Silencing of PROS1 induces apoptosis and inhibits migration and invasion of glioblastoma multiforme cells

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Abstract. Glioblastoma multiforme (GBM) is an aggressive brain tumor and most patients have poor prognosis. Despite many advances in research, there has been no significant improvement in the patient survival rate. New molecular therapies are being studied and RNA interference (RNAi) therapy is one of the promising approaches to improve prognosis and increase survival in patients with GBM. We performed a meta-analysis of five different microarray datasets and identified 460 significantly upregulated genes in GBM. Loss-of-function screening of these upregulated genes using LN18 cells was performed to identify the significant target genes for glioma. Further investigations were performed using siRNA in LN18 cells and various functional assays were carried out on the selected candidate gene to understand further its role in GBM. We identified PROS1 as a candidate gene for GBM from the meta-analysis and RNAi screening. Knockdown of PROS1 in LN18 cells significantly induced apoptosis compared to siPROS1-untreated cells (p<0.05). Migration in cells treated with siPROS1 was reduced significantly (p<0.05) and this was confirmed with wound-healing assay. PROS1 knockdown showed substantial reduction in cell invasion up to 82% (p<0.01). In addition, inhibition of PROS1 leads to decrease in cellular proliferation by 18%. Knockdown of PROS1 in LN18 cells caused activation of both the extrinsic and intrinsic apoptotic pathways. It caused major upregulation of FasL which is important for death receptor signaling activation and also downregulation of GAS6 and other members of TAM family of receptors. PROS1 may play an important role in the development of GBM through cellular proliferation, migration and invasion as well as apoptosis. Targeting PROS1 in GBM could be a novel therapeutic strategy in GBM treatment.

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults with a median survival rate of only 15 months (1,2). GBM originates from the glial cells which subsequently evolved into tumors known as glioma (3). It is the deadliest primary brain tumor and is classified as grade IV astrocytoma by WHO criteria (1,4,5). The standard treatment for GBM consists of surgical removal, radiation followed by chemotherapy. Unfortunately, these treatments only gave minor improvements to the patients' survival particularly for the recurrent GBM (4).

GBM is resistant towards treatment due to the heterogeneous nature of the disease. These are contributed by the dysregulation of the core signaling pathways such as the ErbB, MAPK, mTOR and p53 signaling pathways (6-9). It was shown that the pathogenesis of GBM requires alteration of multiple genetic pathways and each of the primary and secondary GBMs has a unique combination of these genetic changes (5). Primary or de novo GBM frequently showed loss of heterozygosity (LOH) at 10q (70% of cases), EGFR amplification (36%), p16INK4a deletion (31%) and PTEN mutations (25%) (10). Mutations in the IDH1 and IDH2 genes are common in astrocytomas, oligodendrogliomas, oligoastrocytomas and secondary glioblastoma with prevalence of 50-80% of cases (11). In addition, TP53 and PTEN mutations are common in primary and secondary gliomas with a frequency of 28 and 65% for p53, 25 and 4% for PTEN respectively (10). Secondary GBM is generally initiated from diffuse astrocytomas. Some common molecular lesions associated with secondary GBM are TP53 mutations (60-65%) and gain of 7q arm (21-50%) with MET gene gain of function (47% in primary and 44% in secondary glioblastoma) being affected significantly and associated with poor prognosis (12). There are also reports on the involvement of aberrant intrinsic and extrinsic apoptotic pathways and the overexpression of anti-apoptotic proteins such as FLIPs, BCL2 and survivin which contribute to apoptotic resistance (13-17).

Key words: cancer, glioblastoma, RNA interference screening, PROS1

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The therapeutic strategy in GBM could be improved by targeting the multiple pathways involved. RNA interference (RNAi) is one of the attractive approaches and may result in the post-transcriptional knockdown of the genes of interest. Significant impact in in vitro experiments using RNAi has allowed the implementation of therapeutic approach using RNAi gene therapy in vivo (18). One of the major problems in GBM therapy is the difficulty for the drugs to cross the blood-brain barrier (BBB) hindering maximal drug distribution to the tumor site. To date, there are a few strategies being used to efficiently deliver siRNA through the BBB. The RNAi-based nanomedicine platform has been introduced at the pre-clinical stage (18). Based on spherical nucleic acid gold nanoparticle conjugates, which are densely packed, highly oriented siRNA duplexes targeting the oncoprotein Bcl2Like12 (BclL12) were used to neutralize the oncogene expression in GBM (19). There are many delivery systems that form complexes with siRNA including PEGylated immunoliposomes that carry siEGFR, recombinant adeno-associated virus carrying siHec-1 and lentiviral vectors carrying siTRAIL (20-22). An example of a molecular target that has made to clinical trial using siRNA and showed promising results is Tenascin-C (TN-C), which is overexpressed in the extracellular matrix (ECM) of GBM. It has been shown that dsRNA targeting TN-C mRNA could reduce the tumor size significantly and increase the survival rate by 11% (23,24).

Materials and methods

Meta-analysis of microarray datasets. We performed a meta-analysis on five microarray datasets from a cancer microarray database using an integrated data-mining platform, the Oncomine Research Edition (25). Data were filtered based on data source, cancer, the type of datasets and analysis. Candidate genes were selected based on the median rank and p<0.05. Candidate genes obtained from meta-analysis were then screened using synthetic lethal and functional assays were performed 48 h post-transfection.

Preparation of siRNA. ON-TARGETplus SMARTpool™ of PROS1 siRNA (GE Dharmacon) consisting of four different siRNA sequences were used in this experiment. The siRNA sequences used were: i) GCAUGGAAGUGAAAUUA; ii) GCAACAGGGCUUCACAAGUC; iii) UAUUGAGCUCAUCAUGU; and iv) GAAGAGUGUAGGUGU. Lyophilized PROSI siRNA was resuspended with 1X siRNA buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). A total of 25 nM final concentration of PROS1 siRNA and non-targeting siRNA were used with DharmaFECT2. All functional assays were performed 48 h post-transfection.

RNA extraction and qPCR. RNeasy kit (Qiagen, Hilden, Germany) was used to isolate total RNA from cells. The quality and quantity of the isolated RNA were determined using NanoDrop (Thermo Fisher Scientific, Inc.). Briefly, 100 ng of RNA was used to generate cDNA using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). qPCR was conducted using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.) on a Rotor-Gene 3000 (Corbett Life Science/Qiagen, Inc., Valencia, CA, USA) platform. The PROS1 primers used were: forward, 5' -TGCTGGCGTGTCTCCTCCTA -3' and reverse, 5'-GATCTGGCCTGCTCCTCCTA-3' and reverse, 5'-CAGTCTCTCGATGATCCTCTCTTCA-3'. The expression of PROS1-related genes such as GAS6, RhoA, Fasl, Tyro-3, Axl, and Mertk was also quantified using qPCR and results were calculated based on the ΔΔCt method (26). ACTB gene was used as the reference gene. Primer sequences are shown in Table I.

Viability assay. The CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corp.) which is based on the quantification of ATP present in the viable cells was used for viability assay. Cells were cultured for 24 and 48 h post-transfection. Subsequently, CellTiter-Glo® buffer was added onto the CellTiter-Glo® substrate, which was then loaded into the samples. The luminescent signal was captured at 570 nm using SpectraMax® L Luminescence Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Cellular viability was calculated based on the normalization between treated (siPROS1) vs. non-targeting siRNA cells from three independent experiments.

Proliferation assay. Proliferation assay was performed using the bromodeoxyuridine (BrdU) incorporation method (Millipore Corp., Billerica, MA, USA). Transfected cells were cultured for 24 h in the present of BrdU which was incorporated into newly synthesized DNA strand of the proliferating cells. The cells were then fixed, and incubated with anti-BrdU monoclonal antibody (Millipore Corp.) for 1 h. Goat anti-mouse IgG peroxidase was added onto the well. Incorporation of BrdU in the proliferating cells leads to colorimetric changes from clear to blue which was measured using Varioskan Flash Multimode Reader (Thermo Fisher Scientific Oy, Vantaa, Finland) at 450 nm wavelength.

Migration assay. The effect of PROS1 gene silencing on tumor cell invasion was investigated using QCM™ 3 μm 24-well Chemotaxis Cell Migration Assay kit (Millipore Corp.). Cells were seeded in the 24-well inserts at a density of 1x10^4...
The cells were cultured overnight in serum-free media. The cells were transfected with siPROS1 or non-targeting siRNA. Cells were harvested using a standard protocol as indicated in the Cyclestest™ Plus DNA Reagent Kit protocol (BD Biosciences, Mississauga, ON, Canada). Subsequently, cells were washed three times with wash buffer. Cells were then suspended in solution A containing trypsin. Solution B with trypsin inhibitor and RNase were then added into the cell suspension. Finally, solution C which contained propidium iodide (PI) was added. Flow cytometric analysis was performed using BD FACSAria™ (BD Biosciences, Franklin Lakes, NJ, USA). Data were analysed using ModFit LT software (Verity Software House, Inc., Topsham, ME, USA). The percentage of arrested cells was measured by the percentage of hypodiploid cells accumulated at the G0/G1, S, G2/M checkpoints of the cell cycle.

**Western blotting.** Protein expression of PROS1 was assessed using western blotting. Cells were treated with siPROS1 and proteins were harvested and extracted using radioimmunoprecipitation assay (RIPA) buffer. A total of 50 µg protein was loaded onto the Mini-PROTEAN® Precast Gels (Bio-Rad Laboratories, Inc.), and then transferred onto the Immobilon transfer membranes (Millipore Corp.). Membranes were then incubated with SuperBlock® (Thermo Fisher Scientific, Inc.) for 1 h at room temperature. After that, membranes were incubated overnight with PROS1 mouse monoclonal antibody (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C. The membranes were then washed three times with TBST. Membranes were then incubated with goat anti-mouse secondary antibody conjugated to alkaline phosphate (1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Prior to protein detection, the membranes were washed three times. Finally, proteins were detected using Pierce ECL and SuperSignal substrate (Thermo Fisher Scientific, Inc.). β-actin was used as an internal control.

**Protein array.** Protein array was conducted using the Human Apoptosis Array kit (RayBiotech, Norcross, GA, USA). Protein samples were extracted from 48 h post-transfection according to the manufacturer's protocol. The quantity of the protein isolated was determined using BCA Protein Assay (Thermo Fisher Scientific, Inc.). Briefly, protein was loaded into the chamber slides coated with 43 different types of apoptosis antibodies. Subsequently, the slides were washed and the membranes were incubated with a cocktail of biotin-conjugated anti-apoptotic protein antibodies. The membranes were incubated with HRP-streptavidin prior to signal detection.

**Statistical analysis.** All data were expressed as the mean ± SD of three independent experiments. Significant differences were defined as p<0.05. All statistical analyses were performed using Microsoft Excel and the Statistical Package for the Social Sciences (SPSS) software.

### Results

**PROS1 as a novel candidate for GBM therapy.** Meta-analysis on five microarray datasets (Bredel Brain 2, Lee Brain, Liang Brain, Shai Brain, and Sun Brain) identified 460 upregulated genes based on the median rank and p<0.05. All datasets were normalized between cancers vs. normal tissues. These 460 genes were used as candidates for RNAi screening. Based on the SSMD and kMAD analyses, 212 hits were identified. After selection, PROS1 was identified as a target gene for validation since the role of PROS1 in GBM has not been documented (Fig. 1).

**PROS1 silencing decreases PROS1 mRNA and protein expressions.** The efficiency of PROS1 silencing was assessed using qPCR. The results showed that ~80% of the PROS1 gene was

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**Table I. Primer sequences used for qPCR analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’→3’)</th>
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<tr>
<td>PROS1</td>
<td>F TGCTGGCCTGCTCCTCTCATA R CAGTCTCTCGATGCTCTTTCA</td>
</tr>
<tr>
<td>Tyro-3</td>
<td>F CACGGTAGAGGTGTCGCAT R TGTTAAACAGTTCAAGGGGA</td>
</tr>
<tr>
<td>Axl</td>
<td>F TTATGTCTACGGCGAATGGG R TGTCCATTAGCAAACCTGGG</td>
</tr>
<tr>
<td>Mertk</td>
<td>F GTCCATCTCAGCATTGCCAC R CCTCACTGATAGCTAGGCC</td>
</tr>
<tr>
<td>Gas6</td>
<td>F ACGAAGCGAGACCGATAAT R GCGAGCTCGTGGTTTTG</td>
</tr>
<tr>
<td>FasL</td>
<td>F CCTTGTTAGATTGGCGCCTG R CTGTGTGCATCTGGCTGGTA</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
Figure 1. Meta-analysis and RNAi screening identified PROS1 as a novel candidate for GBM therapy. (A) Meta-analysis of PROS1 expression on five microarray datasets from Oncomine database. (B) A total of 212 hits overlapped by using SSMD and kMAD methods. (C) High-throughput RNAi screening shows that PROS1 gene silencing significantly reduced cell viability in GBM cells. *P<0.05. (D) The expression of PROS1 in different parts of the brain. RNAi, RNA interference; GBM, glioblastoma multiforme.

PROS1 silencing results in reduced PROS1 protein expression. (A) The efficiency of PROS1 gene knockdown was 80% at 24 h and increased up to ~100% at 48 h. (B) Western blotting of PROS1 was performed to determine the knockdown efficiency at protein level. The results showed that the PROS1 protein expression was depleted 48 h post-transfection.

knocked down after 24 h and it increased up to 100% after 48 h of transfection. This was further confirmed at protein level via western blotting at 48 and 72 h post-silencing as the expression of the PROS1 protein was reduced significantly compared to control (Fig. 2).

**PROS1 silencing reduces cell viability and cell proliferation.** Cell viability assay was conducted using CellTiter-Glo® Luminescent Cell Viability Assay to determine the effect of PROS1 gene silencing. The number of viable cells was reduced in a time-dependent manner. The quantification of proliferating cells by BrdU showed that the proliferation signal was decreased in siPROS1 treatment (18%) compared to the control (Fig. 3).

**PROS1 silencing inhibits LN18 cell migration.** Migration was reduced significantly (p<0.05) in siPROS1-treated LN18 cells. The scratch assay demonstrated the inhibition of the migratory potential of the 24-h post-scratch siPROS1-treated LN18 cells. Surprisingly, the size of wound scratch remained up to 72 h. These data suggest the possible role of PROS1 in GBM cell migration (Fig. 4A and B).

**PROS1 silencing reduces GBM cell invasion.** Cell invasion assay was performed to study whether PROS1 suppression could influence the invasion of LN18 cells. The results showed that the invasion of LN18 cells through the ECM was inhibited with siPROS1-treated cells up to 82% (p<0.01) compared to the control group (Fig. 4C).

**PROS1 silencing significantly induces cell death through apoptosis.** ELISA-based assay was conducted to determine the mode of cell death in siPROS1-treated LN18 cells. Apoptosis was increased compared to the control at 48 h post-transfection.
There was no evidence of cell cycle arrests identified from the cell cycle assay (data not shown). Further validation was conducted using protein array to elucidate the relevant pathways involved in this process.

**Figure 3.** *PROS1* gene silencing significantly reduces viability and cell proliferation. (A) *PROS1* gene silencing resulting in reduced GBM cell viability up to 40% at 48 h post-transfection, *P*<0.05. (B) Treatment of GBM cells with siPROS1 showed decreased cell proliferation at 48 h post-transfection by BrdU assay. GBM, glioblastoma multiforme; BrdU, bromodeoxyuridine.

**Figure 4.** *PROS1* silencing in GBM cells exhibits decreased migratory potential and may become a potential target for cell invasion. Silencing of *PROS1* gene showed a significant reduction in cell migration and invasion in GBM cells at 48 h post-transfection. (A) The morphological observation of *PROS1* gene silencing effects on wound healing assay in GBM cells shows a clear gating in siPROS1 treatment scratched cells at 0-72 h compared to control. (B) *PROS1* gene silencing significantly reduces migratory potential through membrane in GBM cells. (C) *PROS1* gene silencing significantly controls cancer invasiveness by reducing the invasion capability in GBM cells, *P*<0.05. (D) *PROS1* gene silencing reduced the mRNA expression of *Gas6* and *TAM* receptors. (E) Mapping of physical interaction between *PROS1*, *Gas6* and *TAM* receptors was generated by GeneMANIA. GBM, glioblastoma multiforme.
Figure 5. The PROS1 gene silencing significantly induces cell death through apoptosis by activating both intrinsic and extrinsic pathways. (A) Silencing of PROS1 resulted in an apoptosis signal at 48 h post-transfection. *P<0.05. (B) siPROS1 treatment induced formation of apoptotic bodies. (C) Most significant hits induced by PROS1 gene silencing in apoptosis array indicate the activation of both intrinsic and extrinsic pathways.

Figure 6. The working model of intrinsic and extrinsic pathways induced by PROS1 gene silencing. In the intrinsic pathway, PROS1 silencing activates BAD which is a BCL2-associated antagonist of cell death and then activates the mitochondrial pathway by releasing SMAC protein. The overexpression of SMAC will suppress the expression of IAP family which consists of XIAP and survivin and then induce apoptosis via downstream of apoptosis caspases. In the extrinsic pathway, an overexpression of FasL/Fas activated caspase-8 and induced apoptosis via downstream of apoptosis caspases. In the presence of PROS1, AKT will be activated and then phosphorylated BAD protein. The activation of AKT also suppresses the expression of FasL/Fas and then blocks the caspase-8 activation. Previously it was shown that the activation of AKT promotes overexpression of apoptosis inhibitors like BCL2 and IAP family and also suppresses the expression of pro-apoptotic proteins like BAD and BAX. IAP, inhibitor of apoptosis protein; FasL, Fas ligand.
**Discussion**

The main aim of this study was to understand the functional role of **PROS1** in GBM by performing silencing experiments coupled with various functional assays. **PROS1** was identified as a potential gene target for GBM from our meta-analysis using five microarray datasets and the loss-of-function RNAi screening of 460 upregulated genes. **PROS1** is a vitamin K-dependent plasma protein and is known to be involved in the anticoagulant cascade. It acts as a cofactor for anticoagulant protease in the blood coagulation system known as the activated protein C (APC) (27). **PROS1** shares ~43% of amino acid identity with GAS6, a γ-carboxyglutamatic acid (Gla)-containing protein, which stimulates cell proliferation through activation of the Axl receptor tyrosine kinase (28-30). **PROS1** and GAS6 are ligands for Axl together with Tyro-3 and Mertk and were reported to be overexpressed in haematological malignancies and solid tumors, suggesting that these molecules activate important autocrine-based oncogenic signaling events in cancer cells (31-34). Overexpression of TAM receptors mediates multiple oncogenic phenotypes in GBM such as in *vitro* proliferation, anchorage-independent growth, xenograft growth, resistance to apoptosis, autophagy, invasion and migration as well as activation of the downstream PI3K and MAPK survival pathways (33). Inhibition of Mertk and Axl by gene knockdown in astrocytoma cells increased invasion and migration of these cells (29). Furthermore, the high-throughput wound healing screening on the epithelial cells revealed the involvement of TAM receptors in cell migration (41). Our results are consistent as we showed that **PROS1** gene silencing delayed the wound enclosure in GBM cells and significantly reduced the capability of cells to migrate. This might be due to the involvement of the extracellular domains of TAM receptors that contain adhesion molecule-like motifs which controls cell-cell contacts and actin cytoskeleton regulation (42). Our results showed that silencing of **PROS1** expression also led to a significant reduction in cell invasion through the ECM in GBM cells. These findings suggest that **PROS1** may provide a survival advantage for advanced stage cancer like prostate and GBM by controlling cancer cell migration and invasion. In summary, we showed that silencing **PROS1** reduces survival, migration and invasion of GBM cells (as detailed in Fig. 6). It also activates apoptosis in GBM cells by activating the intrinsic and extrinsic apoptotic pathways. Further validation using *in vivo* studies are needed to enhance our understanding on the mechanistic role of **PROS1** in GBM cells. This will hopefully allow the development of **PROS1** gene therapy as a possible approach to increase patient survival and improve the treatment of GBM patients.

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**References**