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Original Article

Expression of *miR-125b* in the new, highly invasive glioma stem cell and progenitor cell line SU3

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Abstract

MicroRNA (miR)-125b has been shown to play a potential role in the development of glioma stem cells. However, the relationship between miRNA and glioma stem cells is still elusive. This study was designed to elucidate this potential relationship. We established a highly invasive glioma stem cell and progenitor (GSCP) cell line SU3. SU3 cell suspensions were injected into nude mice brains *in situ*, and the invasiveness of graft tumors was analyzed using hematoxylin and eosin staining as well as immunohistochemistry. Real-time polymerase chain reaction (PCR) was used to measure the expression levels of *miR-125b* in SU3 and other cells. *In vitro*, SU3 cells expressed CD133 and nestin as well as differentiation markers glial fibrillary acidic protein (GFAP) and β-tubulin III, which were consistent with the characteristics of glioma stem cells. Scratch assays indicated that the migration ability of SU3 cells was stronger than that of U251 stem cells (U251s). *In vivo*, SU3 cells invaded into each part of the mouse brain from the caudate nucleus in a diffuse pattern and highly expressed invasive and proliferative cell markers matrix metalloprotease 2 (MMP2), MMP9, and Ki-67. Real-time PCR results revealed that the levels of *miR-125b* and *MMP9* were significantly higher in SU3 and SU2, also a highly invasive GSCP cell line we established before, than in U251s. High expression of *miR-125b* both in newly established GSCPs, SU3, and long-term cultured GSCPs, SU2 suggests that *miR-125b* exhibits oncogene-like behavior. This behavior should be considered in further studies of *miR-125b* in cancer stem cells. Furthermore, *MMP9*, which plays a role in cancer stem cell invasion, may be a target gene of *miR-125b*.

Key words Tumor stem cells, tumor invasion, miRNA, metalloprotease

Reya *et al.*[1] reported the first study of cancer stem cells 10 years ago, and glioma stem cells (GSCs) have been under investigation for 8 years[2]. Presently, GSCs are being investigated as "seed cells" of tumor occurrence, progression, and resistance to drugs and radiotherapy. In addition, many studies have been undertaken to identify novel functions of GSCs. Despite the great progress in understanding GSC biology, controversy still exists[3]. Although GSCs share extensive similarities, individual discrepancies should be noted. These differences probably serve as a factor provoking controversy because the GSCs used in experimental studies are neither controlled by the same standard nor from the same source. In order to analyze the invasiveness of GSCs, we cloned GSCs within disseminated tumor lesions from a patient with glioma to establish a highly invasive glioma stem cell and progenitor (GSCP) cell line and analyzed the relationship between the invasiveness of GSCPs and microRNA

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(miRNA) by using *miR-125b* as the target gene.

Materials and Methods

Materials

NC nude mice from Japan Nagoya Health University were bred by Professor Qiang Huang (the Second Hospital Affiliated to Soochow University) and reared in the Experimental Animal Center of Soochow University [License No. SY×K (Jiangsu Province) 2007–0035] [4]. The fluorescence microscope, stereoscopic orientation device, and real-time polymerase chain reaction (PCR) device were supplied by Olympus, Huaibei Zhenghua Bio-instruments Co. Ltd., and iCyderiQ Company, respectively. DMEM/F12 medium and the medium supplements B27, basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were purchased from Invitrogen. Primary antibodies CD133, nestin, and Ki-67 were purchased from Abcam and glial fibrillary acidic protein (GFAP) was from BD Biosciences Company. β-tubulin III, matrix metalloprotease 2 (MMP2), and MMP9 were purchased from Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd. The miRNA-125b kit, SYBR Premix Ex Taq™ Perfect Real Time Kit, was purchased from TaKaRa. GSCP cell line SU2 was established by our lab.

Development of the highly invasive human brain glioma stem cell and progenitor cell line SU3

A neurosurgical specimen of human brain glioma was collected at the Second Hospital Affiliated to Soochow University. The specimen was obtained from a 50-year-old man with recurrent glioma that was pathologically diagnosed as glioblastoma multiforme after surgery. The SU3 cell line was established according to the methods by which we established SU2 as previously reported [5]. Briefly, living tissue was collected from the disseminated glioma lesion. After the vessels and piamater layer were removed, the sample was sheared with a sharp tool, digested with 0.25% trypsin for 10–20 min, filtered through a 70-μm sieve (BD Biosciences), treated with Lympholyte-M (Cedarlane company) to remove red blood cells, prepared for single cell suspension, and incubated under serum-free conditions. The tumor spheres in suspension were screened by CD133 immunomagnetic beads to obtain stem cells and then single cells were pumped by using capillary pipe (fishing method) under microscope for subsequent cloning to assure that the SU3 cell line was originated from one single cell. Sorted cells were then incubated in serum solution. After attachment, cells were collected along with the tumor spheres incubated under serum-free conditions. These cells were subjected to immunohistochemical methods to measure the concentration of marker proteins for progenitor cells and cells in different phases of differentiation. Their *in vitro* proliferation and *in vivo* tumorigenicity were also assessed. The cells qualified for cell line establishment were stored in liquid nitrogen for subsequent analysis.

In vitro invasiveness test

U251 is a glioma cell line known to have stable biological characteristics and low invasiveness, making it a useful comparison for SU3 cells on invasive biological characteristics. Based on the method we previously reported[6], U251 and SU3 cells were cultured using stem cell conditions. Resultant cell spheres cultured in serum-free condition (named U251s or SU3s) and attached cells cultured in 10% FBS (named U251d or SU3d) were collected and digested into single cells for the scratch assay as described in the following steps. Horizontal lines spaced approximately 0.5–1.0 cm apart were evenly marked across the wells on the back of the 6-well plates using a color pen. At least 5 lines passed through each well. Approximately 5×10^5 cells were injected into each well; the number of cells varied according to the type of cell. The cells were incubated in serum-containing solution and transferred to stem cell conditions 2 h later. The next day, a tip was positioned with a ruler to ensure, to every possible extent, that it was placed perpendicular to the horizontal lines drawn on the back of each plate. The cells were rinsed with PBS three times. The detached cells were discarded and the remaining cells were cultured in media containing serum. The cultured cells were kept in an incubator at 37℃ in an atmosphere containing 5% CO_2. Samples were obtained and photographed 0, 3, 6, and 12 h after incubation.

In vivo invasiveness test

To test their invasiveness *in vivo*, SU3 cells were inoculated into the caudate nucleus of NC nude mice. The mice were anesthetized via intraperitoneal injection of 10% chloralic hydrate (200 mg/kg), and a 5-mm incision was made longitudinally at the center of the cranic top. At 1 mm in front of the bregma and 2.5 mm right from the halfway line, a cranium drill with a 1-mm diameter was inserted until the endocranium was reached. Using a stereoscopic orientation device for assistance, a 25-μL microsyringe was used to slowly inject (10 min; insertion needle 3.5 mm) an SU3 cell suspension solution with 1×10^5 cells in 15 μL into the right caudate nucleus of the mice. The syringe was retained in place for 2 min after injection and then slowly removed. The keyhole was sealed using bone wax, and

the skin was closed using size 1 sutures. Four to five weeks after cancer stem cell inoculation, the treated mice presented response retardation, reduced food intake, and emaciation, and they gradually exhibited cachexia. The mice were then euthanatized. The whole brain was prepared for further analysis.

Real-time PCR of *miR-125b* and *MMP9*

Total RNA was extracted from U251s, SU2, and SU3 cells with Trizol, respectively. The samples were treated using PrimeScript™ RT Reagent Kit (TaKaRa), and a reverse transcription was performed with a gradient PCR device. The obtained cDNA templates were used for real-time PCR amplification, and U6 was used as internal reference. The reaction mixture contained 10 μL SYBR Premix Ex Taq™, 0.4 μL PCR forward and reverse primers, and 1–2 μL total cDNA and dH_2O (added to a final volume of 20 μL). Forward and reverse primers for the reaction were as follows: 5′-ACTGATAAATCCCTGAGACCCTAAC-3′ (forward) and 5′-TATGGTTGTTCTGCTCTCTGTCAC-3′ (reverse) for *miR-125b* (GenBank accession number NM_171992); 5′-GCTCTTCCCTGGAGACCTGA-3′ (forward) and 5′-TGCCACCCGAGTGTAACCAT-3′ (reverse) for *MMP9* (GenBank accession number Y00047) . Real-time PCR results were analyzed using the $2^{-\triangle\triangle CT}$ method[7].

Statistical analysis

GraphPad Prism 4 software was adopted for statistical analysis and chart drawing. All data are expressed as mean ± standard deviation. Comparisons among groups were analyzed using ANOVA. *P* values <0.05 were considered significant.

Results

Growth characteristics of highly invasive SU3 cells

In vitro, SU3 cells grew as suspended spheres in the conditional media containing growth factors, but they attached to the walls of the culture dish and differentiated, displaying star-shaped, fusiform, and other morphologies in the culture media containing serum. Using immunofluorescence, we detected SU3 cells were positive for stem cell marker CD133; differentiated SU3 cells cultured in serum condition were positive for the progentior cell marker nestin and differentiation markers GFAP and β-tubulin III. Both of the cells possessed indefinite proliferation proficiency (Figure 1). In scratch tests, both SU3s and differentiated SU3d cells migrated

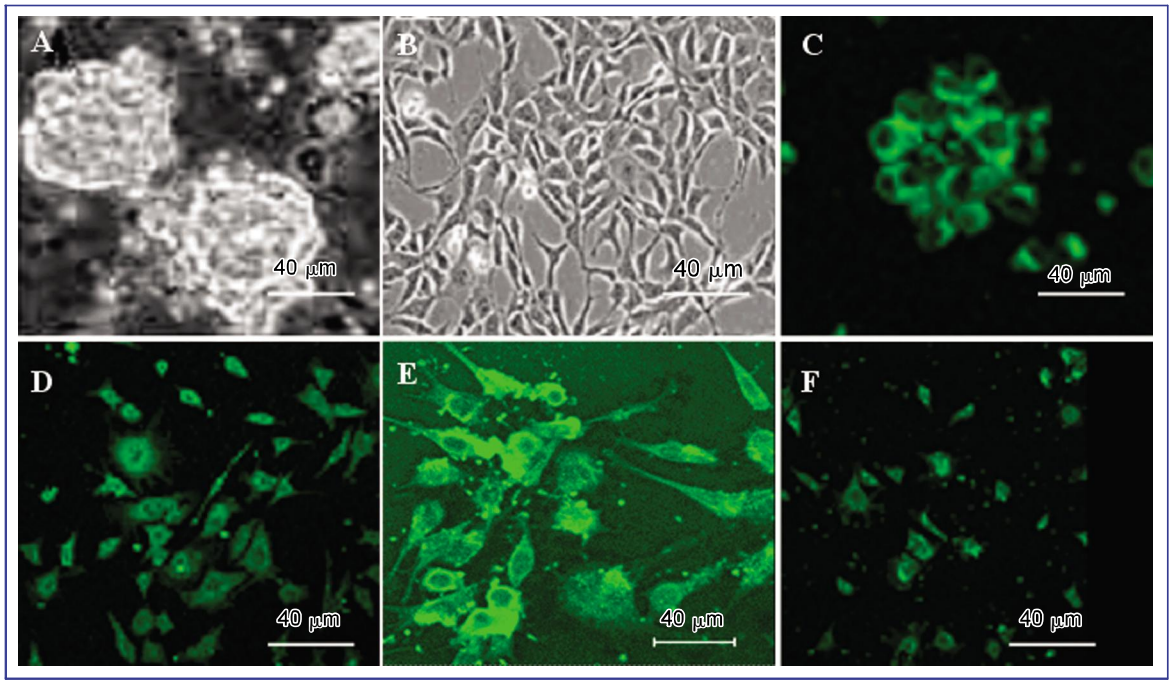

Figure 1. **Primary culture of the SU3 glioma stem cell line and its growth features (original magnification, ×200).** A, fresh tumor tissues were dissociated into single cell suspension, cultured in serum-free conditions, and tumor spheres were formed 7 days later. B, in contrast, cells were adherent when cultured in serum condition containing 10% FBS. Tumor spheres reflected brightly, and differentiated tumor cells exhibited astroid, fusiform, or irregular morphologies (A and B). C-F: immunostaining of SU3. Tumor stem cells were positive for CD133 (C), and differentiated tumor cells were positive for nestin (D), GFAP (E), and β-tubulin III (F).

toward scratch site after 6 h, and the number of migrated cells dramatically increased 12 h later. The migration ability of SU3 cells was significantly higher than that of U251s or U251d cells (Figure 2).

In the *in vivo* experiments, tumor-bearing mice typically experienced cancer cachexia 30 days after cell inoculation at the right caudate nucleus. Anatomical dissection showed that the tumor invaded into the skull inner plate, whole right hemisphere and whole olfactory lobe. Hematoxylin and eosin staining showed darkly stained tumor cells with visibly deformed nuclei that were densely distributed and stretched toward normal brain tissue in a chicken feet pattern (Figure 3). These results suggest that SU3 is a highly invasive tumor cell line with severe grade malignancy.

To determine the molecular basis of the observed invasiveness, SU3- and U251s-transplanted tumor tissues were subjected to immunohistochemical analysis to detect the expression of MMP2, MMP9, and Ki-67 proteins. The results indicated that SU3-transplanted tumor tissues expressed these proteins at significantly higher levels than did U251s tumor tissues (Figure 4).

In vitro expression of *miR-125b* and *MMP9*

To investigate whether the invasiveness of glioma stem cells and progenitors is correlated with the overexpression of *miR-125b* and *MMP9* (*MMP2* was not included because the PCR results were not stable), we performed real-time PCR on SU3 spheres, U251s, and SU2, a GSCP cell line previously confirmed as highly invasive. The results showed that SU3 and SU2 cells had significantly higher levels of *miR-125b* than did U251s control cells (Figure 5A). Likewise, the concentration of *MMP9* in SU3 and SU2 cells was elevated compared to U251s cells. The level of *MMP9*

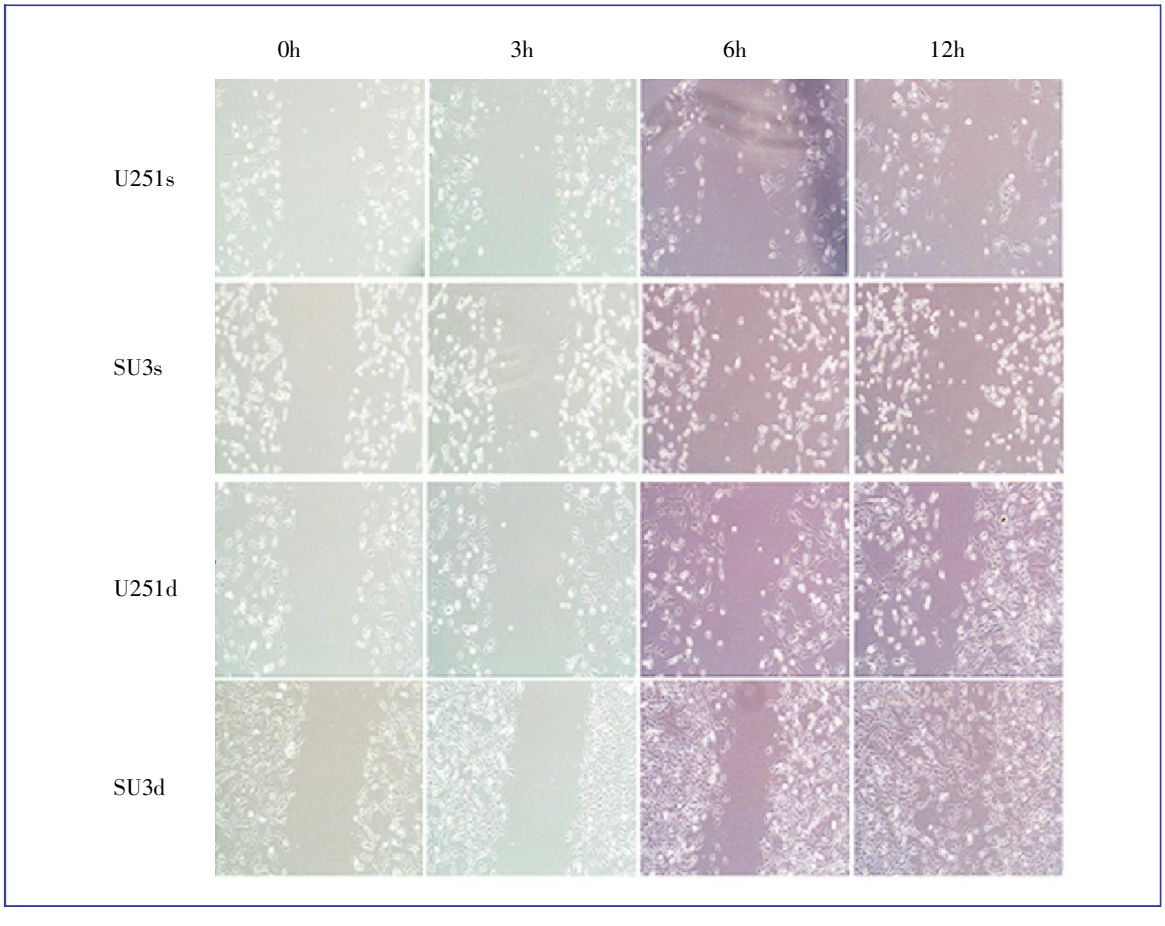

Figure 2. ***In vitro* invasion assay of SU3s and its differentiated cells (scratch test, original magnification, ×100).** U251s was single cell suspension of tumorspheres derived from U251 cells cultured in stem cell conditions. U251d and SU3d were differentiated cells of U251s and SU3s, respectively. Results showed that all cells migrated into the scratch after 6 h, and this migration was most obvious after 12 h. However, the migration ability of SU3s and SU3d were higher than that of U251s or U251d.

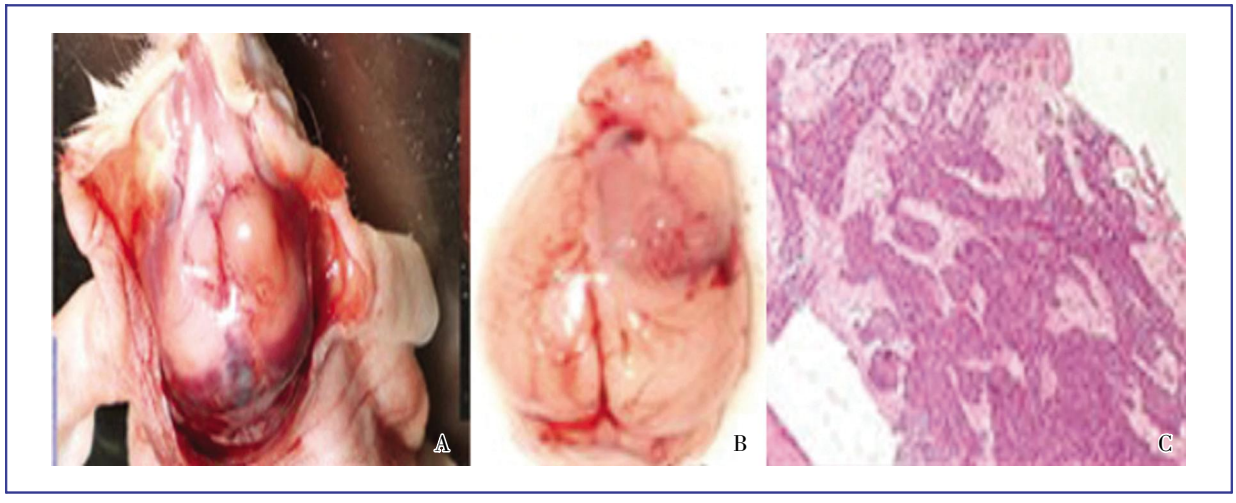

Figure 3. ***In vivo* invasiveness assay of SU3.** SU3 cells were injected into the mouse brain, and tumor cells grew invasively from the injection site (right caudate nucleus) to the surrounding areas, including the inner plate of the skull (A) and the surface of frontal lobe (B). C, hematoxylin and eosin staining shows anachromasis and reveals that tumor cells stretched into the normal brain tissue like chicken feet (original magnification, ×100).

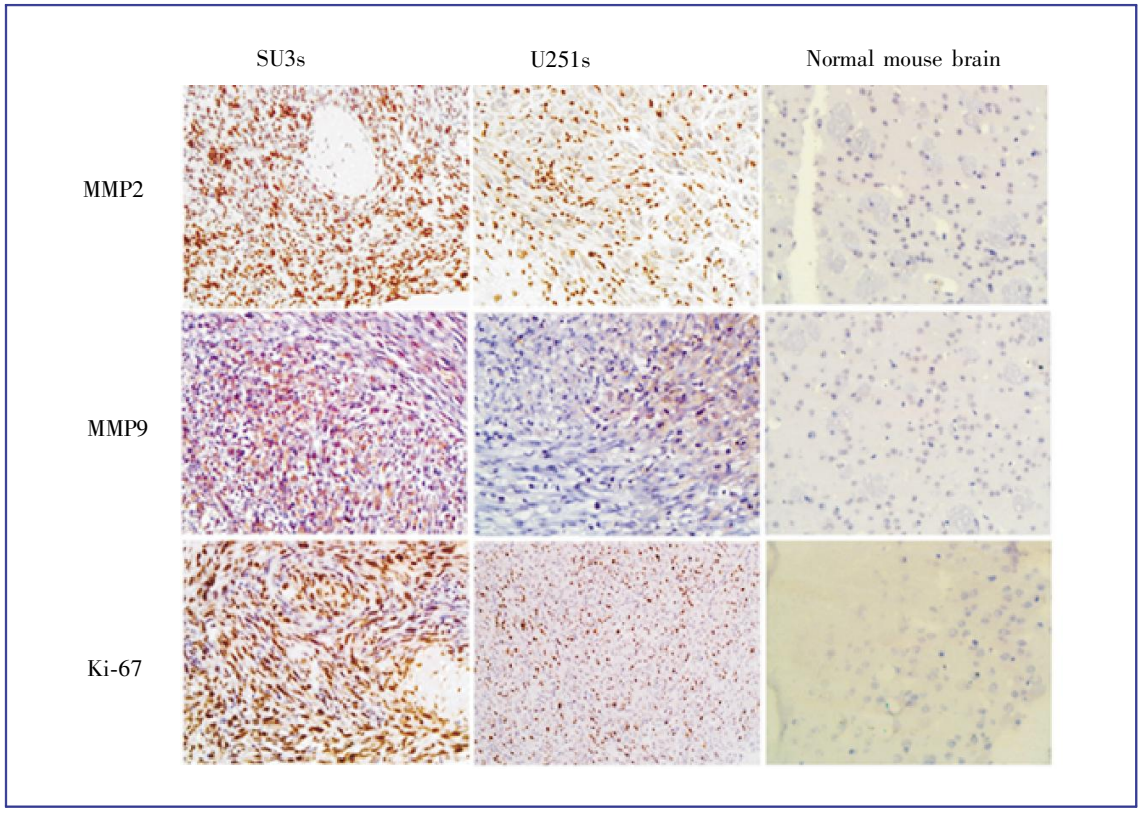

Figure 4. **Expression of molecular markers of invasion and proliferation in transplanted tumors (original magnification, ×100).** Tissue of SU3-transplanted tumors highly express invasion markers MMP2 and MMP9, and proliferation marker Ki-67 (first column). Tissue of U251s-transplanted tumors express MMP2, MMP9 and Ki-67 as well, but the expression is weak (second column). Normal mouse brain tissue is negative for MMP2, MMP9, and Ki-67 (third column).

was significantly higher in SU2 cells than in U251s cells (Figure 5B). *MMP9* levels were not significantly different between SU3 and U251s cells, possibly due to small sample size; however, the absolute value of *MMP9* increased by 5-fold, which is a significant increase in SU3 cells.

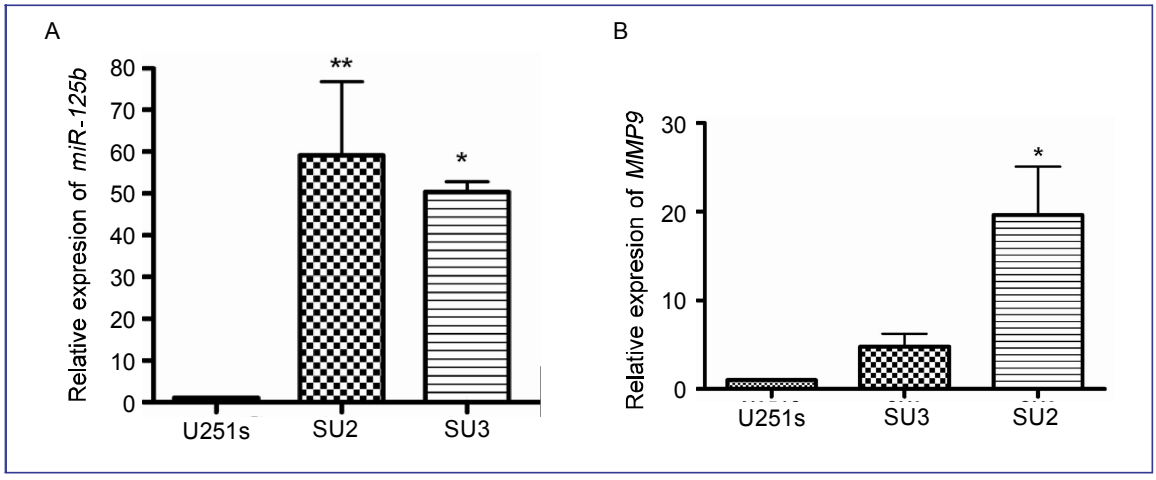

Figure 5. **Levels of *miR-125b* and *MMP9* in SU2, SU3, and U251s cells detected with real-time PCR.** The $2^{-\triangle\triangle CT}$ method was used to analyze the relative changes in gene expression. Statistical comparisons between groups were performed with ANOVA, followed by Student-Newman-Keuls analysis. Data were converted to graph using Prism 4. A, the level of *miR-125b* was significantly higher in SU2 and SU3 cells than in U251s cells ($P < 0.01$, $P < 0.05$); B, compared to U251s cells, SU2 cells exhibited significantly higher expression of MMP9 ($P < 0.05$); no significant difference is observed between SU3 and U251s cells.

Discussion

GSCP cell line SU2, which presents highly invasive characteristics and has been consecutively passaged for more than 2 years, was successfully generated by us from a biopsy sample collected 5 years ago from a patient with recurrent malignant glioma [5,8]. However, whether the highly invasive SU2 cell line established by long-term passage *in vitro* is stable remains to be elucidated. Thus, we newly established SU3 cell line in this study. Our results confirmed the high invasiveness of SU3 cells *in vitro* and *in vivo* by comparison with the vigorously proliferative but lowly invasive cancer stem cell line U251s [9]. We then aimed to analyze the mechanism underlying the high invasiveness of SU3 cells.

miRNAs are stable, non-coding, small RNAs that play a role in cellular processes including differentiation, apoptosis, and metabolism via post-transcriptional regulation of target gene expression [10]. Unbalanced regulation of miRNAs may lead to tumor occurrence [11]. Benard *et al.*[12] reported that specific miRNAs can induce tumorigenesis and resistance to apoptosis in glioma cases. Chan *et al.*[13] found that the expression of *miR-21* was significantly up-regulated in human gliomas, and blocking *miR-21* expression markedly reduced proliferation ability. Ciafre *et al.*[14] found that *miR-221* was significantly up-regulated in both glioma cells and tissues, whereas the *miR-181* family was down-regulated. Dews *et al.*[15] revealed that the up-regulation of certain miRNAs was closely correlated with the tumor angiogenesis; however, whether the abundant blood supply network in malignant glioma cases is correlated with miRNAs remains unknown. Shi *et al.*[16] noted that low expression of *miR-181a*, *miR-181b*, and *miR-181c* was detected in glioma tissues. Thus, miRNAs have been suggested to have differing roles in gliomas.

miR-125b, which was used in this study, is a miRNA mainly accumulated in cerebral tissues [17]. Currently, study outcomes regarding the function of *miR-125b* are divergent. Ciafre *et al.*[14] reported that the expression level of *miR-125b* in malignant glioma tissue was higher than that in normal brain tissue. Xia *et al.*[18] found that the overexpression of *miR-125b* accelerated the proliferation of glioma cells and inhibited retinoic acid-induced apoptosis. Le *et al.*[19] suggested that overexpression of *miR-125b* suppressed the expression of tumor suppressor gene *p53* and blocked p53-mediated apoptosis. These re sults seemingly indicate that *miR-125b* functions as an oncogene. However, Shi *et al.*[20] showed that the up-regulation of *miR-125b*

inhibited the proliferation of U251 stem cells, probably playing the role of a tumor suppressor gene. Our results showed that the expression of *miR-125b* in highly invasive SU3 and SU2 cells was significantly higher than that in U251s and U251d cells, suggesting that the high expression of *miR-125b* was consistent with the malignancy grade of tumor cells. Although inconsistent with the results reported by Shi *et al*.[20], our findings are in accord with those reported by Ciafre *et al*.[14], Xia *et al*.[18] and Le *et al*.[19], as well as Klusmann *et al*.[21], who reported that *miR-125b* played a role in accelerating the progression of leukemia.

Recent studies revealed that *miR-125b* has multiple target genes, including *p53* [19], *TNF-*α [22], *Bmf*[18], and *DICER1* and *ST18* [21]. No studies focusing on whether *miR-125b* regulates the expression of *MMP9*, which is related to invasiveness of tumor cells, have been reported. In this study, we detected high levels of both *miR-125b* and MMP9 in highly invasive SU3 and SU2 cells, indicating that *miR-125b* may regulate the invasiveness of cancer cells by mediating the expression of *MMP9*.

Currently, miRNA-related studies mainly focus on tumor tissues and cancer cell lines. However, few studies explore stem cells, which play an important role in the occurrence and progression of tumors. Although we detected highly expressed *miR-125b* and *MMP9* in SU2 and SU3 cells with real-time PCR, these results must also be validated at the protein level. In addition, the expression of *miR-125b* should be inhibited using a knockout approach to determine whether *miR-125b* plays a role similar to an oncogene in the occurrence and progression of cancer. Furthermore, studies are needed to identify whether the *miR-125b* effects occur during the stem cell stage or the differentiation stages.

Acknowledgments

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