Downregulation of miR-205 contributes to epithelial–mesenchymal transition and invasion in triple-negative breast cancer by targeting HMGB1–RAGE signaling pathway

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Our aim was to study the regulatory molecule networks involved in the epithelial-to-mesenchymal transition and thus promoting the early onset of metastasis in triple-negative breast cancer (TNBC). Forty pairs of human TNBC and their adjacent normal breast tissues were analyzed by real-time PCR and immunochemistry to demonstrate the correlation between the miR-205 expression and clinicopathological characteristics. *In vitro*, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide assay, cell migration, and invasion assay were used to detect the cell growth and invasive ability of TNBC cells after upregulation or downregulation of miR-205 expression. Luciferase reporter assay was used to confirm the potential target directly influenced by miR-205. Our results showed that miR-205 abnormal expression may be involved and associated with the biological traits of TNBC. Ectopic expression of miR-205 not only inhibited cell growth, but also suppressed migration and invasion of mesenchymal-like TNBC cells. In addition, we found that overexpression of miR-205 significantly suppressed HMGB1 by binding its 3′-untranslated region, and that miR-205 was inversely correlated with the expression of HMGB1 and RAGE in cell lines and clinical samples. Our study illustrated that miR-205 was a tumor suppressor in TNBC, which attenuated the viability and the acquisition of the epithelial-to-mesenchymal transition phenotype TNBC cells at least partially exerted through targeting of HMGB1–RAGE signaling pathway. *Anti-Cancer Drugs* 30:225–232

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Introduction

Breast cancer is the most worldwide common malignancies among women. Approximately 15–20% of the cases are of the triple-negative breast cancer (TNBC) subgroup [1–3]. In Asia, TNBC patients are comparatively burdened with larger invasive tumors, high histologic grade, and early high node metastasis [4,5], which suggested more aggressive biological behavior and poor prognosis of TNBC patients present in Asian countries. Although doxorubicin-based adjuvant chemotherapy has been widely used as the first-line chemotherapeutic agent for TNBC, the response and the development of resistance are still the main obstacles against optimal therapy in clinical application [6,7]. Reports showed that high metastatic TNBC tumors possess the traits of harboring epithelial–mesenchymal plasticity, which could initiate tumor cells migration, invasion, and even chemoresistance [8–10]. Therefore, clarifying the molecular mechanisms that regulate biological characteristics in TNBC is vital to develop an efficient strategy for the treatment of TNBC.

MicroRNAs (miRNAs) are a class of short noncoding RNAs comprising 19–25 nucleotides, which are important cellular regulators that modulate gene expression at the transcriptional and at translational level by repressing target genes [11,12]. Abnormally expressed miRNAs have been discovered and validated for clinical application potential in cancer therapy [13,14]. So far, a number of miRNAs have been showed to possess the ability of regulating the epithelial-to-mesenchymal transition (EMT) and EMT-mediated drug resistance [15,16]. For instance, miRNA-200 family members including miR-200a/b/c, miR-141, and miR-429 are required to maintain an epithelial cell morphology and involved in cell migration and invasion by targeting of the EMT-inducing transcription factors Zeb1 and Zeb2 [17–21]. Under hypoxia, miR-205 was remarkably induced and could directly repress ASPP2 expression in cervical and lung cancer cells, and by that

promoted tumor cell proliferation and EMT process [22]. These studies fully demonstrate that certain miRNAs expression are crucial and may contribute to cancer EMT progression.

A different alteration of miR-205 expression has been reported in some kinds of malignancies, including breast cancer [23–26]. And miR-205 was an important tumor suppressor and its ectopic expression could resensitize drug-resistant breast cancer cells to doxorubicin and taxol [27]. However, the function of miR-205 in TNBC progression, in particular EMT and subsequently invasion, metastasis and EMT, remains to be investigated. Herein, we aimed to evaluate the underlying roles of miR-205 in TNBC and its regulation signaling network mechanisms behind. In this study, we investigated that the expression level of miR-205 was significantly lower in TNBC tissues than in the adjacent normal breast tissues, and inversely associated with tumor TNM stage and distal metastasis of TNBC. Re-expression of miR-205 could suppress tumor invasion and EMT process by targeting HMGB1/RAGE. All above observations indicate that miR-205 might be of therapeutic value in the prevention and treatment via targeting HMGB1/RAGE in TNBC.

Methods

Cell lines and tissue specimens

The human TNBC cells, MDA-MB-231, MDA-MB-453 and MDA-MB-468 and non-TNBC cells MCF-7 and MCF-10F were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They are cultured and passaged under strict instructions from American Type Culture Collection.

Forty paired TNBC specimens and corresponding adjacent nontumor tissues were collected from tumor surgical resection in Bethune International Peace Hospital and the Fourth Hospital of Hebei Medical University. In addition, all the human tissue samples were obtained with informed written consent and this study was approved by the Medical Ethics Committee of Bethune International Peace Hospital and the Fourth Hospital of Hebei Medical University, respectively.

RNA extraction and real-time PCR

Total cellular RNA was extracted by TRIzol method (Invitrogen, Carsbad, California, USA) following the manufacturer's instructions. After RNA extraction, an amount of 0.25 μg of total RNA was ready to be reversely transcribed to cDNA by using the miScript II RT Kit (Qiagen Tec, Shanghai, China). Then, we performed miScript SYBR Green qPCR Kit to complete the qPCR reaction on the Applied Biosystems 7500 FAST real-time PCR System (Applied Biosystems, Foster California, USA). Specific primers and probes for mature miR-205 and small nuclear RNA RNU6B (endogenous reference) were purchased from Sangon Biotech (Shanghai, China). All reactions were detected in triplicate. The amount of miR-205 was obtained by normalizing to small nuclear RNA RNU6B as control. The primers for detecting HMGB1, RAGE and GAPDH were as follows: 5′-CATCTCAGGGCCAAACCGAT-3′ (forward) and 5′-TGACATTTTGCCTCTCGGCT-3′ (reverse) for human HMGB1; 5′-GCTTGGAAGG-TCCTGTCTCC-3′ (forward) and 5′-CCTCTGAC-ACACATGTCCCC-3′ (reverse) for human RAGE; 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse) for human GAPDH. The relative fold changes of candidate genes were analyzed by using $2^{-\Delta\Delta C_t}$ method.

Cell transfection

All the miRNA mimics, inhibitor and siRNAs were purchased and synthetized from Genepharma Company (Shanghai, China). We employed Lipofectamine 3000 transfection reagent (Invitrogen, Carsbad, California, USA) to transfect these plasmids and oligonucleotide in TNBC cells, according to the protocol provided by the manufacturer. After 24–48 h transfection, the cells were collected and used for further detecting assays.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide assay

After transfection, cell proliferation was determined by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) assay under the manufacturer's instructions. Cells were seeded into 96-well plates at a density of 5000 cells per well. After 24 h, cells were prepared and transfected with miR-205 mimics or inhibitors, respectively. After the cells were co-cultured additional 24 h, 20 μl of 5 mg/ml MTT solution was added into each well and incubated for another 4 h in a humidified incubator. Then, 200 μl of DMSO was added to each well to dissolve the formazan. The optical density was determined using a microplate reader at 490 nm.

Western blotting

Cell proteins were lysed and isolated using RIPA buffer (Sigma-Aldrich, St. Louis, Missouri, USA) with complete protease inhibitor, and separated by SDS-PAGE and then electro-transferred onto nitrocellulose membrane (Bio-Rad, Hercules, California, USA). The membranes were incubated with the primary antibodies: HMGB1, RAGE, E-cadherin, vimentin and GAPDH, all purchased from Abcam company (Cambridge, UK). Membranes were probed with appropriate secondary antibodies by following the manufacturer's protocol. Immunoreactive bands were finally visualized by using ECL Western Blotting Substrate (Pierce Biotechnology Inc., Rockford, Illinois, USA).

Luciferase reporter assay

We designed and cloned the sequence of the wild type (WT) or mutant seed region of HMGB1 into empty pRL-TK Renilla luciferase vector (Promega, Madison, Wisconsin, USA). Seeded into a 96-well plate, different TNBC cells were prepared to transfect with WT, mutant and the empty vectors along with 50 nmol/l miR-205

mimics, inhibitors, and their respective NC. After 6 h transfection, the culture medium was substituted as complete DMEM, and dual-luciferase reporter assay (Promega, Madison, Wisconsin, USA) was performed under the manufacturer's instructions.

Cell migration and invasion assay

Cell migration and invasion assays were determined using the Transwell chambers assay (Costar, Corning Inc., Corning, New York, USA), with or without coated Matrigel (BD Biosciences, San Jose, California, USA). At the upper chamber, cells were seeded into 24-well at a density of 1×10^5 per well without serum. At the lower chamber, 500 µl DMEM supplemented with 10% fetal bovine serum was filled in the Transwell device. After incubation for 24 h, noninvading cells were removed from the top well with a cotton swab, while the bottom cells were trypsinized and re-seeded into 96-well to detect cell viability by using MTT assay. Then, the optical density of each well was measured at a wavelength of 490 nm using a microplate reader (Bio-Rad Hercules, California, USA).

Immunohistochemistry staining and evaluation

For IHC detecting, 5-µm slides of FFPE tumor samples were prepared and retrieved by citrate and EDTA buffer solution. Ten percent BSA was used to attenuate nonspecific binding influence. Then, the slides were washed and incubated with primary antibody against HMGB1 and RAGE overnight. On the next day, anti-mouse/rabbit secondary antibodies were added for 1 h incubation at 37°C. Color development with DAB substrate was performed and counterstaining with hematoxylin was conducted.

Evaluation of HMGB1 and RAGE staining in tumor cells was evaluated by authorized pathologists who were blind to the patients' clinical status and outcome. HMGB1 and RAGE expression was semiquantitatively scoring based on the percentage of stained cells and the staining intensity. The intensity of HMGB1 and RAGE positive staining was categorized as 0–3 (negative = 0, weak = 1, moderate = 2, or strong = 3), and the percentage of positively stained cells was categorized as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The final staining score was the sum of the intensity and percentage, which included 0–7. For statistical analysis, the scores of 0, 1–3, 4–5, and 6–7 were designated as the sum indexes −, +, ++, and +++, respectively. Sum indexes − and + were defined as low HMGB1 and RAGE expression, while sum indexes ++ and +++ were defined as high HMGB1 and RAGE expression. Each tissue section was evaluated independently by three pathologists.

Statistical analyses

All experiments were performed three times and data were analyzed with SPSS 20.0 (IBM Company, New York, USA). We used a two-tailed *t*-test to compare the mean values between the two groups and a one-way analysis of variance to compare the mean values among three groups. Qualitative variables were compared using Fisher's exact test. The correlation between miR-205 expression and HMGB1, as well as RAGE levels in human TNBC tissues were analyzed using Spearman's rank test. The differences were considered to be statistically significant at P value less than 0.05.

Results

MiR-205 was decreased and correlated with advanced clinical stage and prognosis in triple-negative breast cancer

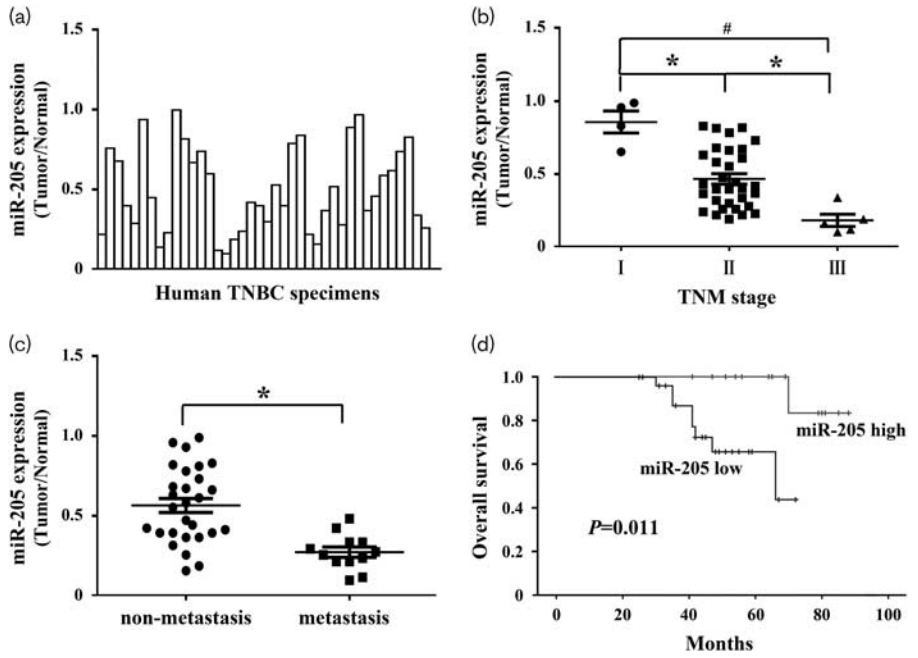
We first assayed the expression levels of miR-205 in 40 pairs of human TNBC and their adjacent normal breast tissues. As shown in Fig. 1a, we could demonstrate a significant downregulation of miR-205 displayed in the most human TNBC in comparison with adjacent normal breast tissues. Forty cases of TNBC tissues were divided into two groups: a high miR-205 expression group (above the median miR-205 expression, $n = 20$) and a low miR-205 expression group (below the median miR-205 expression, $n = 20$). Statistical analysis showed that the miR-205 expression level was reversely correlated to advanced TNM stage (Fig. 1b). Moreover, we found that the expression levels of miR-205 were lower in TNBC with lymph node metastasis compared with those without metastasis (Fig. 1c). To determine the potential relationship between miR-205 expression and the patients' prognosis, Kaplan–Meier analysis was used to evaluate the effects of miR-205 expression on overall survival. The results indicated that patients with higher miR-205 expression had a significantly better prognosis compared with patients with lower miR-205 expression ($P = 0.011$; Fig. 1d).

MiR-205 negatively regulated cell growth, invasion, and the epithelial-to-mesenchymal transition of triple-negative breast cancer cells

To determine the effect of miR-205 on TNBC cell malignancy, the expression levels of miR-205 were detected in three human TNBC cell lines (MDA-MB-231, MDA-MB-453, and MDA-MB-468) and two non-TNBC cells MCF-7 and MCF-10F, respectively. The miR-205 expression levels were extremely low in these three TNBC cell lines, comparatively the lowest in MDA-MB-231 and the highest in MDA-MB-468 (Fig. 2a). Then, we chose MDA-MB-231 and MDA-MB-468 cells for subsequent function experiments accordingly. MDA-MB-231 cells with lower endogenous miR-205 expression levels were applied in gain-of-function studies using miR-205 mimics, whereas MDA-MB-468 cells with higher miR-205 levels were applied in loss-of-function studies using anti-miR-205 inhibitors.

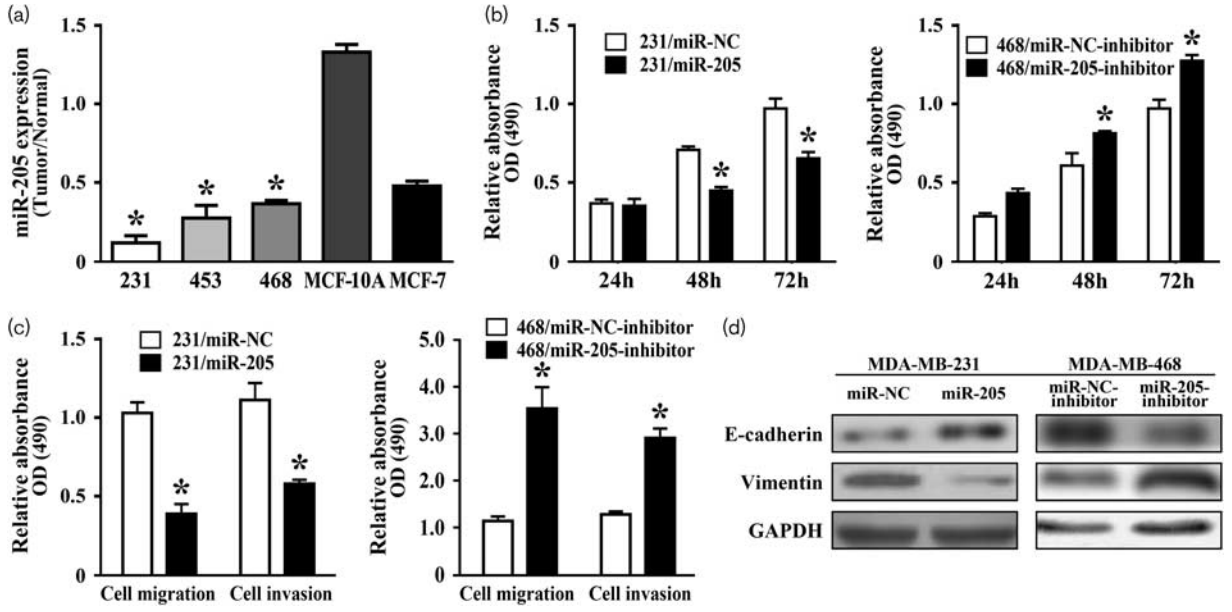
The cell proliferation was determined by MTT assays to predict the effects of miR-205 in TNBC cells. Results

Fig. 1



MiR-205 was frequently downregulated and associated with tumor metastasis and poor clinical outcomes in triple-negative breast cancer (TNBC). (a) The relative mRNA levels of miR-205 were detected by qRT-PCR and normalized against an endogenous control in 40 pairs TNBC specimens. (b) Relative expression levels of miR-205 were shown in different TNM stages of TNBC specimens. (c) Overall survival curves for 40 TNBC patients with high or low miR-205 expression by using the Kaplan–Meier method. (d) Relative expression levels of miR-205 were shown in 40 pairs of primary TNBC tissues and their corresponding lymph node metastases. $^{*,\#}$ Significant difference at $P < 0.05$.

Fig. 2



MiR-205 negatively regulated the cell growth, migration, invasion and the epithelial-to-mesenchymal transition (EMT) of triple-negative breast cancer (TNBC) cells. (a) The expression levels of miR-205 were detected by qRT-PCR in TNBC cell lines and non-TNBC cell lines. (b) Cell growth was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) method after miR-205 upregulated or downregulated. (c) The levels of cell migration and invasion in indicated TNBC cells were analyzed using the Transwell chambers assay after miR-205 upregulated or downregulated. (d) Western blot analysis of EMT markers E-cadherin and vimentin were shown in MDA-MB-231 and MDA-MB-468 cells, respectively. Data represent mean ± SD of three replicates. *Significant difference at $P < 0.05$.

showed that transfected miR-205 mimics in MDA-MB-231 cells attenuated cell proliferation, whereas transfected miR-205 inhibitor in MDA-MB-468 cells promoted cell growth in a significant manner (Fig. 2b). In addition, overexpression of miR-205 reduced cell migration and invasion ability of MDA-MB-231 by 55 and 38%, respectively, compared with control cells. In contrast, inhibition of miR-205 in MDA-MB-468 cells could increase cell migration and invasion by almost three-fold (Fig. 2c). In addition, when transfected with miR-205 mimics, EMT markers including E-cadherin and vimentin were significantly increased or decreased in MDA-MB-231 cells and MDA-MB-468 cells with opposite alteration (Fig. 2d). These observations suggested that miR-205 may be involved in the cell proliferation and EMT of TNBC cells.

MiR-205 negatively and directly regulates HMGB1 expression

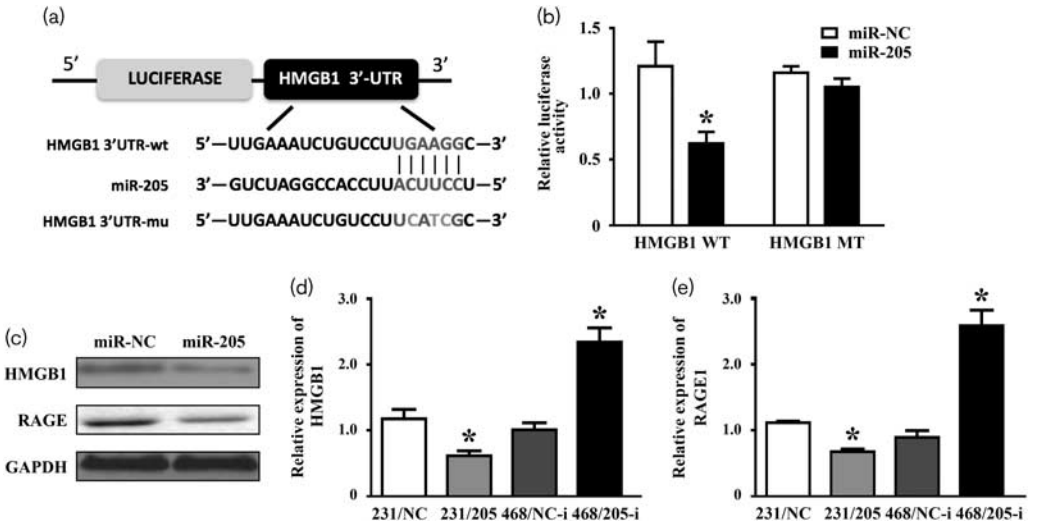
In order to fully elucidate the molecular mechanism by which miR-205 interferes with the EMT transition of TNBC cells, different bioinformatics tools were employed and performed to identify its potential target genes. Analyzing results indicated that HMGB1 might be a meaningful target under miR-205 regulation (Fig. 3a), and HMGB1 has been reported to be relevant to EMT and metastasis in tumors.

Next, we designed and synthesized 3′-untranslated region (UTR) sequences containing wide-type or mutant miR-205-binding sites of HMGB1, and then cloned them into pRL-TK dual-luciferase vector, respectively. For low-expression miR-205 MDA-MB-231 cells, miR-205 mimics or miR-NC were applied and transfected. MiR-205-transfected cells showed a remarkable inhibition of luciferase activities of HMGB1 reporter with WT binding sites, but not with mutant binding sites (Fig. 3b). qPCR and western blotting analysis showed that overexpression of miR-205 could downregulate HMGB1 and RAGE gene expression both at mRNA and protein level, and vice versa (Fig. 3c–e). These data illustrated that miR-205 could negatively and directly regulate HMGB1 expression via targeting its 3′-UTR regions.

Expression and the correlation of miR-205, HMGB1 and RAGE in triple-negative breast cancer tissues

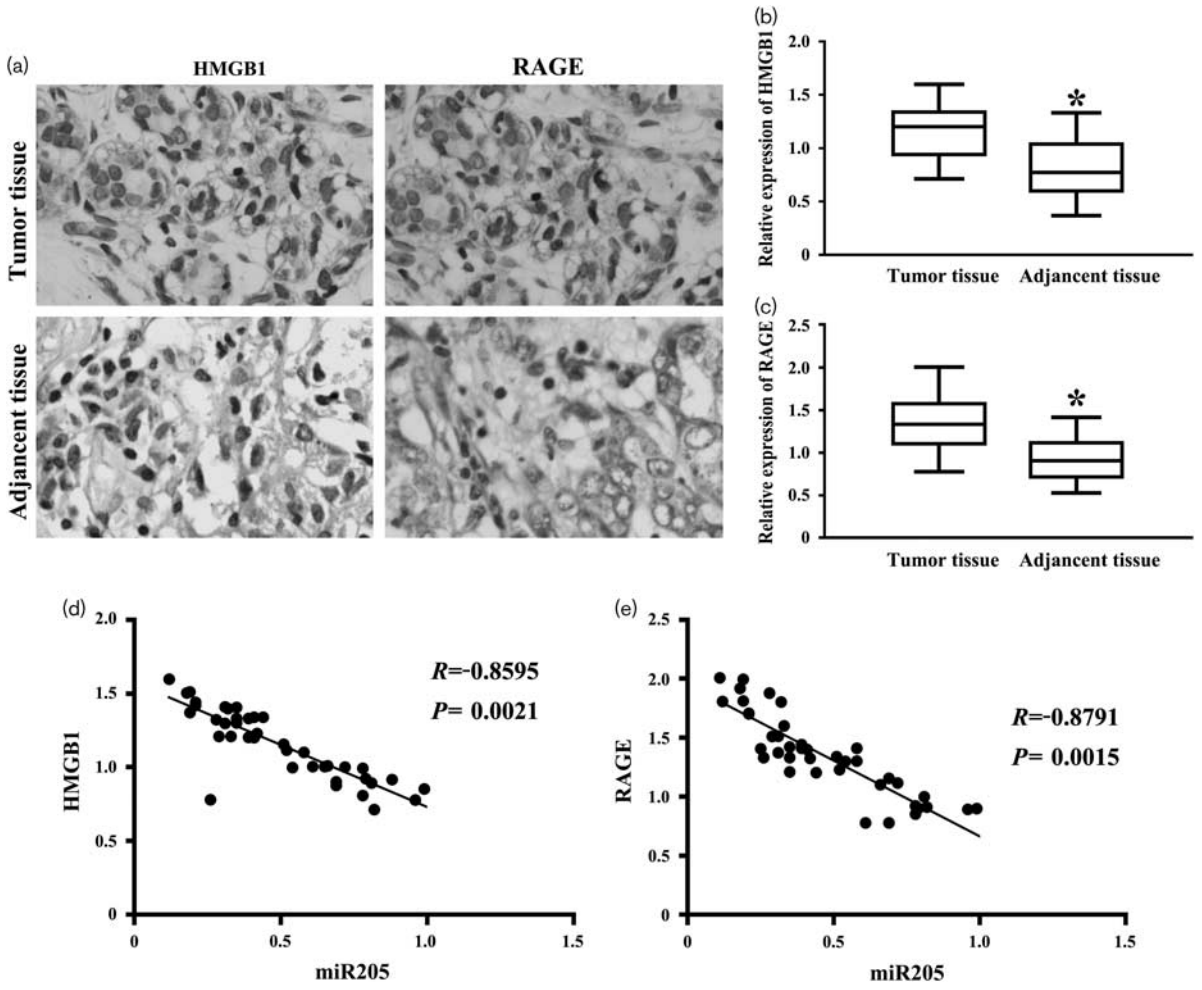
To explore the possible clinical relevance of these findings, we investigated the mRNA and protein expression levels of HMGB1 and RAGE in the same human TNBC tissues. The mRNA levels of HMGB1 and RAGE were much higher in TNBC tissues than those in adjacent nontumor tissues. The similar result tendency was also showed from IHC analyzing data (Fig. 4a–c). Furthermore, Spearman's correlation analysis was employed and demonstrated that both HMGB1 and RAGE levels were inversely associated with miR-205 expression levels (Spearman's correlation $r = -0.8595$ and -0.87918, respectively, Fig. 4d–e). Collectively, we demonstrated that miR-205 could suppress TNBC cell EMT transition and invasion through HMGB1/RAGE pathway.

Fig. 3



MiR-205 directly targets HMGB1. (a) The miR-205-binding site predicted in the 3′-untranslated region (UTR) regions of HMGB1 mRNAs. 3′-UTR fragments of HMGB1 mRNAs containing wild type (WT) or mutant of the miR-218 binding sequences were cloned into the downstream of dual-luciferase reporter vector. (b) MDA-MB-231 cells were transfected with pRL-TK reporter vectors containing either WT or mutant HMGB1 3′-UTRs (indicated as HMGB1 WT and HMGB1 MT) with either miR-NC or miR-205. (c) The protein levels of HMGB1 and RAGE in MDA-MB-231 cells were examined by western blot analysis. (d, e) qPCR analysis indicated that transfection of MDA-MB-231 cells or MDA-MB-468 cells with miR-205 mimics or miR-205 inhibitor led to a significant alteration in HMGB1 and RAGE messenger RNA (mRNA) levels. 468/NC-I indicated 468/miR-NC-inhibitor, 468/205-i indicated 468/miR-205 inhibitor. *Significant difference at $P < 0.05$.

Fig. 4



The correlation of miR-205 with HMGB1 and RAGE in triple-negative breast cancer (TNBC) tissues. (a) The protein expression levels of HMGB1 and RAGE in 40 paired tumor tissues and adjacent normal tissues were determined by using immunohistochemistry. (b, c) The mRNA expression levels of HMGB1 and RAGE in 40 paired tumor tissues and adjacent normal tissues were determined by using qPCR. (d, e) The correlations between miR-205 and HMGB1 and RAGE, respectively, were analyzed by using the Spearman's rank correlation analysis. *Significant difference at $P < 0.05$.

Discussion

Noncoding RNAs are not translated into proteins, but take positive part in the physiological and pathological processes of various cancers, and even drive specific cells' biological responses and fates [28,29]. Among them, miRNAs is a specific class of 'small noncoding RNA' which can alter expression of oncogenes and tumor suppressor genes as well as disrupt cellular functions via binding to the 3′-UTR with target genes [30,31]. Deregulation of miR-205 has been reported to be present in gastric cancer, prostate cancer, pancreatic cancer, and even breast tumor [25,26,32,33]. Gain-of-function of miR-205 restores chemosensitivity of breast cancer cells to chemotherapy (doxorubicin and taxol) by suppressing both vascular endothelial growth factor A and fibroblast growth factor 2 [27]. In this study, we initially examined miR-205 expression in a medium panel of 40 paired TNBC tissues and adjacent normal tissues. MiR-205 was remarkably decreased in TNBC tissues cohort compared with the adjacent normal tissues. Consistently, the expression level of miR-205 was obviously lower in TNBC cell lines compared with the non-TNBC breast cancer cells and normal breast cell lines. According to the clinical characteristics in 40 cases of patients with TNBC, we found that miR-205 expression was associated with tumor stage and lymph node metastasis, and could be useful as independent risk factors of overall survival. Moreover, miR-205 might accelerate the TNBC cell growth and EMT transition and metastasis through promoting the invasion and migration of TNBC cells. However, the mechanism by which miR-205 affects the biology of TNBC still remains to be validated.

HMGB1 is the most abundant member of the HMGB protein family, which could bind to DNA and regulate

DNA reprogramming and gene transcription in a wide variety of physiological processes, including inflammation, immune responses, apoptosis, and cancer [34,35]. HMGB1 can be stimulated and released by damage, inflammation, and necrotic cancer cells, thus activation and proliferation of the remnant cancer cells and metastasis via interaction with its receptor RAGE [36,37]. Besides, serum HMGB1 levels were found significantly higher in metastatic renal cell cancer patients and associated with clinicopathological characteristic and prognosis [38]. HMGB1/REGE axis played an important role in inducing EMT transition in colorectal and gastric cancers [39,40]. These findings enlightened us that HMGB1–RAGE axis may be involved in the development of TNBC, such as the metastasis of TNBC cells. Herein, we further validated whether miR-205 could act on HMGB1/RAGE to affect TNBC cell invasion. In fact, we observed significantly reduced protein levels of HMGB1 and RAGE in miR-205-mimics transfected TNBC cells.

To further validate that miR-205 regulated HMGB1 pathway in TNBC cell lines, we detected the protein expression levels of HMGB1 and RAGE in response to the effect of miR-205 inhibition and overexpression. After miR-205 is upregulated, the protein levels of HMGB1 and RAGE were significantly upregulated in MDA-MB-231 cells, whereas the two molecules were decreased in MDA-MB-468 cells after miR-205 inhibition. According to clinical cohort information, miR-205 expression was downregulated, while HMGB1 and RAGE expression was upregulated. MiR-205 was inversely correlated with HMGB1 and RAGE, respectively, which further confirmed that miR-205 might regulate HMGB1/RAGE axis expression to promote TNBC cell invasion.

The identification of a sensitive and specific biomarker predicting the lethal metastasis of TNBC can enable detection of a relapse as early as possible to promote the survival of patients and reduce treatment costs. In this study cohort, miR-205 was universally downregulated in most TNBC samples and it is negatively associated with progression and metastasis of TNBC. Overexpression of miR-205 could suppress the cell growth and EMT biological features of TNBC cells partially through direct targeting HMGB1/RAGE. However, there was a limitation of the number of collected patients. Therefore, future studies need to expand the cohort and clarify the potential mechanism behind leading to miR-205 downregulation, such as epigenetics modification, circRNA regulation, etc., which would further explains the function of miR-205 in TNBC and provides the crucial development of targeted therapies.

Acknowledgements

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Fu-biao Kang contributed to the design of the experiments and analyzes all the data. Chao Yang collected clinical samples and their information. Juan Wang and Dong-wei He performed the cell experiments *in vitro*. Ling Wang helped design the experiment, provided materials and revised the manuscript.

Conflicts of interest

There are no conflicts of interest.

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