Original paper

Folia Neuropathologica

Neuregulin 2 (NRG2) is expressed in gliomas and promotes migration of human glioma cells

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Folia Neuropathol 2021; 59 (2): 189-197 *DOI: https://doi.org/10.5114/fn.2021.106460*

Abstract

Introduction: *Glioma is the most common primary brain tumour in adults. Numerous studies have shown that neuregulins (NRGs) may be involved in the formation of glioma. Although NRG1 has been extensively studied in glioma, the functions of NRG2 in glioma development remain elusive.*

Material and methods: *In the present study, we investigated the expression of NRG2 in different grades of human glioma samples, and analysed the functional effects of NRG2 in glioma cells mainly using wound healing assay and transmigration assay.*

Results: *We found that NRG2 was differentially expressed in different grades of human glioma/glioblastoma tissues. The data from wound healing assays demonstrated that NRG2 can differentially promote the migration of SHG44 human glioma, and U251 and U-87 MG human glioblastoma cells at different time points. The results of cell transmigration assays showed that, compared with the vehicle control, the number of cells that migrated to the underside of the insert was increased significantly for all the 3 cell lines treated with 5 nM of NRG2 for 12 hours.*

Conclusions: *In conclusion, our results demonstrated that NRG2 is expressed in gliomas to varying extents, and it may play roles in the migration of glioma cells in vitro. These data suggest that treatment targeting NRG2 signalling may partly reverse the migration-based metastasis of glioma cells.*

Key words: *glioma, glioblastoma, neuregulin 2 (NRG2), migration, wound healing assay.*

Introduction

Malignant gliomas are the most common primary malignant tumour type in the nervous system. They account for ~70% of human primary malignant brain tumours with a poor survival prediction [27]. Gliomas are histologically derived from abnormal proliferative glial cells [23]. Despite the application of combined glioma management consisting of surgical resection, radiotherapy, and chemotherapy [5], the overall survival rate remains limited due to potential recurrence [27]. Migration-based invasion and metastasis constitute the most devastating difficulties in the treatment of glioma. Therefore, it is imperative to identify the pathogenesis and regulation mechanism, including the role of cytokines, in the development and progression of gliomas.

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Cytokines have been reported to play pivotal roles in tumour biology [17,18,26]. Among these cytokines, neuregulins (NRGs) have been reported to be associated with the malignancy of a wide spectrum of tumours. Neuregulins belong to the epidermal growth factor (EGF) superfamily and they are a class of polypeptide-like nerve growth factors that regulate cell growth and differentiation. NRG family proteins all contain similar domains, including the immunoglobulin-like loop structures, the EGF-like domain, the transmembrane domain, and the intracellular domain of varying lengths. Four main subtypes of neuregulin, including NRG1, NRG2, NRG3, and NRG4, have been identified. Among them, NRG1, also known as differentiation factor (Neu differentiation factor – NDF), heregulin (HRG), glial growth factor (GGF), acetylcholine receptor-inducing activity (ARIA), and sensory and motor neuron-derived factor (SMDF) are the major members of the NRG family [14].

NRGs can bind and activate the members of the ErbB tyrosine kinase receptor family, which is composed of ErbB1-ErbB4. The activation of ErbB receptors can initiate a variety of downstream signalling transduction pathways responsible for cellular proliferation and differentiation, apoptosis, migration, and adhesion [1,29]. Ritch *et al*. [22] demonstrated that glioma cell survival is enhanced *via* either an autocrine or a paracrine NRG1/ErbB receptor signalling pathway. We previously demonstrated that NRG1 is highly expressed in low- to middle-grade gliomas, in which it can regulate the expression of L1 cell adhesion molecule and close homologue of L1 (CHL1) [11,31] for the migration and metastasis of glioma cells. However, the role of NRG2 in glioma malignancy is still poorly understood.

Neuregulin 2 has been identified as the second member of the neuregulin family, and it is structurally similar to NRG1 [15,16,28]. Although both NRG1 and NRG2 are strongly expressed in the early development of the brain, especially in the germinal layer, they are expressed in different cell populations, showing differential subcellular distribution [3,12]. This unique temporal and spatial expression pattern of NRG2 suggests that it may function differentially both physiologically and pathologically. Astrocyte-derived NRG2 can bind to neuronal ErbB3 receptor, thus promoting neuronal survival and axon extension *in vitro* [15]. Our study is intended to investigate the role of NRG2 in the migration of glioma cells by using wound healing assay and transmigration assay, which may facilitate our understanding of its roles in glioma malignancy.

Material and methods

Cells and cell culture

The human glioblastoma U-87 MG cell line (cat. no. CL-0238) and the human glioma U251 cell line (cat. no. CL-0237) were provided by Procell Life Science and Technology Co., Ltd. The human glioma cell line SHG44 (cat. no. SHG44) was obtained from Guangzhou Jennio Bioech Co., Ltd. All cell lines were authenticated using short tandem repeat analysis. Human SHG44 and U251 glioma cells were maintained in Dulbecco's Modified Eagle's medium (HyClone; Thermo Fisher Scientific, Inc.) supplemented with 50 U/ml penicillin/streptomycin mixture (Beijing Solarbio Science and Technology Co., Ltd.) and 10% foetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.), and human U-87 MG glioblastoma cells maintained in RPMI 1640 medium (HyClone; Thermo Fisher Scientific, Inc.) containing 10% foetal bovine serum and 50 U/ml penicillin/streptomycin mixture. The cells were routinely cultured in 75 cm^2 cell culture plates (Corning Inc.) at 37˚C in a humidified incubator with 5% CO_2.

Immunohistochemical staining

Immunohistochemical staining of paraffinized sections was performed as previously described [11]. Human glioma tissue microarray sections (5 µm thick; cat. no. DS-Bra01027, Avila Biotech, Xi'an, Shaanxi, China) were deparaffinized and rehydrated through a graded ethanol series from 100% to 70%. Antigen retrieval was performed using 10 mM citrate buffer (pH 6.0) at 99˚C for 1 h, and endogenous peroxidase was cleared by incubation in 3% H_2O_2 at room temperature for 10 min. The sections were then blocked with 10% normal goat serum in PBS at room temperature for 10 min, and the samples were incubated at 4˚C overnight with a mouse-anti-human NRG2 antibody (1 : 50; cat. no. SC-398594, Santa Cruz Biotech, Santa Cruz, CA, USA). The antigen-antibody complexes were visualized using the avidin-biotin-peroxidase complex method using an AEC kit (cat. no. ZLI-9036, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.). The NRG2 staining intensity level on each tissue point was evaluated as the aver-

age grey value ranging from 0 to 255 using Image-Tool (IT) software (version 3.0; Department of Dental Diagnostic Science at the University of Texas Health Science Center [UTHSCSA]; original magnification, 400×], which was used to indicate the protein level of NRG2 in the cancer adjacent normal (CAN) tissue and glioma tissues graded from I to IV.

Wound healing assay

SHG44 and U251 human glioma and U-87 MG human glioblastoma cells were routinely cultured until the cell density was close to 90%. Cells were then treated with 0.25% trypsin-EDTA for digestion. Digested cells in the serum-containing medium were centrifuged at 900 rpm for 3 min. The medium was then discarded, and cells were resuspended in a fresh serum-containing medium. Cells were seeded onto the 96-well plate at a density of 5×10^4 cells per well. After routine culture overnight, fully seeded cells were scratched using a sterile 10 µl micropipette tip to create a wound ±500 µm across the monolayer. The culture medium was subsequently replaced with 100 µl of serum-free medium containing recombinant NRG2β (rNRG2β; cat. no. NRG2-363H Human, Creative BioMart, Shirley, NY, USA) dosed at 0, 2, 5, and 10 nM. The migration rate varied with the tumour malignancy. For U-87 MG cells, wound healing was observed at 0, 6, 12, and 24 h time points. For U251 cells, wound healing was observed at 0, 12, and 24 h time points, and for SHG44 cells at 0, 24, and 48 h time points. The migration rate was evaluated using the formula healing rate = (initial scratch area – final scratch area)/initial scratch area × 100%.

Transwell cell migration assay

Since NRG1 has been reported to potentiate the migration of neuronal cells in our previous report [31], we hypothesized that NRG2 may also promote glioma cell migration. We then tested the potential of NRG2 for inducing glioma/glioblastoma cell migration using the transwell cell migration assay. For the transwell migration assay, all the 3 cell lines were pretreated individually with either vehicle control or rNRG2β (5 nM) in serum-free culture medium for 12 h. Then, the culture medium was aspirated, and the cells were resuspended in DMEM and seeded onto the upper chamber (1×10^5/well) of each transwell insert consisting of a filter (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 8 µm pores. The underside of the membrane was pretreated with 100 µg/ml of fibronectin. The lower chamber was loaded with 500 µl of either DMEM or RMPI 1640. Eighteen hours after plating, the cells that had failed to move to the underside of the transwell membrane were removed using a cotton-tipped applicator. The retained cells were rinsed 3 times with PBS and fixed in 4% paraformaldehyde solution in PBS. The migrated cells were stained with 0.1% crystal violet in acetic acid for morphological observation [31]. Stained cells were photographed using a widefield microscope (IX51, Olympus Corporation, Tokyo, Japan) at 100× magnification under a bright field. The number of cells in each field was counted using Image Tool software.

Statistical analysis

In vitro experiments were repeated at least 3 times using independent culture preparations. All numerical data are presented as the group mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad prism 9.0 software and plotted with GraphPad prism 9.0 software. The comparison between the CAN and each glioma/glioblastoma tissue group in NRG2 staining intensity, and the comparison between the vehicle control and treatment groups in transmigration assay was performed by independent Student's *t*-test. The comparison among multiple groups in wound healing assay was performed by one-way analysis of variance (One-Way ANOVA) and Tukey's test. P values < 0.05 were considered statistically significant.

Results

Immunohistochemical staining of NRG2 in the glioma tissue microarray

Human tissues from a commercial glioma tissue microarray were subjected to immunohistochemical staining of NRG2. Representative staining from the CAN tissue and tissues of different grades is shown in Fig. 1. NRG2 was weakly stained in the CAN tissue, where it was mainly localized in morphologically microglia-like cells. In contrast, enhanced NRG2 staining signals were detected in more cells in glioma/glioblastoma tissue samples graded from I to IV (Fig. 1). As was indexed by the average grey value, the staining intensities of NRG2 in I-II and III-grade glioma tissue samples were both significantly higher

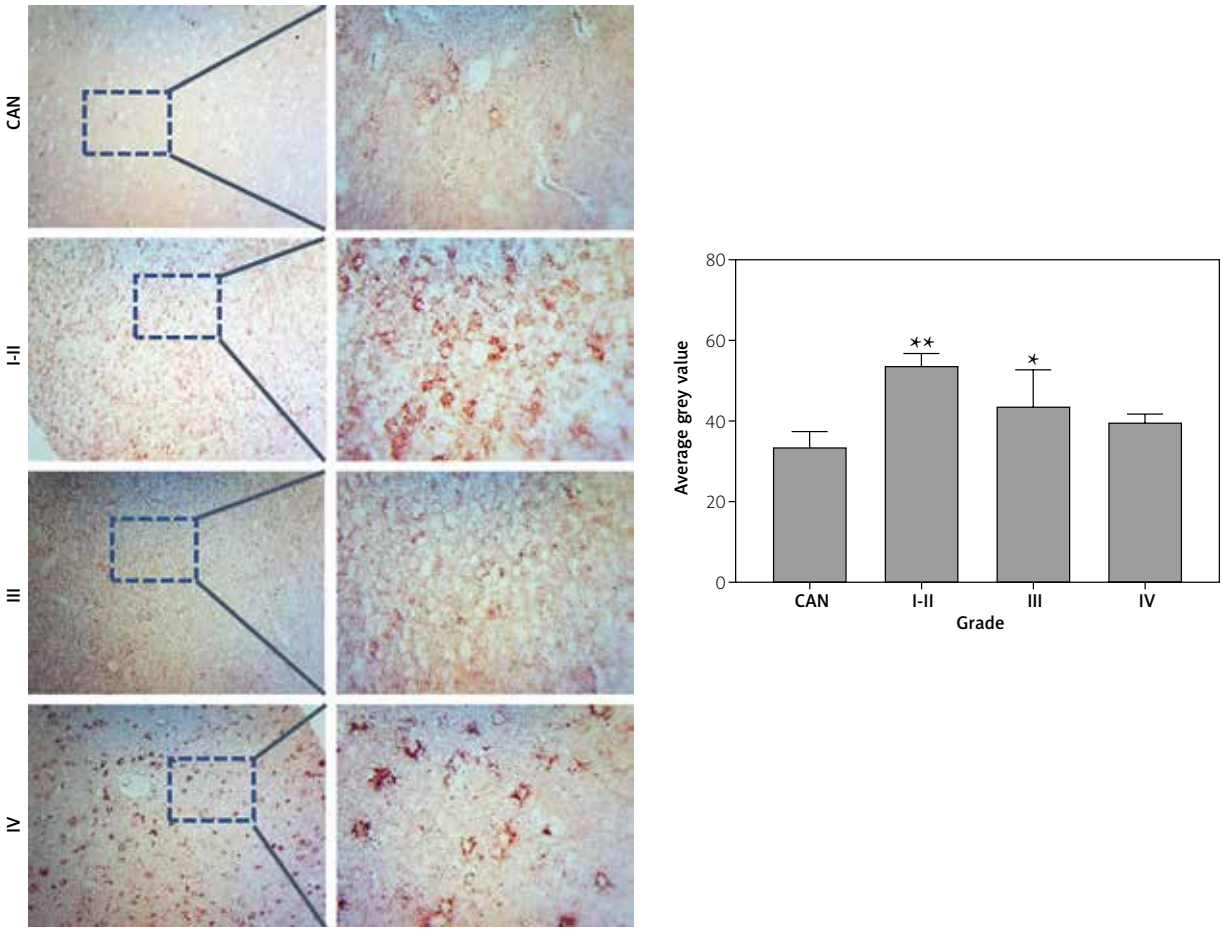

Fig. 1. Immunohistochemical staining of NRG2 in cancer adjacent normal tissues and glioma/glioblastoma tissues graded from I to IV. Independent Student's t-test was used to detect statistical differences between each grade of glioma tissue group and the cancer adjacent tissue (CAN) group. Data are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$, vs. the CAN tissue group.

than that in CAN tissues ($p < 0.01$ for grade I-II and $p < 0.05$ for grade III) (Fig. 1). No significance was found for the staining intensity in grade IV glioma tissue samples when compared to CAN tissue samples, although the average staining intensity was higher than that in CAN tissues ($p = 0.11$) (Fig. 1).

The wound healing effect of NRG2 in scratched glioma/glioblastoma cells

At the time point of 6 h, NRG2 showed no significant effect on the migration of U-87 MG cells at each dose. At 12 h, NRG2 can slightly but significantly enhance the migration of cells at both 5 nM and 10 nM ($p < 0.01$ and $p < 0.05$ vs. control for 5 nM and 10 nM, respectively). However, migrated cells covered the scratched area in each group at 24 h, suggesting the pronounced wound healing ability of U-87 MG cells as one of the most malignant glioblastoma cell lines (Fig. 2A).

We then tested the effect of NRG2 on the wound healing ability of U251 cells, a glioma cell line with less malignancy. Our results demonstrated that NRG2 significantly promoted the migration of cells at doses of 2 nM and 5 nM at the time point of 12 h ($p < 0.05$ and $p < 0.01$ vs. control for 2 nM and 5 nM, respectively). At the time point of 24 h, all doses of NRG2 significantly enhanced the migration of scratched cells, with the most pronounced effect observed in the 2 nM group ($p < 0.01$ vs. control for all groups at 24 h) (Fig. 2B).

Finally, we tested the effect of NRG2 on the wound healing ability of SHG44 cells, the least

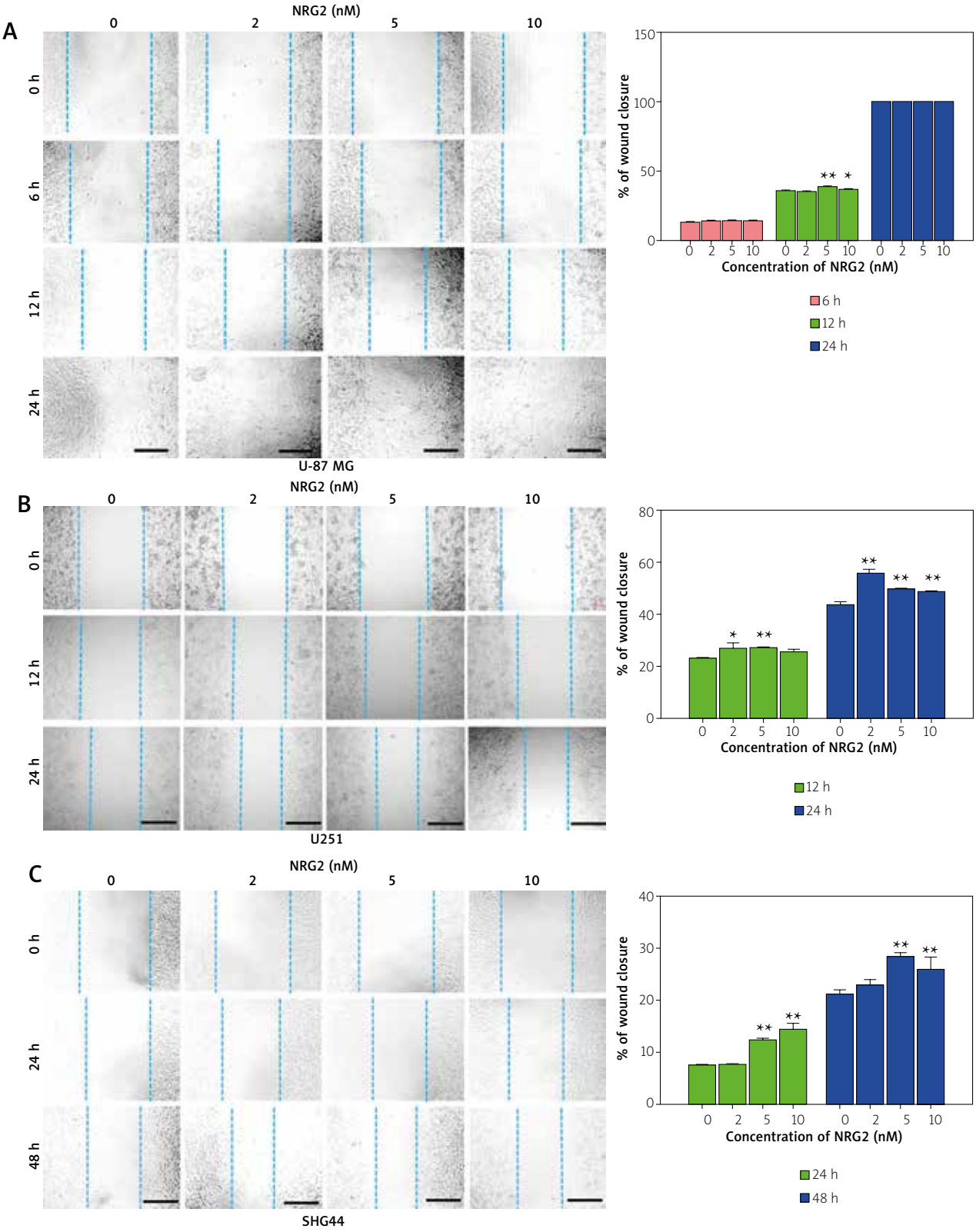

Fig. 2. Scratch wound healing assay to evaluate time- and dose-dependent regulation of cell migration in response to NRG2 in glioma/glioblastoma cells. U-87 MG (**A**), U251 (**B**) and SHG44 (**C**) cells were treated with human recombinant NRG2β (rNRG2β) at concentrations of 0, 2, 5, and 10 nM, respectively. Wound scratch was induced by using a 10 µl micropipette tip, and the scratch was photographed at the designated time points. The experiment was performed from three independent sample experiments. One-way ANOVA was used to detect statistical differences between control group and each NRG2-treated group. Data are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$, vs. control. Scale bars, 250 µm; original magnification, 100×.

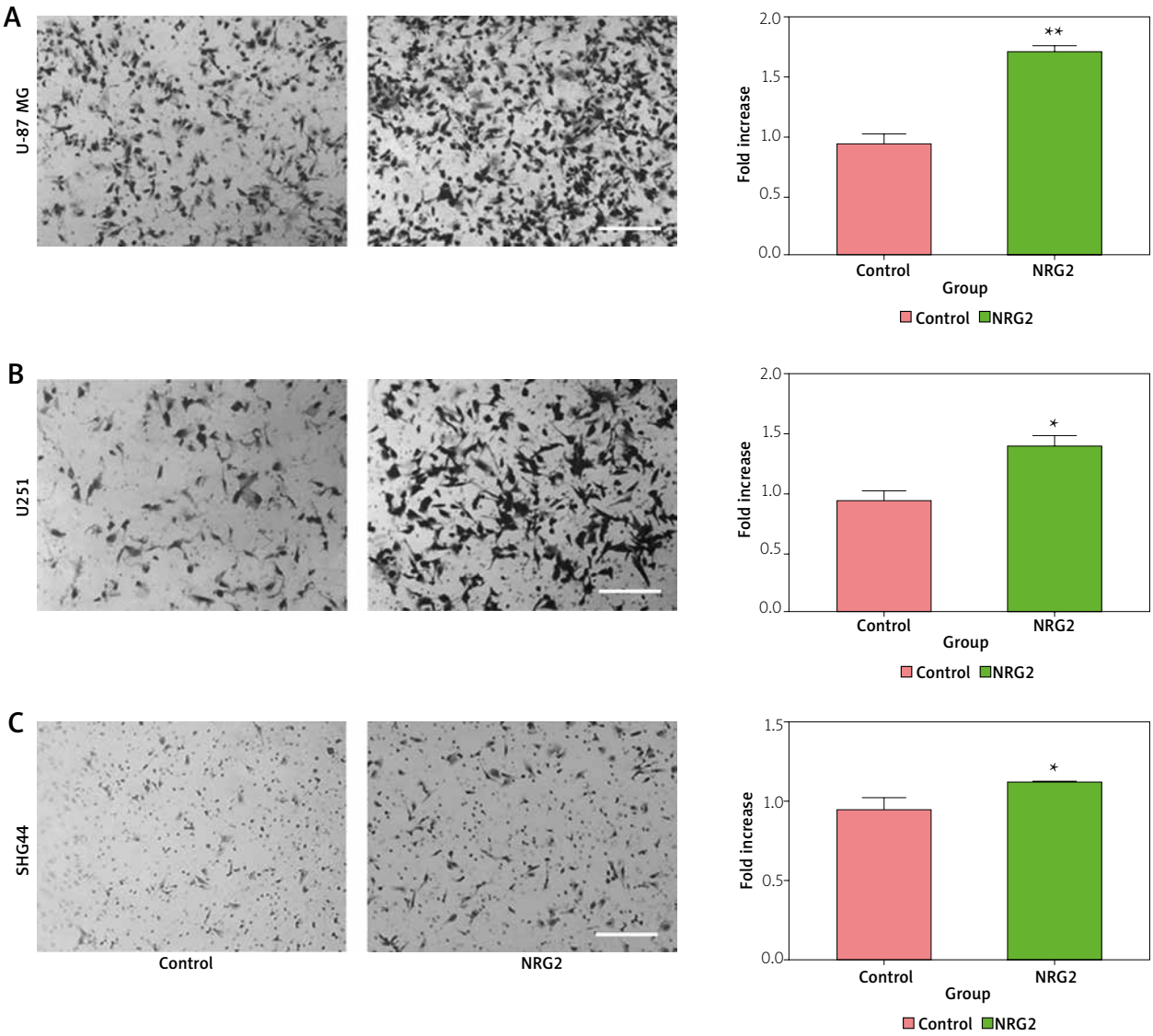

Fig. 3. The effect of NRG2 on the migration of glioma/glioblastoma cells by cell transmigration assay. Cells transmigrated to the underside of the membrane were fixed using 4% paraformaldehyde and stained with 0.1% crystal violet overnight. Pretreatment of NRG2 significantly increased the transmigration ability of U-87 MG (**A**), U251 (**B**), and SHG44 (**C**) cells, as was indicated by the number of crystal violet stained cells attached to the underside of the membrane. The experiment was performed from 3 independent sample experiments. Independent Student's *t*-test was used to detect statistical differences between control group and the NRG2-treated group. Data are expressed as mean ± SEM. *$p < 0.05$ and **$p < 0.01$, vs. control. Scale bars, 200 µm; original magnification, 100×.

malignant among all the 3 cell lines. The scratched SHG44 cells showed no apparent migration until 24 h, at which point pronounced wound closure was found in cells treated with NRG2 at 5 and 10 nM ($p < 0.01$ vs. control for both groups). A similar migration promotion effect was found in cells treated with 5 and 10 nM at the time point of 48 h ($p < 0.01$ vs. control for both groups) (Fig. 2C).

Cell transmigration assay of NRG2

Based on the results of the scratch wound healing assay test, we used cell transmigration assay to further verify the effect of NRG2 on the migration of glioma/glioblastoma cells. All the 3 cell lines were pretreated with 5 nM of rNRG2β for 12 h before the transmigration assay was performed. Our results demonstrated that at the time point of 18 h, the number of transmigrated NRG2-treated glioma/glioblastoma cells stained by crystal violet was significantly increased in comparison to that without treatment ($p < 0.01$ vs. control for U-87 MG cell, and $p < 0.05$ vs. control for both U251 and SHG44 cells) (Fig. 3).

Discussion

The invasion and migration of tumour cells is the fundamental reason for the difficulty in radiological treatment and surgical resection glioma/glioblastoma [19]. According to the histopathological origin, glioma can be subdivided into astrocytoma, astroblastoma, and oligodendroglioma and glioblastoma, etc., among which glioblastoma is the most malignant type (grade IV, World Health Organization [WHO]), and its incidence increases with age [13]. Although great improvements have been achieved, the treatment of malignant glioma remains a challenge. To elucidate the regulation mechanism of the occurrence and development of glioma has become one of the research hotspots of glioma management. In the present study, we investigated the expression of NRG2, one member of the NRG family, in human glioma/glioblastoma samples, as well as its role in regulating the migration of glioma cells, which may provide therapeutic clues for further study of glioma treatment and therapeutic drug screening.

Neuregulins (NRGs) act as important signalling proteins that mediate cell-cell interactions in the nervous system, heart, breast, and other organ systems [6]. The "Forward" signalling from NRG-producing cells to NRG-responsive cells involves the binding of NRG to the extracellular domain of the receptor tyrosine kinase ErbB3 or ErbB4, thus resulting in either homodimerized or heterodimerized ErbB receptor formation [6]. NRG-ErbB receptor-based intercellular signalling is a fundamental feature of multicellular organisms, and it plays an important role in developmental diversification and mediation of different cellular homeostasis [8]. NRG1 is the most investigated form among all the NRG members. NRG1 (also known as modulator, NDF, GGF, and ARIA) is a family of membrane-bound or secreted proteins produced by neurons and mesenchymal cells that have multiple effects on a variety of cell types [10].

Ritch *et al*. found that autocrine or paracrine NRG1/ErbB receptor signalling can enhance the survival of glioma cells, which is dependent on the activation of ErbB2 receptor and phosphatidylinositol-3 kinase (PI3K) [22]. ErbB receptor activation by NRG1 enhances cell motility, which involves the activation of focal adhesion kinase, suggesting that NRG1 plays an important modulatory role in glioma cell invasion [21]. Recent research demonstrated that miR-125a-3p can perform an inhibiting role in glioma development by directly targeting the expression of NRG1, further supporting NRG1 as a potential target for the diagnosis and treatment of malignant glioma [30]. NRG1 can regulate L1 expression as an upstream early signalling molecule and promote cell migration, suggesting that blocking the NRG1 signalling pathway may reduce L1 expression in glioblastoma and inhibit tumour invasion and recurrence [31,32]. Unlike previous reports showing that the highest expression of both NRG1 isoforms were found in grade III and IV glioma/glioblastoma tissues [31], we observed the highest expression of NRG2 in lower grade glioma tissues. These observations suggest that NRG2 may function differentially in glioma development and progression.

The role of NRG2 in tumour cell migration has rarely been depicted. Intraventricular infusion of NRG2 can increase the number of Sox2 and GFAP-positive precursors in the subventricular zone (SVZ) in the adult mouse brain, leading to an increase in the number of newly generated migrating neuroblasts in the olfactory bulb [7]. Buonanno and Fischbach [2] compared the structure and expression pattern of NRG2 to the NRG1 protein and found that NRG2 triggers a different receptor phosphorylation activation mode by specifically activating homodimerized ErbB4 receptors [4,25]. In addition, direct binding of NRG2β to ErbB3 and ErbB4 can transactivate ErbB2 and lead to the activation of PI3K signalling pathway [15,24]. Although NRG2α is derived from the splicing isoform of the same gene also encoding NRG2β, it cannot function as an efficient ErbB4 agonist [9]. Based on these data, rNRG2β was used for the migration evaluation of glioma cells. The results of scratch wound healing assay showed that NRG2 can differentially promote

the migration of U-87 MG, U251, and SHG44 cells. The migration-promoting effect of NRG2 is more pronounced in U251 and SHG44 cells than in U-87 MG cells. We hypothesize that glioblastoma-derived U-87 MG cells can express and secret more NRG2 for migration in both autocrine and paracrine manners. This may partly counteract the effect of exogenous NRG2. Cell kinase-mediated signalling pathways, including PI3K/Akt/mTOR and Ras/MEK/MAPK pathways, can enable the growth, motility, and invasion of glioma cells [20]. Thus, targeting NRG2 may serve as a therapeutic means against glioma.

Conclusions

In summary, our results demonstrated that NRG2 can affect the migration of glioma/glioblastoma cells and significantly increase the rate of cell migration of gliomas. These results indicate that NRG2, like NRG1, can function by promoting the migration of glioma of different grades. In summary, this study lays a theoretical foundation and provides a useful reference value for the management of glioma by targeting NRG2.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81471279 and 81171138 to WZ). Research start-up fund of Jiangnan University (1285081903200020 to WZ). Research start-up fund of Wuxi School of Medicine, Jiangnan University (1286010242190060 to WZ).

Disclosure

The authors report no conflict of interest.

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