http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2022.345.352

Research Article
Effects of Oncogene Neuregulin 1 on Breast Cancer Cells

[1]Qian Li, [1]Biqing Zhu, [1]Jing Chen, [1]Hairong Wang, [2]Yuan Wu, [3]Hanbo Chen and [1,4]Xia He

[1]Department of Radiation Oncology, Jiangsu Cancer Hospital and Jiangsu Institute of Cancer Research,
The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing 210000, Jiangsu Province, China
[2]Department of Chemotherapy, Jiangsu Cancer Hospital and Jiangsu Institute of Cancer Research,
The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing 210000, Jiangsu Province, China
[3]Lianyungang Clinical College of Nanjing Medical University, The First People's Hospital of Lianyungang, Lianyungang 222000, Jiangsu, China
[4]Jiangsu Key Lab of Cancer Biomarkers, Prevention and Treatment, Collaborative Innovation Center for Personalized Cancer Medicine,
Nanjing Medical University, Nanjing 210000, Jiangsu, China

Abstract

Background and Objectives: The NRG1 fusion protein is a driving factor for the occurrence and development of many tumours. We aimed to evaluate the effects of oncogene Neuregulin 1 (NRG1) on the proliferation and migration of breast cancer cells. **Materials and Methods:** Target gene NRG1 was transfected into breast cancer cells using the gene transfection technique and the migration ability of cells was observed by wound healing assay. The migration and invasion abilities of cells were further observed by Transwell assay and cell apoptosis was observed by TUNEL staining. The cell cycle distribution of breast cancer cells was detected by flow cytometry. **Results:** The wound healing assay exhibited that breast cancer cells overexpressing NRG1 exhibited stronger migration ($p = 0.0047$). More breast cancer cells of up-regulating NRG1 penetrated the transwell chamber, showing enhanced invasion ability ($p = 0.0029$). The TUNEL assay and flow cytometry demonstrated that NRG1 inhibited cell apoptosis and made them enter the active division stage. **Conclusion:** The NRG1 can promote the malignant function of breast cancer cells by augmenting migration and invasion abilities. High expression of NRG1 remarkably suppressed the apoptosis of breast cancer cells.

Key words: Neuregulin 1, breast cancer, cell, proliferation, migration, apoptosis, invasion

Citation: Li, Q., B. Zhu, J. Chen, H. Wang, Y. Wu, H. Chen and X. He, 2022. Effects of oncogene neuregulin 1 on breast cancer cells. Pak. J. Biol. Sci., 25: 345-352.

Corresponding Author: Xia He, Department of Radiation Oncology, Jiangsu Cancer Hospital and Jiangsu Institute of Cancer Research,
The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing 210000, Jiangsu Province, China

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The incidence rate of breast cancer is rising annually, which is closely associated with factors such as hormone levels, living habits and heredity. There are many treatments for breast cancer, including surgery, radiotherapy, chemotherapy and targeted or hormone therapy[1]. As research on the pathogenesis of breast cancer deepens, the treatments for breast cancer have been also updated year by year, from total mastectomy in the past to breast-conserving surgery as the first choice currently[2]. Moreover, the radiation range of radiotherapy has been continuously improved and reduced and multiple studies on targeted drugs have also entered clinical trials. Comprehensive treatment of breast cancer especially when combined with targeted drugs has become a global trend[3].

Neuregulin 1 (NRG1), belonging to the NRG family, is the specific ligand of Erb-b2 receptor tyrosine kinase 3 (ERBB3) and ERBB4 and these two Epidermal Growth Factor Receptors (EGFRs) can also be called human epidermal growth factor receptor 3 (HER3) and HER4, respectively[4]. The other members of the ERBB family include ERBB1 (EGFR) and ERBB2 (HER2)[5]. Members of the ERBB family can form homodimers or heterodimers to activate downstream Mitogen-Activated Protein Kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signalling pathways. Most NRG1 exist in the inactive form of NRG1 precursor (pro-NRG1)[6]. After the juxtamembrane domain is hydrolyzed, the soluble NRG1 (sNRG1) encodes different transmembrane proteins through alternative splicing of different transcriptional promoters and mRNAs[7]. The ERBB family is involved in the occurrence and progression of many tumours. Meanwhile, NRG1 serves as a ligand for ERBBs. So far, it has been found in many studies that NRG1 fusion protein is also a driving factor for the occurrence and development of many tumours. The incidence rate of NRG1 fusion is the highest in non-small cell lung cancer, which is about 0.3%[8,9] and it has also been found in other cancers such as ovarian cancer, pancreatic cancer, renal cell carcinoma, gallbladder cancer, urinary bladder cancer and colorectal cancer. Since NRG1 has pro-cancer potential, it is necessary to investigate its function in breast cancer which can provide a new treatment target.

The purpose of this study was to investigate the effect of the NRG1 gene on the proliferation, migration and invasion of breast cancer cells.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Radiation Oncology, Jiangsu Cancer Hospital and Jiangsu Institute of Cancer Research, The Affiliated Cancer Hospital of Nanjing Medical University, China from December, 2018-2019.

Transfection of target gene: Cells were pre-inoculated into a 6-well plate. The control plasmids and NRG1 overexpression (OE) plasmids were mixed with culture medium and Lipofectamine 2000 and then added into the 6-well plate. Later, a Quantitative Polymerase Chain Reaction (qPCR) was conducted to verify the transfection.

Wound healing assay: The 6-well plate was covered with breast cancer 4T1 cell lines and the bottom of the culture plate was scratched vertically and linearly with the pipette tip. After washing with PBS, the cells were cultured for 24 hrs and then photographed.

Cell migration assay: The culture medium was added into the lower chamber of the transwell plate and the cell suspension was added into the upper chamber. After 24 hrs of culture, the cell suspension was taken out of the upper chamber, fixed in the culture plate containing paraformaldehyde, stained with Giemsa's staining solution and observed.

Cell invasion assay: The culture medium was added into the lower chamber of the transwell plate pre-laid with Matrigel and the cell suspension was added into the upper chamber. After 24 hrs of culture, the cell suspension was taken out of the upper chamber, fixed in the culture plate containing paraformaldehyde, stained with Giemsa's staining solution and observed.

Detection of apoptosis and cell cycle: The cells were pre-laid on the cover glass and fixed with paraformaldehyde and the following procedures were following the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay kit (Beyotime Institute of Biotechnology, China). After 1 mL of DAPI staining solution was added, the cells were sealed and photographed. The cell cycle was detected by flow cytometry using the flow cytometry kit (Beyotime Institute of Biotechnology, China). After the staining agent was added, the cells were detected in dark.

RESULTS

Verification of NRG1 plasmid expression by qPCR: The NRG1 is closely related to the proliferation and metastasis of tumour cells in lung cancer, ovarian cancer, pancreatic cancer, renal cell carcinoma, gallbladder cancer, urinary bladder cancer and

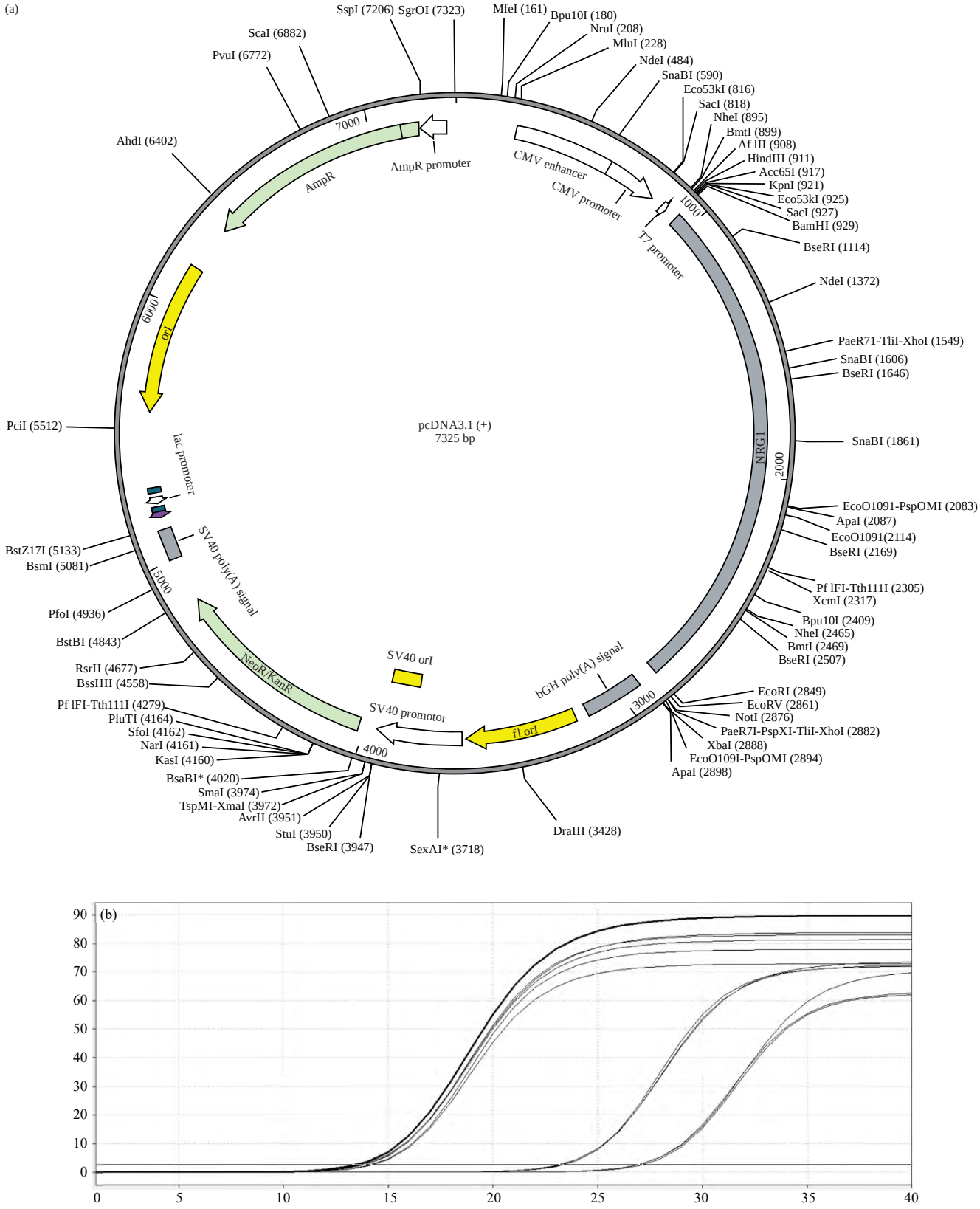

Fig. 1(a-c): Continue

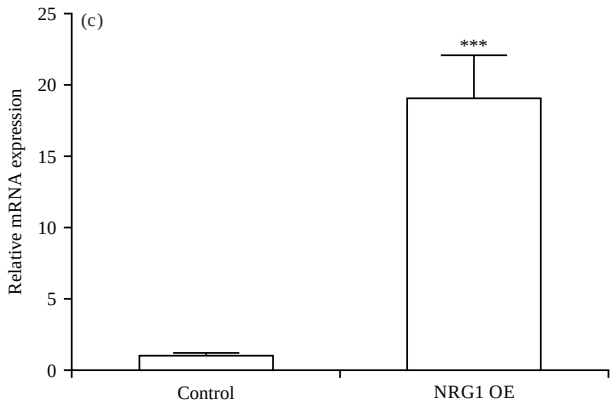

Fig. 1(a-c): (a) NRG1 OE plasmids were constructed and transfected into breast cancer 4T1 cells and (b, c) Transfection process was verified by qPCR

Results of qPCR suggested a high expression of NRG1 after transfection, indicating that the transfection was successful and ***p<0.05, control vs. NRG1 OE plasmids

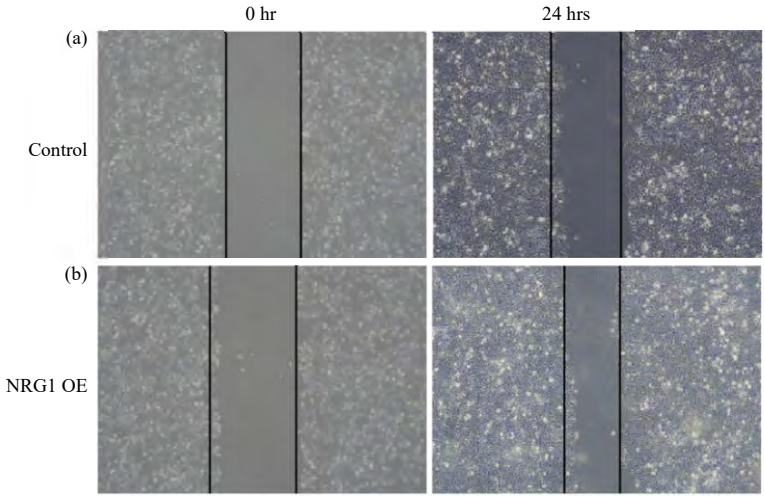

Fig. 2(a-b): Breast cancer cells transfected with overexpressed NRG1 gene, (a) Control group and (b) NRG1 OE

Results of the wound healing assay showed that migration was significantly enhanced in the breast cancer cells with NRG1 OE compared with that of the control group and scale bar: 50 μm

Table 1: Primer sequences of NRG1

Primer	Sequence
NRG1-forward	GCAAGTGCCCAAATGAGT
NRG1-reverse	ATGATGCCGACCACAAGG

colorectal cancer. In this study, therefore, whether NRG1 can promote tumour progression was verified in breast cancer 4T1 cells. The NRG1 OE plasmids were constructed and transfected into 4T1 cells (Fig. 1a-c). The NRG1 plasmids were inserted between BamHI and EcoRI and the primer sequences of NRG1 are listed in Table 1.

Effects of NRG1 on breast cancer cells: After transfection, the proliferation, migration and invasion abilities of 4T1 cells were observed. The results showed that up-regulating the expression of the NRG1 gene in 4T1 cells enhanced the migration ability ($p = 0.0047$) and invasion ability ($p = 0.0029$) of a malignant tumour. Wound healing assay suggested that the migration speed was higher in the NRG1 OE groups (Fig. 2a, b) and more cells were passing through the Transwell chamber (Fig. 3a-c and 4a-c). The TUNEL assay revealed that the NRG1 OE group had dramatically fewer apoptotic 4T1 cells than the empty vector group

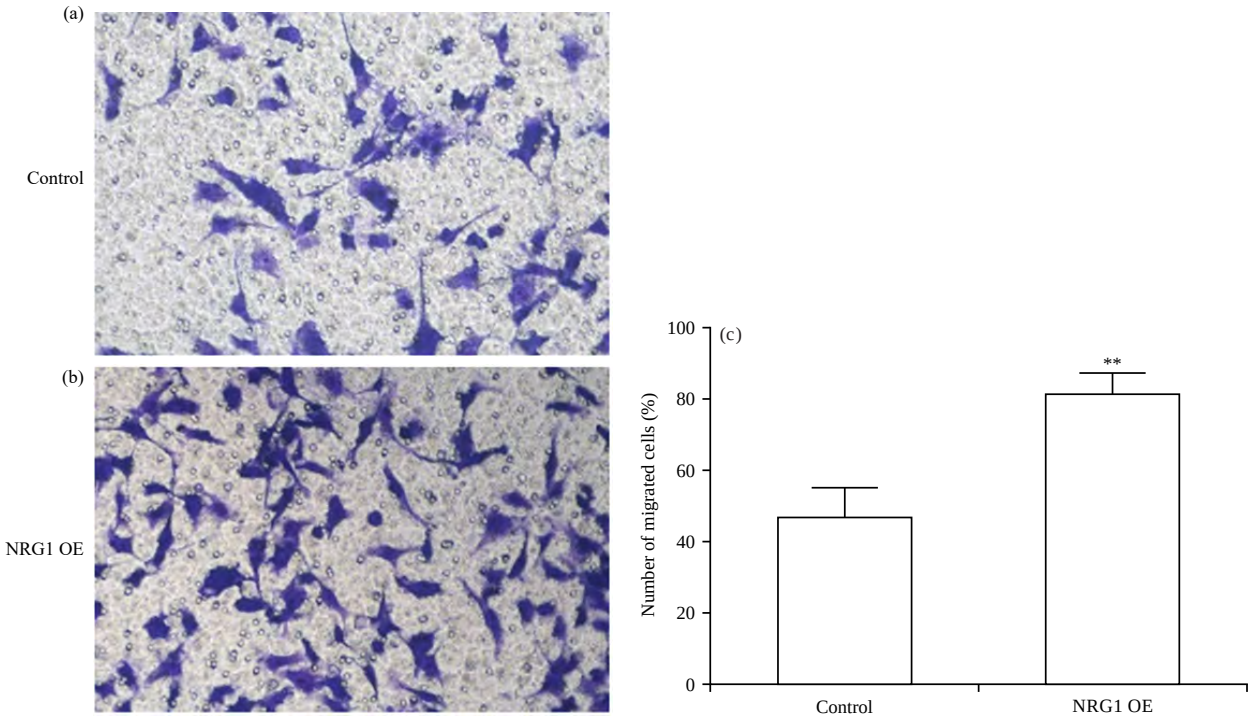

Fig. 3(a-c): (a-b) Number of breast cancer cells with a high expression of NRG1 gene passing through the transwell chamber significantly increased and (c) Enhanced cell migration ability
Scale bar: 200 μm and **p<0.05, control vs. NRG1 OE groups

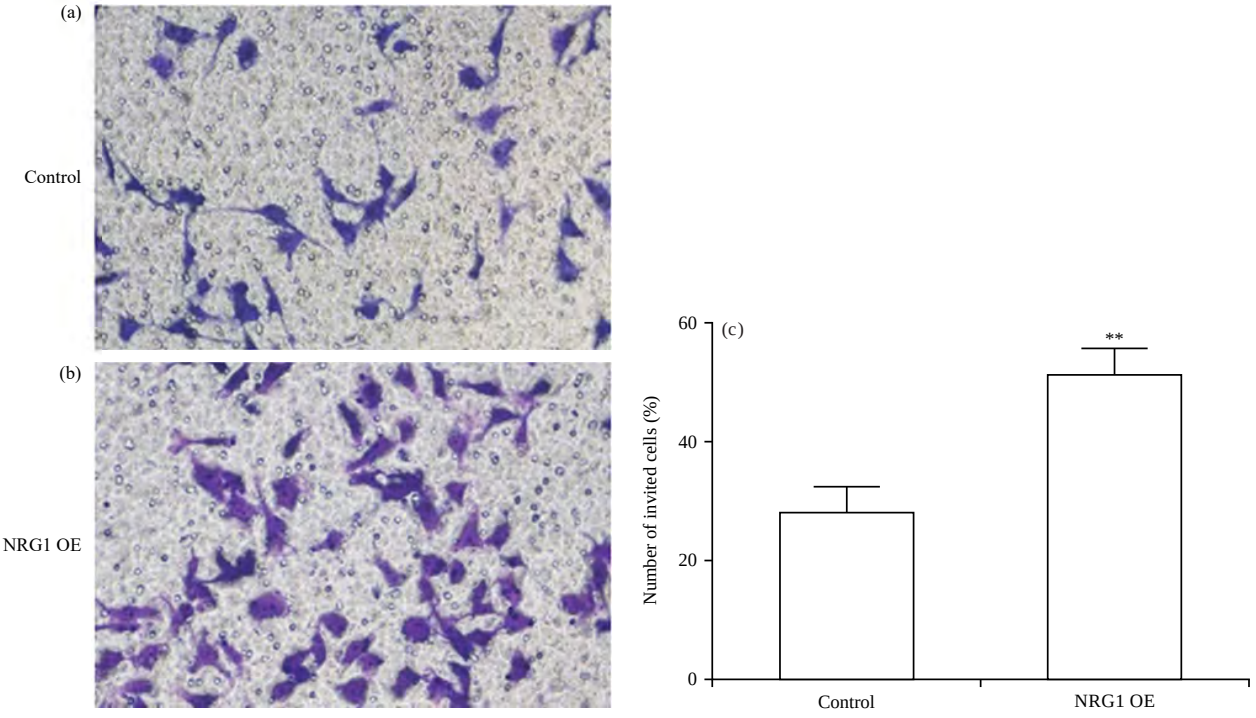

Fig. 4(a-c): (a-b) Invasion ability of cells in transwell chamber and (c) Breast cancer cells with NRG1 OE showed stronger invasion ability
Scale bar: 200 μm and **p<0.05, control vs. NRG1 OE groups

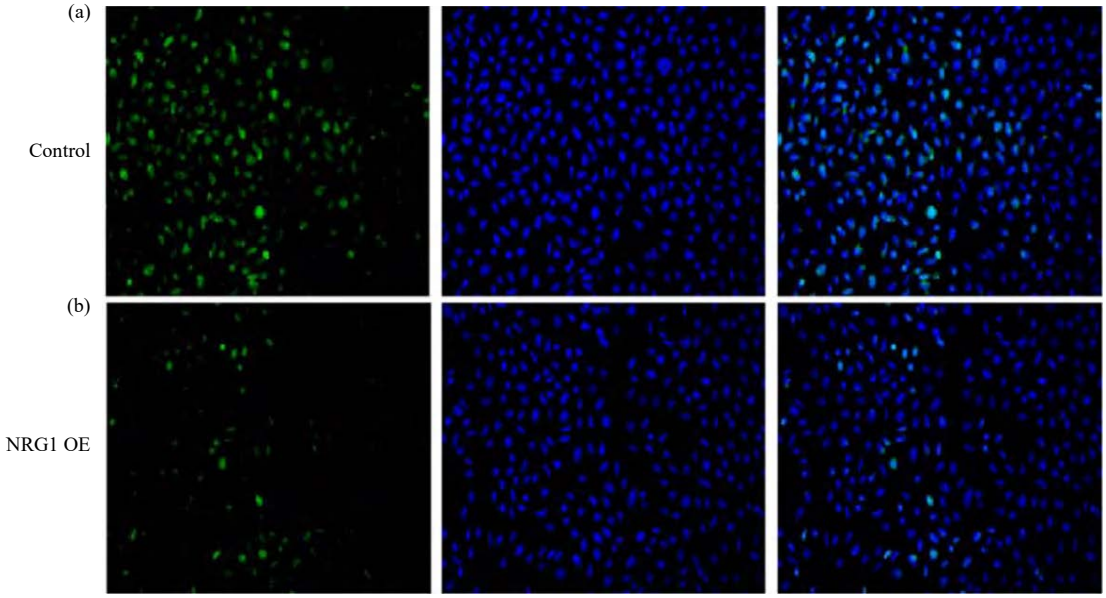

Fig. 5(a-b): Cell apoptosis was detected using a TUNEL assay kit. Cell apoptosis was remarkably reduced in the (a) Control group compared with (b) NRG1 OE group
Scale bar: 100 μm

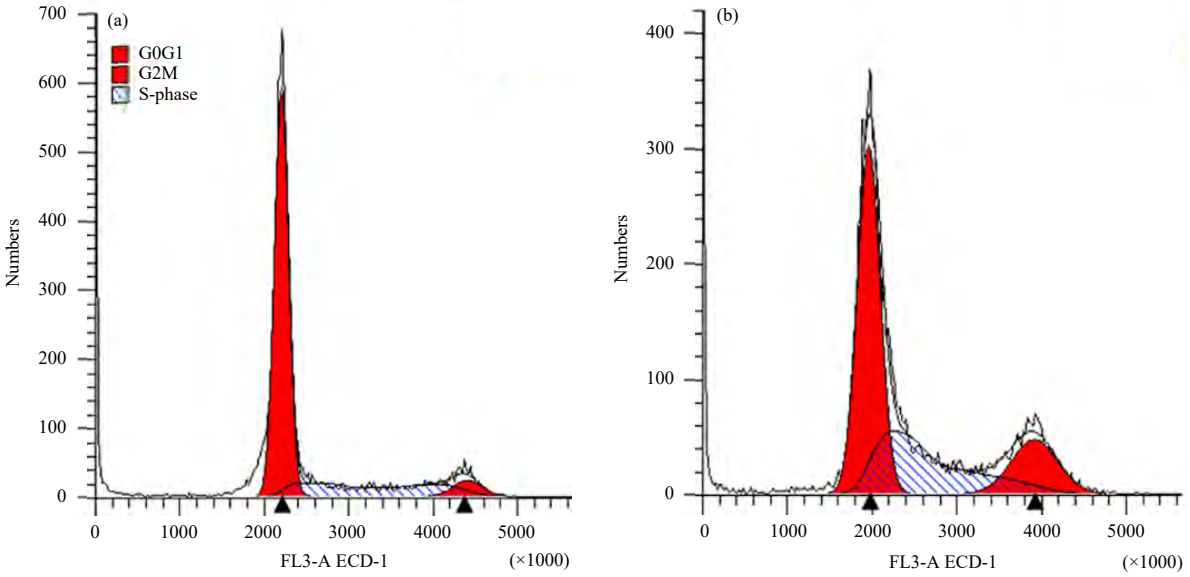

Fig. 6(a-b): Results of cell cycle detection exhibited that mitosis was enhanced and cells were more active in the (a) Control group compared with (b) NRG1 OE group

(Fig. 5a, b). Besides, the results of flow cytometry demonstrated that the NRG1 OE group had remarkably a higher proportion of cancer cells entering the active division stage (Fig. 6a, b). These results suggested that NRG1 is positively correlated with the enhancement of proliferation, migration and invasion abilities of malignant tumour cells.

DISCUSSION

According to the global statistics of female malignant tumours, the incidence rate of breast cancer ranks first, accounting for 28.7% of all new cases[10]. Meanwhile, the mortality rate of breast cancer ranks second among malignant tumours in women and breast cancer has been found in more

and more young women during physical examinations. Breast cancer is induced by many factors. Generally, it is considered that breast cancer is related to lifestyle, heredity, environment and drugs[11,12] and early diagnosis and timely treatment can help improve the survival rate of patients. In addition to the three basic treatments of breast cancer (surgery, radiotherapy and systemic chemotherapy), breast cancer can be classified according to the expression levels of estrogen receptor, progesterone receptor, HER2 and Ki67. The expression of hormone receptors not only determines the treatment method but also predicts the prognosis. Hence, hormone therapy is also currently one of the routine treatments for breast cancer. Due to fewer side effects, low toxicity and strong pertinence, molecular targeted therapy has been widely adopted in clinical treatment and become one of the research hotspots. In recent years, several molecular targeted drugs have been developed and achieved good results in clinical application. The most widely-used trastuzumab and pertuzumab mainly target the members of the EGFR family. Pertuzumab prevents the formation of functional dimers by binding to the ectodomain of HER2[13]. Hence, different from trastuzumab which can only target high expression of HER2, pertuzumab can additionally effectively inhibit homodimers or heterodimers of HER2. In the case of low expression or no expression of HER2, pertuzumab can still play an effective role[14]. In this study, the breast cancer 4T1 cell model was utilized to verify that NRG1 is also expressed in tumour cells and plays an important role in promoting the proliferation, migration or invasion of tumour cells. Such findings provide an idea for searching for molecular targeted therapy of breast cancer.

The NRG family consists of four family members, that is, NRG1, NRG2, NRG3 and NRG4, which are expressed in many tissues. NRGs contain EGF-like domains, so they also belong to the EGF family[6,7,15], which plays an important role in cell proliferation, growth and transformation. As a transmembrane protein, NRG1 has a typical transmembrane domain. The ectodomain of the transmembrane protein is sheared by metalloproteinases, resulting in the ectodomain shedding of the transmembrane protein. Ectodomain shedding is highly important for mediating the extracellular signal transduction into the cells, affecting a variety of biological functions of cells. Ectodomain shedding requires precise proteolysis (otherwise, excessive or insufficient shearing will trigger diseases), in which process many metalloproteinases are involved in, such as a-disintegrin-metalloproteinases (ADAMs), especially ADAM17[16]. After activation, ADAM17 highly expressed in many tissues[17-19] is up-regulated and transported from inside the cell to the cell surface. After reaching the functional site, ADAM17 catalyzes and changes the structure of the ectodomain in the following ways: (1) By degrading the extracellular matrix, it provides space for tumour vascular proliferation and facilitates angiogenesis. (2) It activates vascular EGF, fibroblast growth factor and chemokines, which are closely related to tumour invasion and metastasis. (3) It can also hydrolyze and shear the ligands of many kinds of EGFRs such as Tumour Necrosis Factor-α (TNF-α) and Transform Growth Factor-α (TGF-α) on the cell membrane[19,20] and activate the EGFR, PI3K/Akt, MEK/ERK and other signalling pathways[21], influencing the invasion and metastasis of cancer cells. In recent years, a lot of research achievements have also been made on NRG1 in the field of oncology and it has been confirmed in many studies that NRG1 fusion is closely related to the occurrence and development of tumours. Therapies targeting fusion genes have achieved good results in many malignant tumours. For example, Tyrosine Kinase Inhibitors (TKIs) significantly improve the survival rate of patients with chronic myeloid leukaemia. The TKIs target BCR-ABL1 tyrosine kinase produced by chromosome translocation. Currently, the widely used TKIs include imatinib, dasatinib and nilotinib. The NRG1 fusion protein can be either a cancer-initiating event or a subclonal event during the progression of targeted therapy. For example, McCoach *et al.*[22] found that, RALGAPA1-NRG1 fusion protein was a driver of secondary alectinib resistance in a lung cancer patient with ALK fusion. Jonna *et al.*[9] examined the incidence rate of NRG1 fusion in various solid tumours and found that the fusion mostly occurred in stage, more common in *in situ* tumours (68%) than distant metastasis (32%). The NRG1 fusion is the most common in non-small cell lung cancer and CD74-NRG1 is the most common fusion protein. Other fusion chaperone genes include SDC4, SLC3A2, TNC, MDK, ATP1B1, DIP2B, RBPMS, MRPL13, ROCK1, DPYSL2 and PARP8. Fusion genes in other types of solid tumours include COX10-AS1 and ADAM9 for breast cancer, SETD4, ZMYM2 and TSHZ2 for ovarian cancer, ATP1B1 and NOTCH2 for gallbladder cancer, ATP1B1, VTCN1 and CDH1 for pancreatic cancer, RBPMS for renal cell cancer, POMK for colorectal cancer, WHSC1L1 for sarcoma, GDF15 for urinary bladder cancer and HMBOX1 for nasopharyngeal neuroendocrine carcinoma. In this study, it was verified in breast cancer 4T1 cell lines that the malignant tumour cells with NRG1 OE had stronger proliferation and invasion abilities and reduced apoptosis, suggesting that the therapy targeting NRG1 may exert an effective anti-tumour effect. Kim's team found that the anti-HER3 targeted agent lumretuzumab was effective in two patients with NRG1 fusion and the progression-free remission period was 16 weeks after combined use of lumretuzumab and erlotinib[23]. Hence, NRG1 serving as a target has a bright application future in the treatment of solid tumours.

CONCLUSION

In summary, NRG1 can promote the malignant function of breast cancer cells by augmenting migration and invasion abilities. High expression of NRG1 markedly suppressed the apoptosis of breast cancer cells.

SIGNIFICANCE STATEMENT

This study discovers the role of NRG1 in breast cancer cells that can be beneficial for cancer treatment. This study will help the researcher to uncover the critical area of breast cancer therapy that many researchers were not able to explore. Thus, a new theory on the function of NRG1 as a treatment target for solid tumours may be arrived at.

REFERENCES

1. Harwansh, R.K. and R. Deshmukh, 2020. Breast cancer: An insight into its inflammatory, molecular, pathological and targeted facets with update on investigational drugs. Crit. Rev. Oncol. Hematol., Vol. 154. 10.1016/j.critrevonc.2020.103070.
2. Royce, T.J., G.P. Gupta and L.B. Marks, 2020. Breast conservation therapy versus mastectomy for breast cancer. Lancet Oncol., 21: 492-493.
3. Bellon, J.R., N. Tayob, D.D. Yang, J. Tralins and C.T. Dang *et al*., 2022. Local therapy outcomes and toxicity from the ATEMPT trial (TBCRC 033): A phase II randomized trial of adjuvant trastuzumab emtansine versus paclitaxel in combination with trastuzumab in women with stage I HER2-positive breast cancer. Int. J. Radiat. Oncol. Biol. Phys. 10.1016/j.ijrobp.2021.12.173.
4. Kim, H.G., C.K. Lee, S.M. Cho, K. Whang and B.H. Cha *et al*., 2012. Neuregulin 1 up-regulates the expression of nicotinic acetylcholine receptors through the ErbB2/ErbB3-PI3K-MAPK signaling cascade in adult autonomic ganglion neurons. J. Neurochem., 124: 502-513.
5. Hynes, N.E. and J.H. Dey, 2009. PI3K inhibition overcomes trastuzumab resistance: Blockade of ErbB2/ErbB3 is not always enough. Cancer Cell, 15: 353-355.
6. Iwakura, Y., R. Wang, N. Inamura, K. Araki and S. Higashiyama *et al*., 2017. Glutamate-dependent ectodomain shedding of neuregulin-1 type II precursors in rat forebrain neurons. PLoS ONE, Vol. 12. 10.1371/journal.pone.0174780.
7. Kim, J., A. Elias, T. Lee, P. Maurel and H.A. Kim, 2017. Tissue inhibitor of metalloproteinase-3 promotes schwann cell myelination. ASN Neuro, Vol. 9. 10.1177/1759091417745425.
8. Pan, Y., Y. Zhang, T. Ye, Y. Zhao and Z. Gao *et al*., 2019. Detection of novel NRG1, EGFR, and MET fusions in lung adenocarcinomas in the Chinese population. J. Thoracic Oncol., 14: 2003-2008.
9. Jonna, S., R.A. Feldman, J. Swensen, Z. Gatalica and W.M. Korn *et al*., 2019. Detection of NRG1 gene fusions in solid tumors. Clin. Cancer Res., 25: 4966-4972.
10. Islami, F., A.G. Sauer, K.D. Miller, R.L. Siegel and S.A. Fedewa *et al*., 2018. Proportion and number of cancer cases and deaths attributable to potentially modifiable risk factors in the United States. CA: Cancer J. Clin., 68: 31-54.
11. Harbeck, N., F. Penault-Llorca, J. Cortes, M. Gnant and N. Houssami *et al*., 2019. Breast cancer. Nat. Rev. Dis. Primers, Vol. 5. 10.1038/s41572-019-0111-2.
12. Sancho-Garnier, H. and M. Colonna, 2019. Breast cancer epidemiology. La Presse Méd., 48: 1076-1084.
13. Franklin, M.C., K.D Carey, F.F. Vajdos, D.J. Leahy, A.M. de Vos and M.X. Sliwkowski, 2004. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. Cancer Cell, 5: 317-328.
14. Agus, D.B., R.W. Akita, W.D. Fox, G.D. Lewis and B. Higgins *et al*., 2002. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. Cancer Cell, 2: 127-137.
15. Falls, D., 2003. Neuregulins: Functions, forms, and signaling strategies. Exp. Cell Res., 284: 14-30.
16. Willem, M., 2016. Proteolytic processing of Neuregulin-1. Brain Res. Bull., 126: 178-182.
17. Mustafi, R., U. Dougherty, D. Mustafi, F. Ayaloglu-Butun and M. Fletcher *et al*., 2017. ADAM17 is a tumor promoter and therapeutic target in western diet–associated colon cancer. Clin. Cancer Res., 23: 549-561.
18. Baumgart, A., S. Seidl, P. Vlachou, L. Michel and N. Mitova *et al*., 2010. ADAM17 regulates epidermal growth factor receptor expression through the activation of notch1 in non–small cell lung cancer. Cancer Res., 70: 5368-5378.
19. Gao, M.Q., B.G. Kim, S. Kang, Y.P. Choi, J.H. Yoon and N.H. Cho, 2013. Human breast cancer-associated fibroblasts enhance cancer cell proliferation through increased TGF-α cleavage by ADAM17. Cancer Lett., 336: 240-246.
20. Scheller, J., A. Chalaris, C. Garbers and S. Rose-John, 2011. ADAM17: A molecular switch to control inflammation and tissue regeneration. Trends Immunol., 32: 380-387.
21. Zheng, X., F. Jiang, M. Katakowski, Y. Lu and M. Chopp, 2012. ADAM17 promotes glioma cell malignant phenotype. Mol. Carcinog., 51: 150-164.
22. McCoach, C.E., A.T. Le, K. Gowan, K. Jones and L. Schubert *et al*., 2018. Resistance mechanisms to targeted therapies in *ROS1*$^{+}$ and *ALK*$^{+}$ non–small cell lung cancer. Clin. Cancer Res., 24: 3334-3347.
23. Kim, H.S., J.Y. Han, D.H. Shin, K.Y. Lim and G.K. Lee *et al*., 2018. EGFR and HER3 signaling blockade in invasive mucinous lung adenocarcinoma harboring an NRG1 fusion. Lung Cancer, 124: 71-75.