch assay, we observed that upregulation of NPRA dramatically promoted the wound closure in both MCF-7 and SKBR3 cells compared with control cells (Figure 3D and 3E). Meanwhile, using transwell migration assay, we further confirmed that tumor cells with NPRA overexpression displayed improved ability of migration compared with control cells (Figure 3F). Consistently, the number of MCF-7 and SKBR3 cells, which invaded through Matrigel from upper chamber was increased remarkably after NPRA upregulation (Figure **3G**). In brief, these results suggested that NPRA overexpression enhanced breast cancer cell proliferation, migration and invasion in vitro.

NPRA regulates MMP9 expression

Next, we investigated the potential mechanism that how NPRA regulated breast cancer cell migration and invasion. It's known that breast cancer expressed high level of MMP9, which played an important role in tumor cell migration and invasion [12, 13]. We evaluated whether alteration of NPRA expression in breast cancer cells affected MMP9 protein expression. Results of Western Blot showed that knocking down NPRA in both MDA-MB-231 and MDA-



NPRA promotes breast cancer development

Figure 2. Silencing of NPRA reduced cell proliferation and inhibited their ability of migration and invasion in breast cancer cell lines. (A and B) The MDA-MB-231 and MDA-MB-436 breast cancer cell lines were transfected with two different NPRA short hairpin RNAs (sh#1 and sh#2) and one control empty vector (shCtrl). NPRA expression were analyzed by Western Blot (A) and qRT-PCR (B). (C) MTT assay showed that downregulation of NPRA significantly influenced the growth of MDA-MB-231 and MDA-MB-436 cells. (D and E) Wound healing assay indicated that NPRA downregulation inhibited MDA-MB-231 (D) and MDA-MB-436 (E) cell migration. (F and G) Decreased NPRA expression attenuated the migration and invasion in MDA-MB-231 and MDA-MB-436 cells. Cells were seeded into the Transwell up chamber coated without (F) or with Matrigel (G) and incubated in low chamber with medium containing 10% FBS. Representative images and the number of migrated or invaded cells are shown. (*P<0.05; **P<0.01; ***P<0.001).

MB-436 cells dramatically decreased the expression of MMP9 compared with the control shRNA cells (Figure 4A). At the same time, upregulating NPRA expression in both MCF-7 and SKBR3 cells could also remarkably enhanced MMP9 expression compared with the vector control cells (Figure 4B). Furthermore, to investigate whether NPRA modulated MMP9 through the transcriptional level, we assessed the MMP9 mRNA level by gRT-PCR and found that knocking down NPRA in both MDA-MB-231 and MDA-MB-436 cells significantly reduced the mRNA of MMP9 compared with the control shRNA cells (Figure 4C). Consistently, MCF-7 and SKBR3 cells with NPRA overexpression have higher MMP9 mRNA level than vector control cells (Figure 4D). These results showed that NPRA might positively regulate the MMP9 expression through the transcriptional level.

NPRA upregulates MMP9 via STAT3 activation to promote breast cancer cell migration and invasion

To investigate how NPRA modulated MMP9 through the transcriptional level, we used ST-RING (https://string-db.org/) to analyze potential mechanism involved in this regulation and found that STAT3 could be the downstream signal of NPRA and STAT3 was the only transcription factor in these potential molecules (Figure 4E). Some studies also reported that activating STAT3 could enhance MMP9 expression [15, 21, 22]. To validate whether NPRA regulated MMP9 expression through active STAT3. We first tested p-STAT3 expression after knockdown NPRA using Western Blot, results showed that in both MDA-MB-231 and MDA-MB-436 cells, NPRA knockdown could decrease both MMP9 and p-STAT3 expression compared with the control shRNA cells (Figure 4A and 4F). Then we assessed p-STAT3 expression in NPRA overexpression cells by Western Blot and found that upregulating NPRA in both MCF-7 and

SKBR3 cells could enhance p-STAT3 expression compared with the vector control cells (Figure 4G). Furthermore, siRNA was used to downregulate STAT3 in NPRA overexpression MCF-7 and SKBR3 cells and we found both protein and mRNA expression of MMP9 decreased obviously compared with the siRNA Control cells (Figure 4G and 4H). Besides, using siRNA to downregulate STAT3 in NPRA overexpression MCF-7 and SKBR3 cells significantly inhibited wound closure and invasion of breast cancer cells compared with NPRA overexpression control siRNA cells (Figure 5A and 5B). Collectively, these results demonstrated that NPRA might enhance the migration and invasion ability of breast cancer cells by activating STAT3 to increase MMP9 expression and promote breast cancer development.

Positive correlation of NPRA and MMP9 expression in MDA-MB-436 nude mice xenografts model

To further validate our observations in vivo, MDA-MB-436 nude mice xenografts model was established. We tested whether NPRA depletion from MDA-MB-436 cells could inhibit tumor growth. We injected control shRNA and NPRA shRNA cells into the fourth mammary fat pads of nude mice and monitored tumor growth. MDA-MB-436 control shRNA cells formed larger tumors within 25 days than MDA-MB-436 NPRA shRNA cells (Figure 5C). Tumor growth curves, generated for 25 days, showed that NPRA downregulation slowed down MDA-MB-436 tumor growth compared with control shRNA tumors (Figure 5D). Consistently, the weight of tumor xenografts derived from MDA-MB-436 NPRA knocking down cells was significantly lighter than those from MDA-MB-436 shRNA control cells at the 25th day since tumor cells injection (Figure 5E). Furthermore, we analyzed xenograft tissues by conducting IHC staining and found that both NPRA and MMP9



Figure 3. Overexpressing NPRA increased cell proliferation and enhanced their ability of migration and invasion in breast cancer cell lines. (A and B) MCF-7 and SKBR3 breast cancer cell lines were transfected with a control empty vector and NPRA overexpression plasmid. NPRA expression was analyzed by Western Blot (A) and qRT-PCR (B). (C) MTT assay showed that upregulation of NPRA significantly accelerated the growth of MCF-7 and SKBR3 cells. (D and E) Wound healing assay indicated that NPRA upregulation enhances MCF-7 (D) and SKBR3 (E) cell migration. (F and G) Increased NPRA expression enhanced the migration and invasion in MCF-7 and SKBR3 cells. Cells were seeded into the Transwell upper chamber coated without (F) or with Matrigel (G) and incubated in low chamber with medium containing 10% FBS. Representative images and the number of migrated or invaded cells are shown. (*P<0.05; **P<0.01; ***P<0.001).

NPRA promotes breast cancer development



Figure 4. NPRA upregulated MMP9 via STAT3 activation. A. Expression of MMP9 protein was inhibited by NPRA shRNA in MDA-MB-231 and MDA-MB-436 cells. B. Overexpression of NPRA significantly increased MMP9 expression in MCF-7 and SKBR3 cells, as confirmed by Western Blot analysis. C. MMP9 mRNA level was reduced dramati-

NPRA promotes breast cancer development

cally after knocking down NPRA in MDA-MB-231 and MDA-MB-436 cells. D. NPRA overexpression enhanced MMP9 mRNA expression in MCF-7 and SKBR3 cells, as confirmed by qQT-PCR analysis. E. STRING analysis showed potential molecules involved in NPRA-MMP9 regulation. F. STAT3 phosphorylation determined by Western Blot in MDA-MB-231 and MDA-MB-436 cells transfected with NPRA shRNA. G. STAT3 phosphorylation and MMP9 expression were confirmed by Western Blot in NPRA overexpression MCF-7 and SKBR3 cancer cells transfected with siSTAT3 and Control siRNA. H. MMP9 mRNA level was detected by qRT-PCR in NPRA overexpression MCF-7 and SKBR3 cancer cells transfected with siSTAT3 and Control siRNA.



Figure 5. NPRA promoted cancer cell migration and invasion through enhancing STAT3 activation and there was positive correlation between protein expression of NPRA and MMP9 in MDA-MB-436 xenografts of nude mice model. (A and B) Silence of STAT3 inhibited the increased ability of migration (A) and invasion (B) in NPRA overexpression

MCF-7 and SKBR3 cancer cells. In MDA-MB-436 xenografts of nude mice model, MDA-MB-436 cells of control shRNA and shNPRA were respectively conducted subcutaneous injection around the fourth mammary fat pad of nude mice. Tumor nodules were measured using a caliper at different times after injection. NPRA knocking down MDA-MB-436 cells showed a greater tumor-inhibiting effect compared with control cells reflected by tumor size (C and D) and weight (E). (F and G) Tumor nodules were subjected to immunohistochemical staining for NPRA and MMP9. Representative immunostaining indicated that NPRA knocking down dramatically reduced the number of MMP9 positive cells. Scale bar =70 μ m. (*P<0.05, **P<0.01).

expression was dramatically lower in all five xenografts from MDA-MB-436 NPRA knocking down group than control shRNA group (**Figure 5F** and **5G**). The data of xenograft experiments validated that NPRA promoted breast cancer development through MMP9.

Discussion

Breast cancer is one of the most common female cancer types, though its 5 year prognosis seems better than other cancer types, the mortality is still at the top [1]. Breast cancer starts as a local disease, however, finally results in systemic disease with progress [3, 23]. Nowadays we have several biomarkers to predict prognosis of breast cancer, however, there are still some limitations when using these traditional biomarkers [24]. Thus, we still need to search more powerful biomarkers for prognosis prediction of breast cancer patients. Herein, we firstly reported the upregulation of NPRA in breast cancer patients and the correlation between NPRA expression and the prognosis of breast cancer patients. We initially assessed the expression of NPRA in 98 breast cancer patients through Western Blot, gRT-PCR and immunohistochemistry and found that NPRA expression was significantly higher in breast cancer tissues compared with matched adjacent normal tissues. Additionally, high NPRA expression was associated with lymph node metastasis, advanced TNM stage and large tumor size. Besides, our data showed that NPRA high expression would predict worse 5-year survival of breast cancer patients. And multivariate Cox repression analysis indicated that NPRA was an independent factor in predicting both OS and DMFS of breast cancer patients. All in all, our data showed the important status of NPRA in breast cancer.

To investigate the function of NPRA in breast cancer cells, we firstly assessed NPRA expression levels in 5 breast cancer cell lines and compared them with that of a non-transformed breast cell line. Consistent with NPRA IHC staining of breast cancer patient tissues, NPRA expression was higher in breast cancer cell lines, especially in more aggressive breast cancer cell lines. And NPRA expression was associated with proliferation, migration and invasion ability of breast cancer cell lines.

Mechanistically, it is widely known that hydrolyzed of the extracellular matrix surrounding tumors is the most common feature of cancer cell invading into adjacent tissues and early metastasis [11]. Members of the MMP family secreted by invasive cancer cells could hydrolyze all essential components of the extracellular matrix. Therefore, these MMPs expression levels effectively reflect the aggressiveness of cancer cells and are related to poor prognosis in various cancers [25, 26]. MMP9 is a critical member of MMP family which plays a key role in degrading basement membrane and has been proved to enhance tumor invasion and metastasis in many different types of cancer [27, 28]. Some studies also reported that the invasive and metastatic abilities of breast cancer cells were reduced by inhibition of MMP9 [13, 29]. Actually, in this study, we observed that reduction of MMP9 expression was associated with the inhibition of breast cancer cells migration and invasion in vitro after knocking down NPRA. Additionally, we established a breast cancer xenograft mouse model and found that MMP9 was decreased in xenografts with low NPRA expression. These results indicated that MMP9 might be upregulated by NPRA in breast cancer.

However, it is unknown how NPRA modulates MMP9 expression. We found that NPRA could regulate the protein and mRNA of MMP9, which hinted that NPRA most likely regulated MMP9 through the transcription level. STRING analysis showed that STAT3 is the only transcription factor candidate. Moreover, studies have reported that activated STAT3 can increase MMP9 expression and promote cancer cells invasion and metastasis [18, 30]. Thus, we investigated whether NPRA could regulate MMP9 through activating STAT3. Here we found that NPRA could enhance activated STAT3 and knocking down STAT3 in NPRA overexpression cancer cells also decreased the expression of MMP9, which is increased by NPRA overexpression. In brief, our study firstly pointed out that NPRA could promote breast cancer development by NPRA-STAT3-MMP9 signal pathways.

The detailed mechanisms of NPRA in promoting breast cancer development need to be further investigated. Though we found STAT3 activation by NPRA could contribute to increasing MMP9 expression in the breast cancer progression, how NPRA regulated p-STAT3 still needed to be further studied. Our findings may provide valuable information for the prognosis prediction of breast cancer patients and development of future therapies to more effectively prevent breast cancer migration and invasion.

Acknowledgements

The authors would like to acknowledge the National Natural Science Foundation of China, which supported this study. This study was funded by National Natural Science Foundation of China (No. 81702430).

Disclosure of conflict of interest

None.

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