Effects of Neuregulins on Invasion and Metastasis of Non-overexpression ErbB2 Breast Cancer Cells

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CLC number: R737.9 Document code: A Article ID: 1000-9604(2009)02-0115-07

DOI: 10.1007/s11670-009-0115-7

ABSTRACT

Objective: To explore the effects of neuregulins on ErbB2 receptor signal transduction pathway activation, and invasion and metastasis of non-overexpression ErbB2 breast cancer cell MDA-MB-231.

Methods: The expressions of neuregulin were detected by immunocytochemistry and Western blot. MDA-MB-231 cells were treated with ErbB2 kinase inhibitor AG825. Proliferations were measured with MTT assay. Invasion and metastasis of MDA-ME-231 cells were evaluated with transwell chamber. The enzyme activities of MMP-2 and MMP-9 were detected by gelatin zymography. The expressions of MMP-2 and HIF-1α were detected by Western blot.

Results: MDA-MB-231 cells expressed a relatively higher level of neuregulin. In Western blot, the positive reaction band was found at 44KD which coincides with the molecular weight of NRG. When MDA-MB-231 cells were treated with AG825, the proliferation was inhibited in a time-dose-dependent manner (P<0.01), invasion and metastasis were also depressed (P<0.05). The enzyme activities of MMP-2 and MMP-9 were lower (P<0.05). The expression levels of MMP-2 and HIF-1α were decreased (P<0.05).

Conclusion: Our study indicates that neuregulins are synthesized in MDA-MB-231 cells as transmembrane proteins, neuregulins could activate ErbB2 receptor signal transduction pathway by autocrine or paracrine secretion, and induce invasion and metastasis of MDA-MB-231 cells.

Key words: Non-overexpression ErbB2 breast cancer; Neuregulins; Invasion; Metastasis

INTRODUCTION

ErbB2 (HER2 & neu) gene is a proto-oncogene closely related with the pathogenesis and development of breast cancer. It encodes a protein highly homologous with human epidermal growth factor. ErbB2 belongs to "orphan receptor". It can be activated by its own proliferation or overexpression and induces the formation of homo- or hetero- dimers directly, which can activate the receptor tyrosine kinase domain and enhance the downstream signal pathways that govern proliferation and migration of tumor cells[1]. Overexpression of ErbB2 is closely related to high aggressiveness, poor prognosis, high recurrence rate and low survival rate. ErbB2 overexpression is defined by immunohistochemical method as ErbB2 (+++) or ErbB2 (++) or by fluorescence *in situ* hybridization as ErbB2 (+) in clinical pathology[2]. With the increasing concernment about the pivotal effect of ErbB2-induced signal pathways, an monoclonal anti-ErbB2 antibody has already been used to treat ErbB2 overexpression breast cancer. However, there is approximately 70% breast cancers that do not overexpress ErbB2, most part of which is extremely aggressive. The mechanism needs to be clarified in order to look for effective therapies.

Neuregulins(NRGs), which belong to epidermal growth factors, are the ligands of ErbB3 & ErbB4. They are expressed in many histiocytes and play an important role in the regulation of the development of organs such as the neurvous system and heart[3]. NRGs bind either ErbB3 or ErbB4 directly and induce activation of ErbB2 through the formation of

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ErbB2:ErbB3 or ErB2:ErbB4 heterodimers. As we know so far, it is the only or principal mechanism to activate ErbB2 receptor[4]. Recent evidences suggest that NRGs are overexpressed in nearly 30% of breast cancers which do not overexpress ErbB2[4,5]. Thus, the ErbB2 receptor signal transduction pathway probably is activated by NRGs. It is crucial that the proliferation, invasion and metastasis of non-overexpression breast cancer cells are regulated via NRGs/ErbB2 intracellular signal pathways. There has been some reports on the effect of NRGs in tumors at present[6-8].

In our study, we chose the MDA-MB-231 breast cancer cells which express NRGs and do not overexpress ErbB2[4], used the functional inhibitor of ErbB2 receptor AG825 to explore the effects of neuregulins on activation of ErbB2 receptor signal transduction pathways and the invasion and metastasis of non-overexpression ErbB2 breast cancer cells.

MATERIALS AND METHODS

Materials and Reagents

Breast cancer cells MDA-MB-231 and mouse fibroblasts NIH3T3 were supplied by the department of pathophysiology of Chongqing Medical University. RPMI-1640（Dry Powder Culture Medium）was purchased from Gibco Company, USA. Fetal bovine serum was the product of SiJi Qing Company, Hangzhou. 3-(4,5-demethy-2-thiazolyl)-2, 5-dephenyl -2h-tetrazolium-bromide (MTT) was obtained from Sigma Company, USA. Matrigel was from BD Company, USA. Transwell Chamber (8 μm) was purchased from Costar Company, USA. The monoclonal mouse anti-human neuregulin antibody was the product of Thermo Company, USA. The monoclonal mouse anti-human HIF-1α antibody, mouse anti-human MMP-2 antibody, mouse anti-human β-actin antibody and horseradish peroxidase(HRP)-labelled goat anti-mouse IgG were obtained from Santa Cruz Company. ABC Kits and DAB were supplied by Boster Biological Company, Wuhan. BeyoECL, Western fluorescence detection reagents were from Beyotime Institute of Biotechnology. Automatic ELIASA, PAGE Gel Electrophoresis System, Vertical Electrophoresis Cell and Electrophoresis Gel Image Analysis System were all from Bio-Rad Company, USA.

Cell Culture

Breast cancer cells MDA-MB-231 and mouse fibroblasts NIH3T3 were routinely cultured in RPMI-1640 medium containing 10% newborn bovine serum, 100KU/L penicillin and 100mg/L streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Immunocytochemistry

MDA-MB-231 cells were cultured at a cell density of 5×10^4/ml. After reaching to optimal density the cells were washed with phosphate-buffered saline (PBS) and then fixed by 75% ethanol. Following SP method, immunocytochemistry staining was undergone according to the directory of SP box. The concentration of the monoclonal anti-NRG antibody was 1:40. For negative control PBS solution was used instead of primary antibody. Cells were subjected to hematoxylin staining after DAB coloration and were observed under light microscope.

MTT Assay

Cell proliferation was measured by a modified MTT assay. MDA-MB-231 cells were plated at a density of 5×10^4/well in 96-well plates and incubated for 24h. Cells were grouped as the control group, the AG825 experimental group and the group without reagents. Cells of experimental group were treated with 30,40,50,60 and 70μmol/L of AG825, and cultured in an incubator at 37°C in a humidified atmosphere with 5% CO_2. The supernatant was removed after 24, 48 and 72 h respectively. Then 20 μl MTT and serum-free RPMI-1640 200μl were added to each well and the cells were further incubated at 37°C for 4h. Then cells were centrifuged at 2000r/min for 10min, the supernatant was discarded and 200 μl DMSO was added to each well and mixed for 10min. The absorbance at wave length of 570nm (A_{570}) was measured by ELIASA. All MTT assay were repeated three times and the mean was taken. The inhibition rates of MDA-MB-231 cells at different doses and different times were calculated.

$$\text{Inhibition rate}[\%] = 1-(A_{treated}/A_{untreated})\times100\%$$

Where $A_{treated}$ *is the average absorbance in wells treated with AG825 and* $A_{untreated}$ *is the average absorbance in wells without AG825.*

Cell Invasion Assay

To obtain chemotactic factors, NIH3T3 cells were cultured routinely, washed with PBS three times and treated with serum-free RPMI-1640 medium when 80% of them grew to the confluent state. After 24h, the supernatant was collected and centrifuged at 2000r/min and kept at -20°C.

For the invasion experiment, Matrigel was well-distributed on the Transwell Chamber at the concentration of 40μl/well and treated with ultraviolet ray overnight. Both cultured MDA-MB-231 cells in AG825 experimental group and control group were harvested and washed with PBS. The density was regulated at 4×10^5/ml after cells was lysed and centrifuged. And then, 0.2ml was planted in congealed Transwell Chamber. The Transwell Chamber was located in 24-well plates and 0.6ml NIH3T3 cell supernatant was added under it. Transwell Chamber was taken out after 48h, the Matrigel was wiped off, and the chamber was washed three times with PBS and fixed with 95% ethanol. Finally it was subjected to HE staining and Transwell Chamber was unveiled and stuck to the glass slide. Under the high-power field (×200), random observations were performed in 3 fields of each Transwell Chamber membrane and the experiment was repeated three times. The mean was taken as the final result for analysis.

Cell Migration Assay

The steps of this experiment are similar to the cell invasion, except without the Matrigel.

Gelatin Zymography

The enzyme activities of MMP-2 and MMP-9 were detected by gelatin zymography. MDA-MB-231 cells were planted at the density of 2×10^5 in the culture flask. Cultured for 24h, they were incubated in the absence or presence of AG825 for 48 h. The supernatant was collected after further culturing in serum-free medium for 24 h. After centrifugation at 2000 r/min for 10 min, the supernatant was reserved at -20°C. 5% stacking gel and 10% SDS-PAGE separating medium containing 1.0 mg/ml gelatin were prepared. The samples from different groups were mixed with Sample Loading Buffer without β-Mercaptoethanol and electrophoresis in ice water bath. The gel was eluted and rinsed in the fluid after electrophoresis and incubated at 37°C for 42 h. Then the gel was stained and decolored until the white bands were come into view. The results were displayed as photos by Bio-Rad gel imaging system, and gray value of the bands were measured by Quantity-One image analysis software.

Western Blot

The expressions of MMP-2, HIF-1α and NRGs were detected by Western blot. Cells were routinely cultured for 24 h and treated with reagent for the following 48 h. The cells from experimental groups and control groups, were collected and lysed. The total protein quantification was performed using Brodford method and the protein was immersed onto SDS-PAGE, and then transferred to PVDF membrane. The PVDF membrane was blocked with fat-free dried milk for 1 h and incubated overnight with the monoclonal mouse anti-human NRGs antibodies at a dilution of 1:150, mouse anti-human HIF-1α antibody at a dilution of 1:300 and mouse anti-human MMP-2 antibody at a dilution of 1:300 at 4°C. The membranes were washed with TBST and then the goat anti-mouse IgG/HRP at a dilution of 1:2000 was added for further incubation at 37°C for 2h. NRGs were visualized using DAB. MMP-2 and HIF-1α were observed using ECL. Photos could be taken using Bio-Rad system and the densitometry was performed by Quantity-One image analysis Software. B-actin was used as an internal standard to normalize the protein expressions. Band intensities were expressed as the percentage of the β-actin band intensity, which was set at 100%.

Statistical Analysis

The data were analyzed by SAS 8.0 software and expressed as $\bar{x}\pm s$. Multiple-factor ANOVA was used for inhibition of AG825 on MDA-MB-231 cell growth and other all data were performed with the student's *t*-test. $P<0.05$ was considered to be statistically significant.

RESULTS

The Expression of NRG in MDA-MB-231 Cells

The expression of NRG in MDA-MB-231 cells is shown in Figure 1 as brown granules distributed in the cytoplasm and membrane, also a few in the nucleus of MDA-MB-231 cells.

The Expression of NRG Protein in MDA-MB-231 Cells

The expression of NRG protein in MDA-MB-231 cells was detected by Western blot and were shown in Figure 2 as the positive reaction band with a molecular weight of 44KD.

Inhibition of AG825 on MDA-MB-231 Cell Growth

Cell growth was measured by MTT assay. The result demonstrated that the proliferation of MDA-MB-231 cells was inhibited in a time-dose- dependent manner(Table 1). The median-effect concentration (IC_{50}) was calculated using the Linear Regression

Equation Y=1.11X-12.94. Since the median-effect concentration IC_{50} was 56.59 μmol/ L when cells were treated with AG825 for 48 h, we chose the concentration of 40 μmol/L in the following experiments.

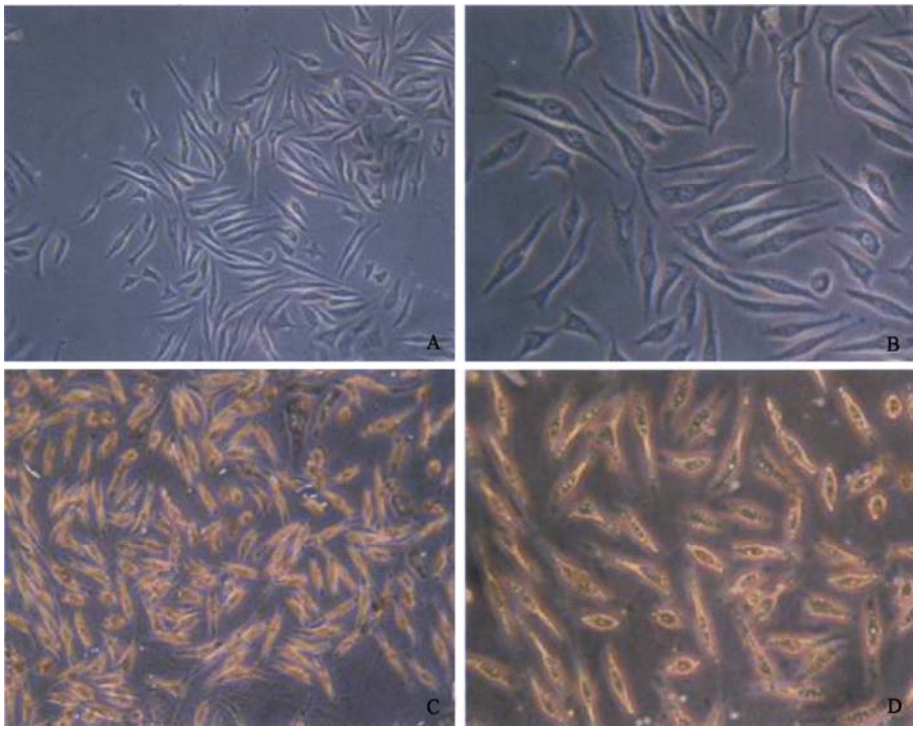

Figure 1. The expression of NRG in MDA-MB-231 cell (IHC：SP-DAB)
A. Control group (×100)；B. Control group (×200)；
C. Experimental group (×100)；D. Experimental group (×200)

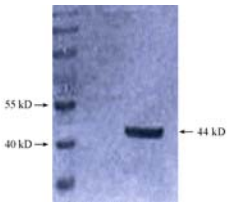

Figure 2. Expression of NRG protein in MDA-MB-231 cell.

Invasion and Metastasis of MDA-MB-231 Cells

As shown in Figure 3, the invasion and metastasis of MDA-MB-231 cells were depressed after being treated with 40 μmol/ L AG825 for 48h. In the AG825 experimental groups, invasion was reduced by 34.27% and metastasis was reduced by 32.62%, when compared with the control groups. $P<0.05$ was considered to be statistically significant.

Table. 1 Inhibition of AG825 on MDA-MB-231 cell growth
(%, n=15, $\bar{x} \pm s$)

AG825 concentration	Inhibition rate (%) 24h	48h	72h	F
30μmol/L	14.74±3.33	17.32±1.76	30.99±2.66	32.74##
40μmol/L	18.23±1.91	33.58±1.59	43.98±2.97	46.41**
50μmol/L	26.26±0.98	40.53±1.94	54.64±1.01	
60μmol/L	33.24±2.22	54.52±1.72	65.67±1.53	
70μmol/L	39.27±1.23	62.14±3.40	75.03±2.28	

##: analysis of variance, $P<0.01$, among different time group; **: analysis of variance, $P<0.01$, among different concentration group

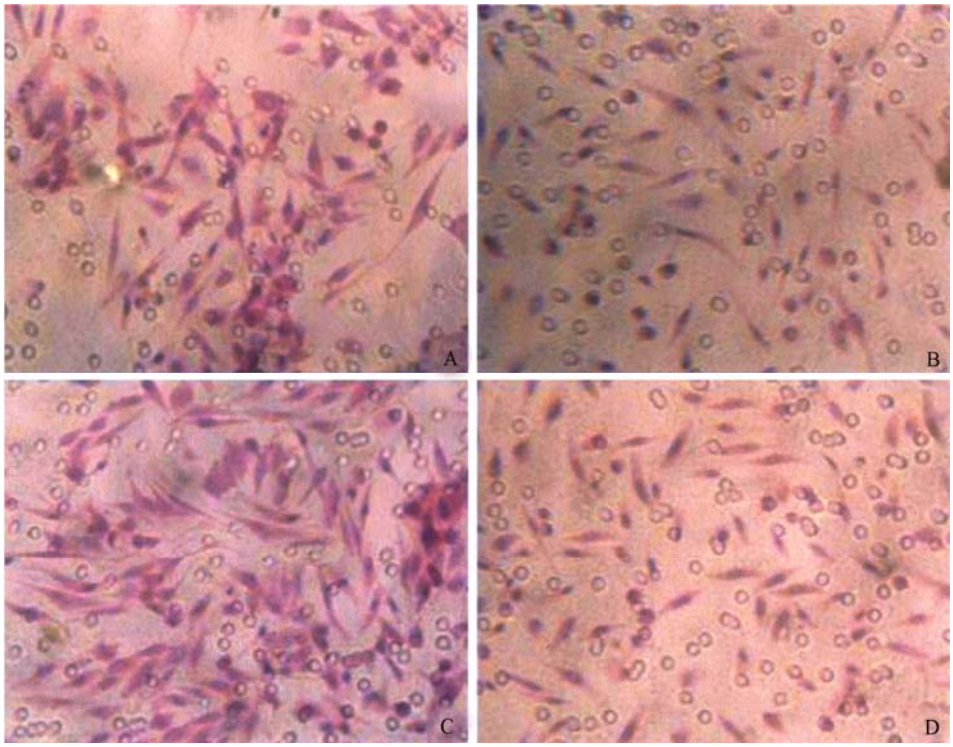

Figure 3. Invasion and metastasis of MDA-MB-231 cells(HE×200)
A: Control group(Invasion); B: AG825 group(Invasion);
C: Control group (metastasis); D: AG825 group (metastasis)

Detection of MMP-2 and MMP-9 Enzyme Activities

MMP-2 and MMP-9 could be detected in the supernatant of cultured MDA-MB-231 cells. The enzyme activities of MMP-2 and MMP-9 are shown in Figure 4. It is shown that the enzyme activity in experimental groups was lower when compared to the control groups, since MDA-MB-231 cells has been treated with AG825. $P<0.05$ was considered to be statistically significant.

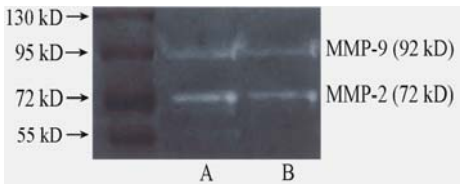

Figure 4. Enzymatic activities of MMP-2 and MMP-9.
A: Control group; B: AG825 group

Expressions of MMP-2 and HIF-1α Proteins in MDA-MB-231 Cells

The expressions of MMP-2 and HIF-1α protein are detected by Western blot. The results were shown in Figure 5. Compared with control groups, the expressions of MMP-2 and HIF-1α protein were decreased by 40.76% and 41.27% respectively. $P<0.05$ was considered to be statistically significant.

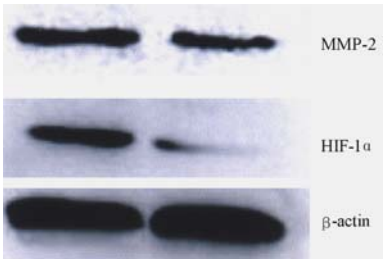

Figure 5. Expressions of MMP-2 and HIF-1α proteins in MDA-MB-231 cells.
A: Control group; B: AG825 group

DISCUSSION

Neuregulins (NRGs), which belong to the epidermal growth factors (EGF), include 4 subtypes: neuregulin 1 (NRG1, NRG), NRG2, NRG3 and

NRG4. NRG is the main member of NRGs family. It has different names such as neu differentiation factor (NDF), heregulin (HRG), glial growth factor (GGF), acetylcholine receptor-inducing acticity (ARIA) and sensory and motor neuron-derived factor (SMDF)[3]. NRGs receptors belong to ErbB receptor which has intrinsic tyrosine kinases activities, including ErbB3/HRG3 and ErbB4/HRG4. NRGs directely bind either to ErbB3 or ErbB4 receptors and indirectely induce activation of ErbB2 through the formation of ErbB2:ErbB3 or ErbB2: ErbB4 heterodimers, which activate the receptor tyrosine kinases and enhance the downstream signal that governs cell proliferation and migration[9]. ErbB2 can be activated by its own overexpression and induces the formation of heter- or homo- dimers directely, which activate receptor tyrosine kinases and downstream signal transduction pathways. It can also be activated through ligand-binding when NRG exists[10]. NRGs/ErbB2 can activate the signal transduction pathways including mitogen-activated protein kinases (MAPK) as well as phosphatidylinostiol 3-linases (PI3K/AKT), and promote cell proliferation and differentiation by regulating myc, jun and fosb genes et al[9,11]. Previous studies showed that NRGs were widely expressed in many human malignancies such as prostate and lung cancers. They regulate the expressions of genes which control proliferation and invasiveness through signal transduction pathways, and play an important role in tumor cell growth and carcinogenesis[4].

AG825 is an inhibitor of ErbB2 receptor function. It inhibits the phosphorylation of ErbB2 tyrosine kinases selectively and blocks ErbB2 receptor activation, thereby hinders the ErbB2-induced intracellular signal transduction pathways and the corresponding effects[12]. Using AG825, we can study the ErbB2 receptor activation state and function of the relevant signal transduction pathways.

In our study, non-overexpression ErbB2 breast cancer cells MDA-MB-231 were selected, the expression of NRGs was detected by immunocyto-chemistry and Western blot. It was observed that MDA-MB-231 cells expressed a relatively higher level of neuregulin and the positive reaction bands were found at the location of NRG. Our study indicates that the NRGs are synthesized and secreted in MDA-MB-231 cells. They are localized in the cytoplasm mainly and also can be found in nucleus. Madlaina Breuleux, et al[13]. considered that NRG slice variant was located in nucleus and nuclear $NRG_{\alpha 1\text{-}241}$ could interact with the nuclear transcription factors. They pointed out that NRG may also act as a nuclear growth factor despite being the extracellular signal molecule, potentially participating in gene regulation, while the biological effects are still open to further research and investigation.

When MDA-MB-231 cells were treated with AG825, the proliferation was notably decreased. The findings suggests that ErbB2 receptor signal transduction pathways are in activated state and it probably exists that NRGs active ErbB2 receptor signal transduction pathways via autocrine or paracrine action, and induce invasion and metastasis of MDA-MB-231 cells. The inhibition of AG825 to the proliferation of MDA-MB-231 cells was in a time-dose-depending manner.

The invasion and metastasis process of tumors includes tumor cells departing from primary foci, passing through basal lamina and extracellular matrix into vessels, arriving at the distance via blood circulation, and finally forming the micrometastasis. It is a key step that the tumor cells depart from primary foci and get through the obstructions. Both MMP-2 and MMP-9 which belong to collagenase type IV, promote the tumor cell breaking through the obstruction by dissolving the extracellular matrix that plays a key role in the tumor invasion and metastasis. HIF-1α is a factor related with tumor aggressiveness. Hypoxia-induced factor-1 (HIF-1) is made from HIF-1α: HIF-β heteromider, the activity of which is determined by HIF-1α. HIF-1 can regulate the expression of vascular endothelial growth factor (VEGF) gene and promote angiogenesis process[14]. We found that both the ability of invasion and metastasis of MDA-MB-231 cells and the enzyme activities of MMP-2 and MMP-9 were depressed after ErbB2 was blocked. The expressions of MMP-2 and HIF-1α proteins were detected further. Using AG825, we found that the expressions decreased. It pointed out that NRGs can active ErbB2 receptor signal transduction pathways, upregulate the expressions of MMP-2 and HIF-1α proteins, increase the power to penetrate the extracellular matrix and angiogenesis and promote the ability to invade and metastasis of tumor cells.

Ella Atlas, et al[15]. reported that NRGs play an especially important role in tumor invasion and metastasis. Our researches confirms their views. It is possible that NRGs are important activators in the deteriorations produced by non-overexpression ErbB2 breast cancer cells and NRGs-targeting strategies would be the ideal therapies to non-overexpression ErbB2 breast cancer.

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