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MicroRNA-100 Inhibits Migration of Glioblastoma Cancer Stem Cells and Reduces the Nuclear Orphan Receptor Family member 77, Nur77

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ABSTRACT

Glioblastoma multiforme (GBM) is the most fatal and most progressive malignant human glioma. In spite of top and excellent standard of care which include total resection, radiation, and chemotherapy, the median survival did not exceed two years. Many microRNAs (miRNAs) have been reported recently as important for stimulating or restraining GBM growth. We previously demonstrated that GBMs possess low expression levels of miR-100 relative to control tissue and that restoring high expression repressed GBM tumorigenesis. It is also known that cancer stem cells are resistant to conventional therapy. In this project, we report that miR-100 internal expression is low in GBM cancer stem cells (CSCs) for both U87 derived CSCs and patient derived primary CSCs. Rescue miR-100 expression decreased CSCs migration ability in all samples. Furthermore, overexpression of miR-100 reduced Nur77 (nuclear orphan receptor family member 77) protein expression which play a major role migration. This finding may contribute to less tumor propagation and better outcome.

Keywords

NR4A1/ Nu77
microRNA
miR
GBM stem cells
Tumor initiating cells

INTRODUCTION

Glioblastoma multiforme (GBM) is the most-deadly form of brain cancer. It accounts for more than 50% of all recognized brain tumors [1, 2]. Every year, more than 15,000 new patients are diagnosed with GBM and CNS malignancies in the USA, and most have an inferior prognosis, with median survival predicted to be 15 months or less [3, 4]. Although tremendous efforts have been made in providing GBM patients with the most effective treatments, including surgery, radiotherapy, immunotherapy and targeted therapy, the idiopathic aspects of the disease, including chemoresistance and metastasis to the surrounding brain tissues, are significant barriers to solve in order to achieve a better prognosis or outcomes in GBM patients [5].

MicroRNAs (miRNAs) are a group of noncoding, short-sequence RNA molecules (16-22 nucleotides long) that post-transcriptionally suppress gene expression by binding to the 3′-untranslated region (UTR) of targeted genes to induce gene or protein degradation [6]. Among the numerous cancer-regulating miRNAs, microRNA-100 (miR-100) has shown to be crucial in the development, diagnosis, and treatment of cancer. miR-100 has been linked to multiple targets that are known to modulate GBM growth and survival, such as fibroblast growth factor receptor 3 (FGFR3), silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), and ATM Serine/Threonine Kinase (ATM; ataxia telangiectasia mutated) [7-9] . Previously, we showed that GBMs express a lower level of miR-100 compared to control tissues and that overexpression of miR-100 in glioblastoma cells reduces tumorigenicity. We also demonstrated that miR-100 decreases cell proliferation and increases the survival of mice bearing

Short communication

orthotopic GBM xenografts via stopping the miR-100 target, SMRT/NCOR2 [7]. Here, we report that miR-100 levels significantly decreased in all tested tumor samples compared to the primary human neural stem cells (hNSCs). Additionally, we show that miR-100 overexpression reduces cell migration of human cancer stem cells (CSCs) derived from glioblastoma cell lines (U87).

MATERIALS AND METHODS

Isolation of patient cancer stem cells

Human tumor specimens were collected after the patient's informed consent and with approval of the Ministry of National Guard-Health Affairs Institutional Review Board (RC13/258/R). Patient-derived cancer stem cells were isolated from GBMs and validated as previously described [10-13]. Tumor tissue transferred to the lab from the operating room was chopped using a scalpel and then homogenized through DNAse (LS006361, Worthington, USA), and collagenase IV (LS004186, Worthington, USA). The Homogenized cells were filtered with cell strainer, 70um, and plated in growth media that contain Dulbecco's modified Eagle medium-high glucose-F12 (11330-032, Gibco), 20% Neuroplex (600-301, Gemini, USA). 1X B27 supplement (12587010, Gibco), 1% penicillin-streptomycin-amphotericin (15240-062; Gibco), and 20 ng/ml epidermal growth factor -EGF- (PHG0311, ThermoFisher), and 20 ng/ml basic fibroblast growth factor -bFGF- (CC-4065J; Lonza, Basel, Switzerland). U87 purchased from ATCC (ATCC- HTB-14, USA) was transformed to U87 (CSCs) as previously described [14]. A tumor-free neural stem cell (hNSC) purchased from Merck, Germany.

Quantitative RT-PCR

Absolute quantitation with real time PCR (7500 real time, Applied Biosystem) was performed according to the recommended protocol and as previously described [7]. RNA isolation kit, probes and primers, were ordered from Life Technologies. 30ng of RNAs were used per reaction and the control (housekeeping RNA) was 18s. The data calculated through ΔΔCT method.

Transfection of microRNA

Previously published procedure was used [7]. microRNA-100 (has-miR-100 #4427975) and scramble (miR control) were purchased from Life Technologies. All transfections were done according to the provided protocol using PepMute (Catalogue #SL100566; SignaGen Laboratories, MD, USA). Scramble and miR-100 were used at 12 pmoles per 500k cells. The achieved transfection efficiency was previously reported [7].

Transwell-based migration assay

Fifty thousand cells were inoculated on transwell (140629; ThermoFisher, USA) as previously described [15]. Cells attracted with complete growth media added to the bottom side of transwell. The Topside of the transwell which contained the seeded cell had no attractant in it and cells were added with DMEM only. All the transwells' plates were incubated for 5 hrs at 37°C then bottom parts were fixed with methanol and stained with DAPI. Later, all samples were imaged with fluorescent microscope (EVOS FL auto reader) and cells were counted automatically.

Protein Extraction and Immunoblotting (Western Blot)

A minimum of 500K cells were used per assay. Immunobloting protocol was published previously [7]. The control scramble cells from U87 and patient CSCs were lysed, and the proteins were quantified. Likewise, miR-100 transfected cells were lysed, and the proteins were quantified. Immunoblot analysis was performed by loading 20µg of protein samples on an SDS-page of 4–12% gels (Catalogue #NP0322BOX; Life Technologies). The Transfers were performed on wet transfer cells (Bio-Rad) with PVDF membranes (LC2001; EMD Millipore). The antibodies used were anti-β-actin, internal control (Catalogue #3700, Cell Signaling Technology, USA), Nu77 (Catalogue #3960, Cell Signaling Technology, USA), Secondary antibodies were goat anti-Rabbit (Catalogue # G-21234, Li-Cor, USA)

Immunoblot Protein expression of Nur77, members of NR4A nuclear orphan receptor family member 77 (Nur77) which also known as nerve growth factor IB

Statistical Analysis

Statistical analyses were done by unpaired Student's t test, using Graphpad Prism5 (Graphpad Prism Software, Inc, USA). Bar graphs represent the means ± SEM (standard error of the mean) Significance level was established at (*) $P < 0.05$. All experiments were performed in triplicate.

DISCUSSION

The involvement of miR-100 expression in limiting tumor progression in GBM tumors was reported previously. However, miR-100 activities within GBM CSCs were not completely understood. It is known that Cancer stem cells are resistant to radio/chemo treatment [16]. In this study, miR-100 level in context of CSCs was investigated. Similar to others, CSCs isolation and culture show sphere-like shapes holding a group of cells, Figure 1A. The level of miR-100 was significantly ($p < 0.05$) low compared to hNSC control, Figure 1B. The internal level of miR-100 expression found less only in malignant neural stem cells. This observation may indicate a potential role in cancer progression. A major problem with malignant CSCs is migration to other organs or other sites within the same organ besides resistance to chemotherapy.

Consequently, both U87 and patient CSCs migration abilities

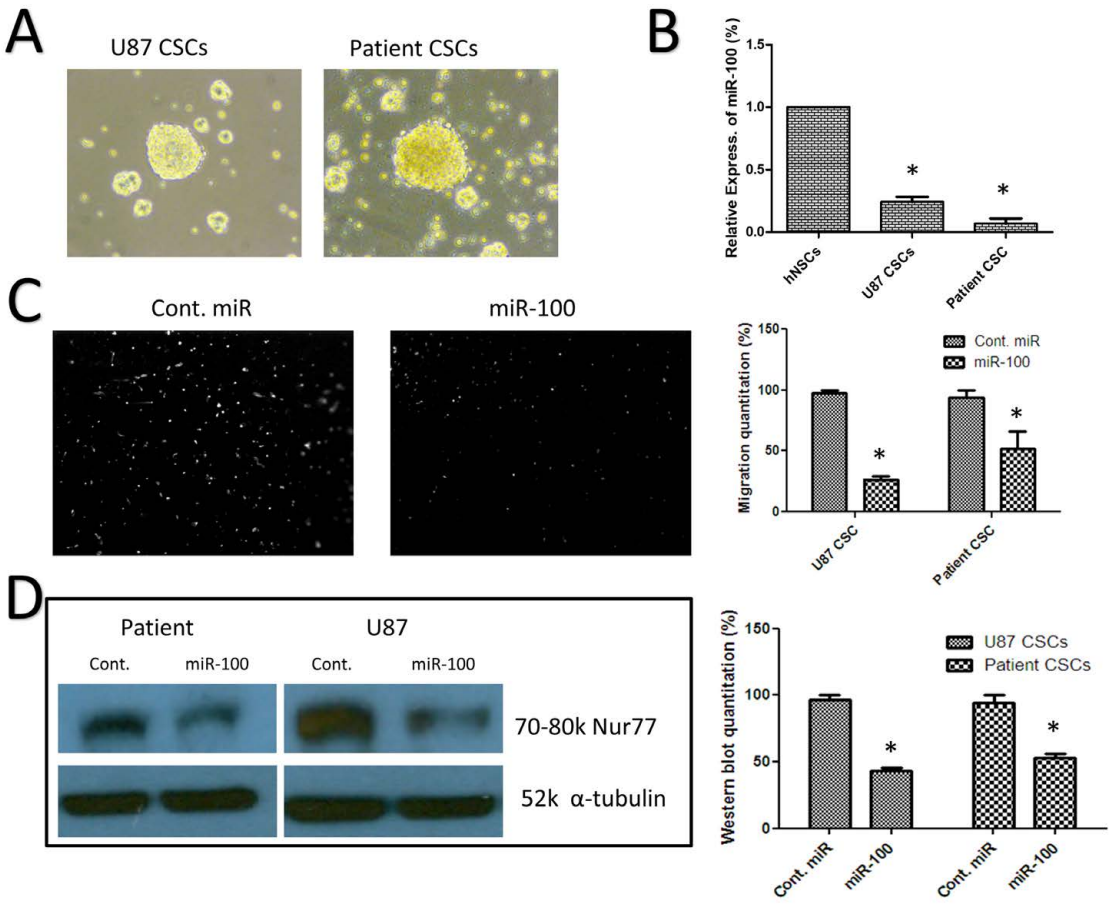

Figure 1: miR-100 ability to reduces migration activity in GBM CSCs

A, depiction shows sphere like shape of both U87 derived CSCs and patient GBM CSCs. B, quantitative PCR showing internal expression of miR-100 in GBM samples. C, reveal reduced migration in GBM samples overexpressed with miR-100. D, immunoblot showing reduced expression of Nur77 in GBM cells overexpressed with miR-100.

were investigated. We hypothesized that overexpression and correction of miR-100 would decrease migration ability for all samples. A Gain of function was assayed by overexpressing miR-100 in both samples resulted in significant decrease ($p < 0.05$) in migration ability, Figure 1C. The reduction in migration ability reached 70% in some occasions. This result validates the importance of miR-100 in regulating cellular migration. The finding most likely helps improve prognosis and recurrence. The nuclear orphan receptor family member 77 (Nur77) has been known for its involvement in migration and promoting cellular motility in stem cells and cancer [17, 18]. Overexpression of miR-100 within cancer samples (U87 and patient CSCs) show a significant decrease ($p < 0.05$) in the Nur77 expression level, Figure 1D. The decrease reached 50%, which most likely affected migration machinery. Thus, we report a possible link between miR-100 activity and migration inhibition that probably takes place through Nur77 inhibition. This finding would help advance targeting to CSCs, which are known to be resistance to conventional cancer therapy. Moreover, considering our data and other reports we conclude that overexpressing miR-100 had an anti-cancer effects on GBM and its CSCs. This suggests miR-100 and its target genes as candidates for novel therapies against GBM. Further studies are needed to rule-out whether interaction between miR-100 and Nurr77 is direct or indirect in addition to assessment of mechanism of interaction between them.

COMPETING INTERESTS

All authors declare no conflict of interest.

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Short communication

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