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MIR-451 and Imatinib mesylate inhibit tumor growth of Glioblastoma stem cells

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ABSTRACT

We examined the microRNA profiles of Glioblastoma stem (CD133+) and non-stem (CD133−) cell populations and found up-regulation of several miRs in the CD133− cells, including *miR-451*, *miR-486*, and *miR-425*, some of which may be involved in regulation of brain differentiation. Transfection of GBM cells with the above miRs inhibited neurosphere formation and transfection with the mature miR-451 dispersed neurospheres, and inhibited GBM cell growth. Furthermore, transfection of *miR-451* combined with Imatinib mesylate treatment had a cooperative effect in dispersal of GBM neurospheres. In addition, we identified a target site for SMAD in the promoter region of miR-451 and showed that SMAD3 and 4 activate such a promoter-luciferase construct. Transfection of SMAD in GBM cells inhibited their growth, suggesting that SMAD may drive GBM stem cells to differentiate to CD133− cells through up-regulation of miR-451 and reduces their tumorigenicity. Identification of additional miRs and target genes that regulate GBM stem cells may provide new potential drugs for therapy.

Glioblastoma (GBM) is a highly infiltrating, aggressive brain cancer with no available curative treatment. Recently, identification of tumor initiating cells in human brain tumors has been reported, using CD133 as a marker. It was demonstrated that as few as 100 CD133+ cells isolated from high-grade GBM could initiate and propagate a tumor when re-introduced into the brain of immuno-deficient mice, whereas even a large number (10^5) of CD133− cells can not transfer the tumor [1]. The tumor initiating cells propagated in vitro as free floating neurosphere-like structures when cultured in serum free medium supplemented with growth factors [2]. Previously, we characterized the properties of cancer stem cells (CSCs) by comparing gene expression profiles in Acute Myeloid Leukemia stem cells [3]. Here, we compare the microRNA (miR) profiles of CSCs with that of the non-stem cell population in GBM.

MicroRNAs (miRs) are a novel group of short RNAs, ~22 nucleotide in length, that regulate gene expression in a posttranscriptional manner by pairing with complementary nucleotide sequences in 3' untranslatable region of target mRNA. Abnormal expression of miRs is linked with various human disorders [4–6], including cancer development and progression [6,7], where miRs can function either as oncogenes or as tumor suppressors in their effect on tumor growth [8,9] and recently miR-7 [10] and miR-21 [8,11] were implicated in GBM by comparing miR expression in tumor and normal tissue.

Since the CD133+ fraction contains the GBM stem cells whereas the CD133− fraction includes partially differentiated tumor cells, we assume that overexpression of miRs in the CD133− cells indicates that they drive CD133+ cells to differentiate and loose their stem cell character. Hence, raising the level of these miRs in CD133+ cells by transfection may inhibit cell growth and tumor proliferation, due to loss of self-renewal potential. We therefore analyzed the effect of the overexpressed miRs in CD133−, by transfection of these miRs to CD133+ cells and by studying their effect on the dispersion of the neurospheres and on the viability and proliferation capacity of the GBM stem cells. Surprisingly, several miRs, and particularly miR-451, at nanomolar concentrations, inhibited the growth and neurosphere formation of A172 cells and synergize with Imatinib mesylate in enhancing this effect. Similar results were also obtained with the CD133+ cells derived from primary GBM. These results suggest that certain miRs can function as differentiation drugs in driving CSCs to the state of inhibited proliferation.

Materials and methods

GBM samples, isolation of stem cells and cell culture. Tumor samples were obtained from surgical resection of adult GBM patients

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after informed consent at the Tel-Aviv Souraski Medical Center (Tel-Aviv, Israel). Cells were prepared and subjected to magnetic bead isolation using the CD133 Cell Isolation Kit (Miltenyi Biotec) as described previously [12]. Cells were labeled with anti-human CD133-2 Phycoerythrin (PE) and control IgG isotype monoclonal antibodies (Miltenyi Biotec, Auburn, CA, USA) and analyzed for purity by flow cytometry with the FACS-Calibur machine (Becton–Dickinson, Franklin Lakes, NJ, USA). For sample fractionation CD133/1 Microbeads were used (Miltenyi Biotec) and Cell separation was carried out on the autoMACS machine (Miltenyi Biotec). Data acquisition and analysis were performed with CellQuest software (Becton–Dickinson).

For neurosphere formation the GBM CD133+ and CD133− cell populations or GBM cell line A172 cells were resuspended in a defined serum-free tumor sphere medium (TSM) [2], containing DMEM/F12, B27 (1:50; Gibco-Invitrogen), Fibroblast growth factor (bFGF, Peprotech, Rocky Hill, NJ, USA), Epidermal growth factor (EGF, Peprotech) Leukemia Inhibitory Factor (LIF, Chemicon), each 20 ng/ml and Heparin (5 μg/ml) and plated in 96-well at 20,000 cell per well. Neurospheres were observed after 24–36 h.

Expression profiling of miRs. For microRNA analysis, total RNA was isolated from three GBM patients using TRIZOL according to the manufacturer's instructions. Custom microarrays were produced using the Rosetta Genomics miRdicator™ microRNA microarray platform and contained DNA oligonucleotide probes representing 688 miRNAs as described [7].

microRNA was labeled in total RNA (1–5 μg) by ligation of an RNA-linker, p-rCrU-Cy/dye (Eurogentec), to the 3' end with Cy3 or Cy5 at 4 °C for 1 h followed by 1 h at 37 °C. The labeled RNA was mixed with 3X hybridization buffer (Ambion), heated to 95 °C for 3 min and then added on top of the miRdicator array. Slides were hybridized for 12–16 h at 42 °C, followed by two washes in room temperature (25 °C) with 1× SSC, 0.2% SDS and a final wash with 0.1× SSC.

Arrays were scanned using an Agilent Microarray Scanner Bundle G2565BA (resolution of 10 mm at 100% power). The data were analyzed using the SpotReader software (Niles Scientific) and triplicate spots were combined, as described in [7]. Differentially expressed miRs were identified by using a filter based on a fold change of 2 combined with *t*-test ($p < 0.01$) for CD133+ versus CD133− comparisons.

Transfection of A172 and primary GBM tumors with microRNAs. Stability-enhanced *miR-451* and control oligo-ribonucleotides were from Dharmacon. Transfection was carried out using Dharmafect (Dharmacon), according to the manufacturer's instructions. Additionally, plasmids containing *miR-451*, *miR-425*, *miR-486* and pGFP were from Dr. R. Agami's lab (R. Nagel and C. LeSage) [13]. A172 cells were plated in 24-well plates, (12×10^5 cells/well) in antibiotic-free medium one day prior to transfection. Transfection with miR-containing plasmids *miR-451*, *miR-425*, and *miR-486* was performed with 100–300 ng and after 24 h fresh medium was added for additional 24 h. For GBM primary tumor cells, transfection was for 4hr without further incubations. A172 cells and GBM primary cells were then transferred to 96-well plates at 6×10^4 cells/well with TSM medium for neurosphere formation. Neurosphere growth was determined 3 days after transfer, viewed and photographed microscopically (Olympus CK2) using a 4X objective. The assay was repeated several times.

Luciferase assay. A pair of specific primers (F-5'CTC GAG AGA CCA TGC TAA GCA GAG'3, R-5'AAG CTT GAT CTC CAG AAT CTG TCT GG'3) were used to amplify the mir-451 putative promoter from human DNA, with added sites of XhoI and HindIII, and cloned into pGEMT vector (Promega, Madison, Wisconsin), denoted *pGEMT1-miR 451*. From this, the miR promoter was released with XhoI and HindIII and cloned into *pGL3-basic* vector at the corresponding sites, denoted *pGL3-miR451*.

A172, H1299, and HCT116 cells were plated in 24-well dishes at 6×10^4 cells/well. All transfections included *pGL3-miR451*, and the SMAD containing plasmids, pFLAG-SMAD3 or pMyc-SMAD4, or both, at 100–300 ng/well and a constant amount of *Renilla* luciferase plasmid (100 ng) for internal control. Twenty four hours post-transfection, cells were incubated for 30 min with 100 μl/well lysis buffer (Amersham) and 35 μl of each lysate was subjected to a dual luciferase assay (Promega) using a Luminoskan Ascent apparatus (Thermo lab systems). Results of triplicate transfection are presented after normalization to *Renilla* luciferase activity. Values are shown as means ± SEM. *Renilla* plasmid was from Promega and the SMAD plasmids were a gift of Dr. Y. Shaul.

Results

Isolation of CD133+ and CD133− fractions from primary GBM and miRNA expression profiling

We used the antibody coated magnetic beads (Miltenyi) to fractionate primary GBM samples to CD133+ and CD133− cells. GBM

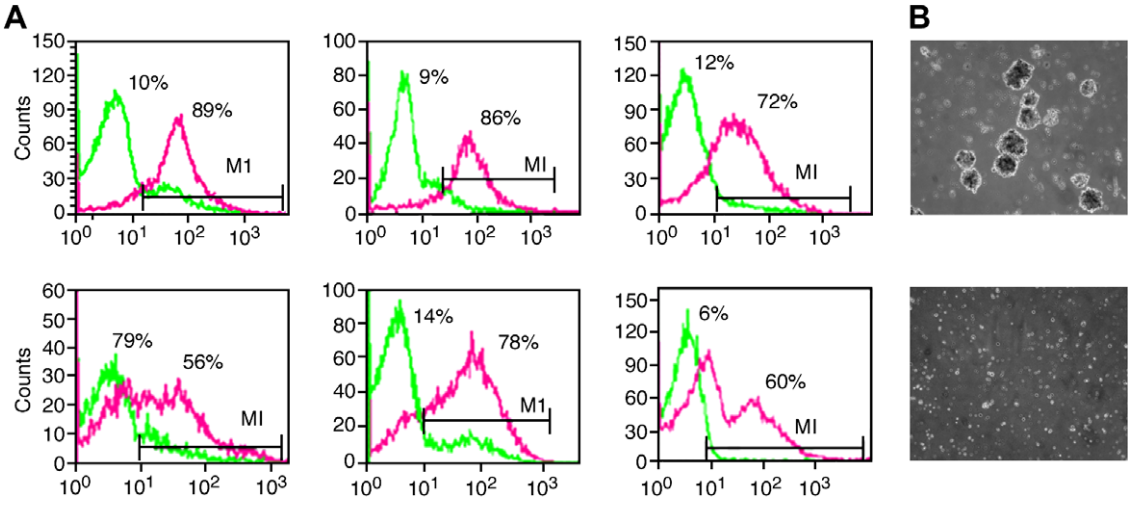

Fig. 1. Characterization of primary GBM by expression of CD133. (A) The expression of CD133 in primary tumor cells, obtained from six GBM patients. The percent of CD133+ cells in the total population, prior (left number) and after magnetic sorting (right number), is indicated. (B) Neurophere-like structures were found in the CD133+ fractions of the primary GBM cells (upper right). The CD133− fractions did not form neurospheres (lower right).

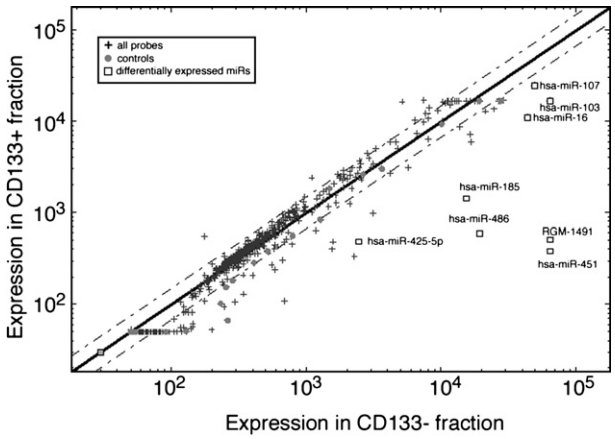

Fig. 2. miR expression in primary GBM cell fractions. Scatter plot of the expression levels of miRs in the CD133+ cells (vertical) vs. the CD133− cells (horizontal) of a primary GBM tumor. Several miRs (marked as empty squares) showed apparent up-regulation in CD133− compared to the CD133+ expression with p-values of $p < 0.01$, using t-test. These findings were consistent in the three GBM samples that were analyzed. RGM-1491 is a probe that overlaps with hsa-miR-451.

samples derived from six patients (p1–p6) were sorted for stem cells (CD133+) and for CD133− cell fractions by one passage through the antibody coated beads (Fig. 1A). After sorting, the content of the CD133+ fraction was enriched up to 89% and each fraction formed neurosphere-like structures within 24–48 h, in the appropriate serum-free medium [2], whereas the CD133− fraction did not exhibit neurosphere formation (Fig. 1B).

We isolated total RNA from the cells of the GBM fractions and analyzed the RNA for the content of miRs, using Rosetta Genomics miRdicator™ microRNA microarrays for three out of six GBM tumors (p1, p2, and p4). Fig. 2 shows the differentially expressed miRs in the CD133+ and CD133− fractions of a particular GBM sample (p1). Interestingly, the modulated miRs were not found to be overexpressed in the CD133+ cells, but rather several miRs were overexpressed in the CD133− fraction of all samples (Fig. 2). These include *hsa-miR-451*, *hsa-miR-486*, *has-miR-425*, *hsa-miR-16*, *hsa -miR-107*, and *hsa -miR-185* (Fig. 2). Similar results were obtained for the other two GBM samples (p2 and p4).

miR-451 and Imatinib mesylate cooperate in targeting GBM stem cells for neurosphere dispersion

We used A172 cells to study the effect of overexpression of miRs on neurosphere formation. Transfection with the miR-containing plasmids *miR-451*, *miR-425*, and *miR-486* inhibited the formation of neurospheres in A172 cells (Supplementary Figure 1A). Next, we focused on miR-451 that showed a considerable neurosphere inhibition effect (Supplementary Figure 1A). We transfected the plastic-attached A172 glioma cells, with the 22-mer oligonucleotide of *miR-451* to study the effect of miR expression on the growth of GBM cells (Supplementary Figure 1B). A172 cells were transfected with either *control-miR* or *miR-451* and assayed for viability for 4 days. Transfection of miR-451 caused a significant drop in cell number, depressing cell viability by more than 80% and inhibited cell growth (Supplementary Figure 1C).

We have shown previously that Imatinib mesylate at 2.5 μM inhibited neurosphere formation and caused neurosphere dispersion [12]. To evaluate the combined effect of miR-451 and Imatinib mesylate, we transfected A172 cells with miR-451 followed by neurosphere formation and the addition of Imatinib mesylate at concentrations of 1 μM and 2.5 μM (Materials and methods). Cells

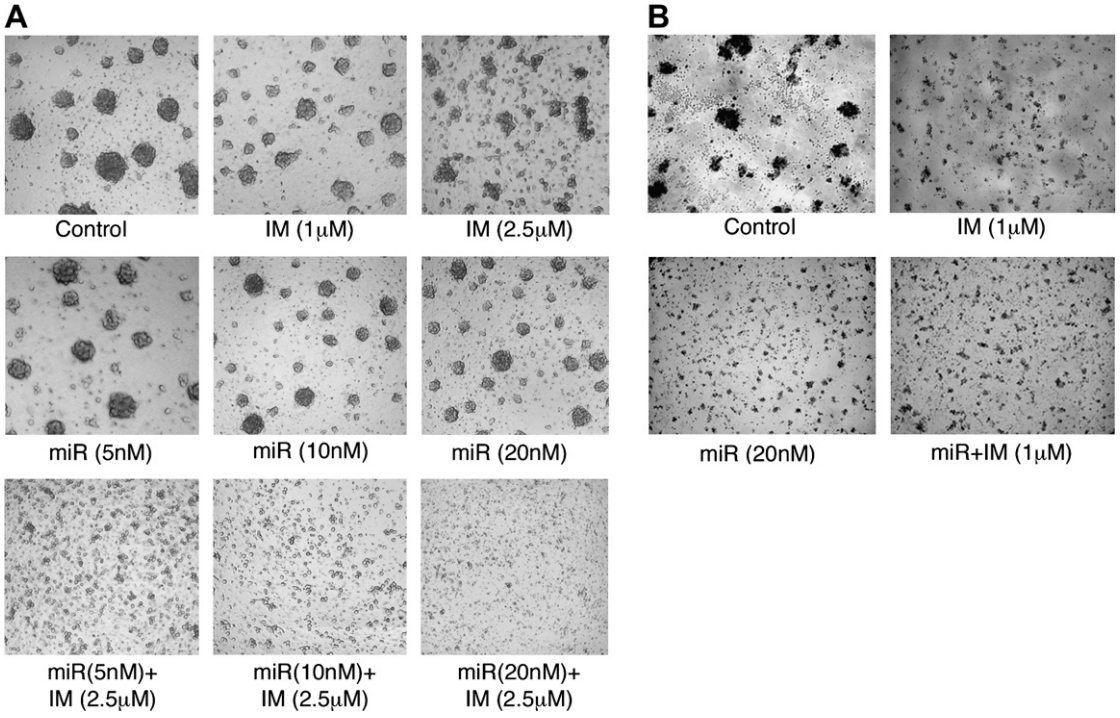

Fig. 3. Combined effect of miR-451 and Imatinib Mesylate treatment on primary GBM and A172 cells. (A) A172 cells were transfected with miR-451 at the indicated concentration and subsequently transferred to 96-well plates in serum free medium for neurosphere formation. Imatinib mesylate was added at concentration of 1 μM and 2.5 μM, 2 h after transfer. Neurospheres were photographed 24 h later. IM, Imatinib Mesylate, miR, miR-451. (B) Transfection of primary GBM with miR-451 and treatment with Imatinib mesylate. Primary GBM cells were fractionated for CD133+ stem cells and transfected with miR-451 at 20 nM. Cells were then transferred to 96-well plates for neurosphere formation. Imatinib mesylate was added at 1 μM and 2.5 μM (data not shown), 2 h after transfer. Combination of miR-451 (20 nM) and Imatinib mesylate (1 μM) shows complete dispersion of neurospheres.

were evaluated for neurosphere formation and growth 3 days later (Fig. 3).

Transfection of miR-451 at concentrations of 5 nM, 10 nM, and 20 nM showed a reduction in neurosphere size and number (Fig. 3A, middle). Dispersion of neurospheres was observed at miR concentrations of 10 nM and 20 nM (Fig. 3A, middle), whereas Imatinib mesylate showed partial dispersion of the neurospheres and reduction in sphere size at 1 μM and the effect was pronounced at 2.5 μM (Fig. 3A, top). The combination of miR-451 and Imatinib mesylate led to complete dispersion of the neuropheres at lower concentrations of both reagents (Fig. 3A, bottom).

This assay was also performed on primary GBM stem cells within 24 h of surgical resection and with similar results (Fig. 3B). The fractionated CD133+ cells were transfected with miR-451 (see Materials and methods) and Imatinib mesylate was added, as described above for the A172 cells. Fig. 3B shows the response of the transfection with miR-451, Imatinib mesylate treatment and their combination on a particular GBM tumor (p5). We demonstrate that the combination of miR-451 and Imatinib mesylate disperse the spheres at 20 nM miR-451 and 1 μM Imatinib mesylate. Although the dispersion of neurospheres is not complete, the effect is similar to that obtained with A172 cells. Furthermore, inhibition of cell growth and reduction in cell viability was observed on attached A172 cells that were transfected with miR-451 (20 nM), followed by the addition of Imatinib mesylate (2.5 μM) 12 h later (data not shown).

Regulation of miR-451 by SMAD

MiR-451 is located on chromosome 17q11.2, a region known to be amplified in certain types of cancers, and is in close proximity to HER2 (17q12) [14,15]. To better understand its potential role in cell signaling, we examined the upstream regions of miR-451 for transcription factor targets, using the Genomatix software [16]. We identified binding sites for SMAD3 (GTCTGGCCT) and SMAD4 (GTCTAGTCT), separated by 157 bp and beginning 1135 bp upstream to the miR-451 sequence (AAACGTTACCATTACTGAGTTT) (see Fig. 4A).

To explore whether these target sites respond to SMAD, we amplified by PCR the miR-451 putative promoter that contained the SMAD targets and cloned it fused to firefly luciferase, to generate the pGL3-miR451 vector (see Materials and methods). SMAD3 and SMAD4 containing plasmids and the pGL3-miR451 or appropriate controls were co-transfected to A172 cells and assayed for luciferase activity. The results showed increased luciferase activity in the presence of SMAD3 or SMAD4, and when both activators were used, 6-fold luciferase inductions were observed (Fig. 4B). Similar results were obtained with H1299 or HCT116 cell lines (data not shown), suggesting that miR-451 transcription is enhanced by SMAD.

To investigate the effect of SMAD3 and SMAD4 on the growth of A172 glioma cell lines, these cells were transfected with SMAD plasmids (see Materials and methods). Cells transfected with SMAD3 or SMAD4 showed a much slower growth rate in comparison to control or GFP transected cells, whereas their combination induced a more profound growth inhibition (Fig. 4C). This is in line with the luciferase assay, suggesting that SMAD3 and SMAD4 may enhance the miR-451 transcription and induce inhibition of cell growth and proliferation.

Discussion

Our work is based on the hypothesis that tumors contain a minor population of Cancer Stem Cells which maintain the proliferation of the tumor due to the self-renewal properties of these cells. This concept was shown to hold true for leukemia, breast, colon, lung, prostate cancer and brain tumors [17]. The therapeutic implications are highly significant since targeting the minor population of CSC may be more effective in eradicating the tumor. We used GBM as a model for testing the differences in expression profiles of microRNA since recent evidence suggests a role for miRs in human cancers [5,18,19].

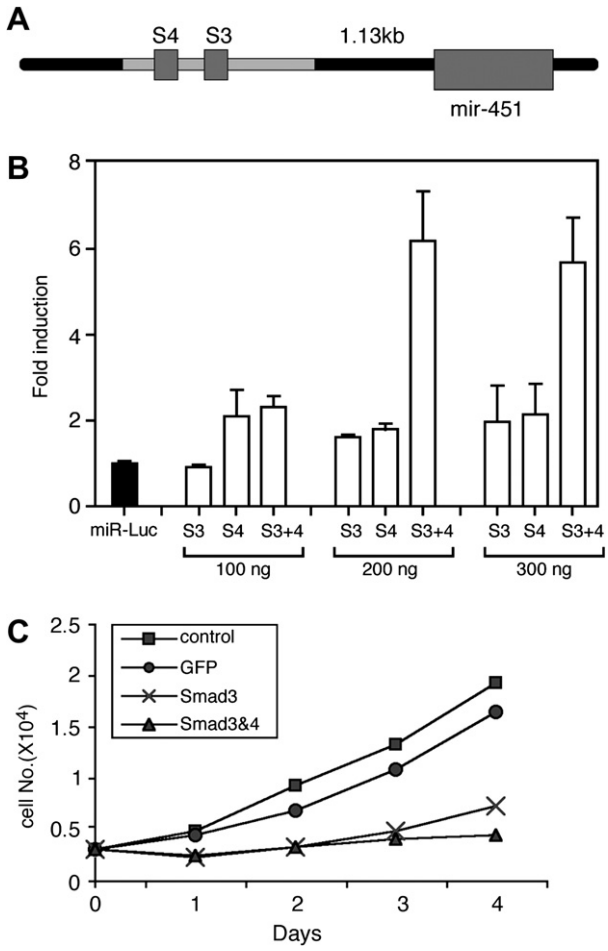

Fig. 4. SMAD effect on miR-451 promoter and GBM A172 cell growth. (A) Schematic view of miR-451 and target sites (S3,S4) for SMAD. (B) A172 cells were transfected with miR promoter-luciferase construct along with SMAD3 and 4 plasmids. Luciferase assay showed activation of miR-451 promoter by SMADs and particularly by combined SMAD3 and 4, reaching up to 6-fold luciferase induction. mir-luc, control transfection of mir-luc alone, S, SMAD. (C) A172 cells were transfected with either SMAD3, SMAD4 or both. Cells were grown for 4 days in multiple wells of 24w plates and cell counts were taken in duplicates each day.

Our analysis of differential expression of miRs in the CD133+ and CD133− of primary GBM tumors showed overexpression of several miRs in the CD133− population. We therefore overexpressed three of the differentially expressed miRs in the A172 cell line and observed their effect on neurosphere formation. Surprisingly, these miRs inhibited neurosphere formation. We focused on miR-451 and used the mature 22-mer long oligonucleotide to transfect A172 cells. We found that miR-451 inhibited cell growth as well as neurosphere formation at a very low concentration (~20 nM). Previously we have shown that Imatinib mesylate (STI-571) inhibits neurosphere formation of primary GBM as well as A172 cell growth [12]. We therefore, applied a combination of miR-451 and Imatinib mesylate to the GBM cell line and primary tumors and found a synergistic effect in inhibiting neurosphere formation since the concentration of miR-451 can be reduced by at least 4-fold to completely disrupt neurosphere growth when Imatinib mesylate is present.

In order to understand the pathway activated by miR-451 in the inhibition of GBM stem cells, we search for transcription factors that may activate miR-451. We identified two target sites for SMAD in the upstream promoter region of miR-451 and showed that SMAD3 and 4 activate the miR-451 promoter-luciferase construct. This suggests that miR-451 may be activated by the SMAD pathway. Indeed transfection of the GBM cell line A172 with SMAD-containing plasmids inhibited their growth, similar to the effect of miR-451. Human miR-451 is a single-copy gene and the sequence of the mature miR-451 is conserved and located on human chromosome 17q11–12, in close proximity to the miR-144, HER2 gene and TRAF4 (TNF receptor-associated factor 4). TRAF4 is a subunit of TNF receptor that interacts with TGF-β1 and this region, including miR-451 is amplified in some cancers. It is very likely that miR-451 is also involved in brain development since it was shown that the medulla oblongata displays enrichment of miR-34a, miR-451, miR-219, miR-338, miR-10a, and miR-10 b [20]. In addition, a recent analysis reported a substantial alteration of the miR-451 level as well as miR-21 and miR-128 levels in Glioma from an in vitro invasion assay [21], suggesting that overexpression of these miRs qualifies them as relevant target genes in GBM. Our analysis of differential expression of miRs in the stem cell and non-stem cell fraction of GBM and using the overexpressed miRs in CD133− as a drug is a novel approach to identify new reagents for modulating GBM tumorigenicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.107.

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