Original Paper

Nuclear SMAD2 Restrains Proliferation of Glioblastoma

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Key Words

Glioblastoma multiforme (GBM) • Transforming growth factor β (TGF β) • SMAD2 • SMAD3 • p21 • Cancer cell growth

Abstract

Aims: Although TGF β receptor signaling has been shown to play a role in regulation of the growth and metastasis of glioblastoma multiforme (GBM), the downstream pathway through either SMAD2 or SMAD3 has not been elucidated. In this study, we investigate whether nuclear SMAD2 can restrain the proliferation of glioblastoma. **Methods:** A total of 23 resected specimens from GBM patients were collected for SMAD2 detection. Human GBM cell line A172, U87mg, D341m and Hs683 were maintained in Dulbecco's modified Eagle's medium and transfected with SMAD2 and SMAD3 shRNA plasmids. Gene expression was detected by RT-qPCR and Western and cell growth were detected by MTT assay. **Results:** Our results showed that the phosphorylated SMAD2 (pSMAD2, the nuclear and functional form of SMAD2) levels in GBM were significantly lower than the paired normal brain tissue in patients. Depletion of SMAD2, but not SMAD3, significantly abolished the inhibitory effects of TGF β 1 on the growth of GBM cells, possibly through pSMAD2-mediated increases in cell-cycle inhibitor, p27. **Conclusion:** Our data suggest that TGF β /SMAD2 signaling cascades restrains growth of GBM.

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Introduction

Glioblastoma multiforme (GBM), which is named as glioblastoma by WHO, is the most common and most malignant primary brain cancer in humans [1-5]. GBM is originated from glial cells and the patients suffer from an extremely low five-year survival ratio, which reasons from the relatively high invasiveness of GBM and the location of the tumor in the relatively

closed central nervous system [1-5]. Hence, understanding of the molecular control of the growth of GBM represents one of the most important questions in the research in GBM, in which it may provide innovative therapy for the disease.

Transforming Growth Factor β (TGF β) receptor signaling pathways play essential and diverse roles in the regulation of many biological events, including cell replication, differentiation, apoptosis, epithelial-mesenchymal transition, and tissue remodeling [6-12]. Binding of a TGF β ligand (1, 2 or 3) to a type II TGF β receptor induces the phosphorylation of a type I TGF β receptor, which subsequently phosphorylates receptor-regulated SMADs proteins (SMAD2 and SMAD3). Phosphorylated SMAD2 or SMAD3 then forms heteromeric complexes with the common-mediator SMAD (SMAD4) to be translocated into the nucleus [6-12], where they regulate the gene transcription, e.g. activation of p21 [13-18] and p27 [12, 19-21]. Although TGF β receptor signaling pathway has been extensively studied and is suggested to play a critical role in controlling growth and invasion of GBM [22-38], the signaling pathway through SMAD2 or SMAD3 has not been distinguished yet.

Here, we showed that the phosphorylated SMAD2 (pSMAD2, the nuclear and functional form of SMAD2) levels in GBM were significantly lower than the paired normal brain tissue in patients. Depletion of SMAD2, but not SMAD3, significantly abolished the inhibitory effects of TGF β 1 on the growth of GBM cells, possibly through pSMAD2-mediated increases in cell-cycle inhibitor, p27.

Materials and Methods

Patient tissue specimens

A total of 23 resected specimens from GBM patients were collected for this study. All specimens had been histologically and clinically diagnosed at Department of Neurosurgery of the First People's Hospital of Zunyi from 2007 to 2013. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell line culture and transfection

Human GBM cell line A172 (GBM) [39], U87mg (GBM/astrocytoma) [40], D341m (medulloblastoma) [41] and Hs683 (Glioma) [41] have been described before and were used in the current study. Since similar results were achieved from these cell lines, only data from A172 cells were shown. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (PAA, Austria). SMAD2 and SMAD3 shRNA plasmids (sc-38374-SH and sc-38376-SH) were purchased from Santa Cruz (St Jose, CA, USA). Transfection was performed with Lipofectamine 2000 reagent (Invitrogen), according to the instructions of the manufacturer. SB431542 [42-44] was purchased from Sigma-Aldrich (St Louis, MO, USA), and used at a dose of 10 μ mol/l.

RNA extraction, reverse transcription and RT-qPCR

Total RNA was extracted from the cultured cells using RNeasy kit (Invitrogen), according to the manufacturer's instruction. For mRNA analysis, complementary DNA (cDNA) was randomly primed from 2 μ g of total RNA using the Omniscript reverse transcription kit (Qiagen, Hilden, Germany). Real-time quantitative PCR (RT-qPCR) was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SyBr green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. All primers were purchased from Qiagen. Data were collected and analyzed using the Rotorgene software accompanying the PCR machine. Relative expression levels were determined using the comparative quantification feature of the Rotorgene software. All mRNA quantification data were normalized to α -tubulin, and then compared to controls.

Western blot

Protein was extracted from the cultured cells by RIPA buffer (Sigma-Aldrich) for Western Blot. The supernatants were collected after centrifugation at 12000 $\times$ g at 4°C for 20min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4 $\times$ SDS

loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100 °C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are anti-human SMAD3, phosphorylated-SMAD3 (pSMAD3), SMAD2, phosphorylated SMAD2 (pSMAD2), p27 and α -tubulin (Cell Signaling, St Jose, LA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson Labs, LA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson Labs, Bar Harbor, ME, USA). Images shown in the figure were representative from 5 repeats. Densitometry of Western blots was quantified with NIH ImageJ software.

MTT assay

For assay of cell growth, the cells were seeded into 96 well-plate at 4000 cells per well and subjected to a MTT kit (Roche, Indianapolis, IN, USA), according to the manufacturer's instruction. The MTT assay is a colorimetric assay for assessing viable cell number, since NAD(P)H-dependent cellular oxidoreductase enzymes in viable cells are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which is in purple color and could be quantified.

Statistical analysis

Each experiment condition contains 5 repeats. All values are depicted as mean $\pm$ standard deviation and are considered significant if $p < 0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferoni correction.

Results

Lower phosphorylated SMAD2 was detected in GBM, compared to paired normal brain tissue in patients

Since TGF β receptor signaling has been shown to play a critical role in controlling growth and invasion of GBM but the levels of the functional form of SMAD2 (phosphorylated SMAD2 (pSMAD2)) that represent the activity of TGF β receptor signaling has not been evaluated in the GBM patients, we thus analyzed pSMAD2 levels in the resected GBM tissue from 23 patients, and compared with the paired normal brain tissue (NBT) by Western blot. Six specimen were randomly selected and shown as representative images (Fig. 1A). We detected significantly lower pSMAD2 levels in GBM, compared to NBT (Fig. 1B). These data suggest a possibility of involvement of SMAD2 signaling in the pathogenesis of GBM.

Fig. 1. Lower phosphorylated SMAD2 was detected in GBM, compared to paired normal brain tissue in patients. Phosphorylated SMAD2 (pSMAD2) levels in the resected GBM tissue from 23 patients were analyzed, and compared with the paired normal brain tissue (NBT) by Western blot, shown by representative images (A), and by quantification (B). * $p < 0.05$.

Fig. 2. Preparation of GBM cells with SMAD2 or SMAD3 depletion. SMAD2, or SMAD3 in GBM cells was depleted by shRNAs. GBM cells that were transfected with scrambled sequence (scr) were used as controls. Modification of SMAD2 or SMAD3 levels in A172 cells was confirmed by RT-qPCR (A), and by Western blot (B). * $p < 0.05$. NS: non-significant.

Fig. 3. Depletion of SMAD2, but not SMAD3, significantly abolished the activation of p27 by TGFβ1. Proteins in GBM cells of either SMAD2 or SMAD3 depletion in responsive to 10ng/ml TGFβ1 were examined by Western blot. A specific TGFβ receptor inhibitor, SB431542 at a dose of 10μmol/l, was applied with TGFβ1 to block the signaling at the level of receptor phosphorylation.

Preparation of GBM cells with SMAD2 or SMAD3 depletions

In order to examine the downstream signaling of activation of TGFβ receptor, we depleted either SMAD2, or SMAD3 in GBM cells by shRNAs, since phosphorylation of each may transduce the signals from TGFβ receptor activation. GBM cells that were transfected with scrambled sequence (scr) were used as controls. We examined 4 commonly used GBM cell lines, with different cell origin. Since similar results were achieved from these cell lines, only data from A172 cells were shown. We confirmed the modification of SMAD2 or SMAD3 levels in A172 cells by RT-qPCR (Fig. 2A), and by Western blot (Fig. 2B). Importantly, depletion of SMAD2 did not affect the levels of SMAD3 in A172 cells, and vice versa (Fig. 2A-B). Thus, these cells with SMAD2 or SMAD3 depletion allow us to evaluate the roles of SMAD2 and SMAD3 in the growth of GBM in responsive to TGFβ receptor activation.

Depletion of SMAD2, but not SMAD3, significantly abolished the growth inhibitory effects of TGFβ1 on the growth of GBM cells, possibly through pSMAD2-mediated increases in cell-cycle inhibitor, p27

Then we examined the GBM cells of either SMAD2 or SMAD3 depletion in responsive to TGFβ receptor activation. TGFβ1 is potential ligand for TGFβ receptor and triggers robust receptor phosphorylation and activation. We thus used 10ng/ml recombinant TGFβ1 to

Fig. 4. Depletion of SMAD2, but not SMAD3, significantly abolished the growth inhibitory effects of TGF β 1 on the growth of GBM cells. In a MTT assay, pSMAD2 activation, but not pSMAD3 activation, seemed to inhibit the growth of GBM cells in responsive to TGF β 1 stimulation. * $p < 0.05$. NS: non-significant. Asterisks and "NS" above the columns are statistical comparisons to same cells without any treatments (TGF β 1- and SB431542-). Asterisks and "NS" under the columns are statistical comparisons to A172-scr that received same treatments (TGF β 1 and SB431542).

Fig. 5. Schematic of the model.

TGF β receptor [32, 45, 46]. We also used a specific TGF β receptor inhibitor, SB431542 at a dose of 10 μ mol/l, to block the signaling at the level of receptor phosphorylation.

We found that TGF β 1 induced significant phosphorylation of both SMAD2 and SMAD3 in A172-scr cells, but only induced significant phosphorylation of SMAD2 in SMAD3-depleted A172-shSMAD3 cells, or significant phosphorylation of SMAD3 in SMAD2-depleted A172-shSMAD2 cells (Fig. 3). The inductions of SMAD phosphorylation were completely inhibited by SB431542-mediated blockage of its upstream TGF β signaling, the receptor phosphorylation (Fig. 3). Interestingly, depletion of SMAD2, but not SMAD3, significantly abolished the pSMAD2-mediated increases in cell-cycle inhibitor, p27 (Fig. 3) [12, 20, 47-49]. Moreover, in a MTT assay, pSMAD2 activation, but not pSMAD3 activation, seemed to inhibit the growth of GBM cells in responsive to TGF β 1 stimulation (Fig. 4), which was possibly mediated by p27 upregulation (Fig. 5).

Discussion

The TGF β receptor signaling plays a critical role in the tumorigenesis of various human tumors in that it controls cancer cell growth, invasion and metastasis [6-12, 22-31]. Since growth of GBM accounts for its major malignancy and detrimental effects on patients [1-5], here we aimed to elucidate the precise downstream signaling of an activated TGF β receptor signaling on the growth of GBM cells [6-12, 22-31].

Four GBM cell lines that represent different cancer cell type of origin were analyzed, showing similar results within the scope of the current study. Thus, a cell-line dependent possibility was excluded. Since SMAD2 and SMAD3 are the only two direct targets of an activated TGF β receptor signaling, we depleted each and checked the effects on the cancer cell growth in responsive to TGF β 1 stimulation, the effect of which was validated by a receptor phosphorylation inhibitor upstream of SMAD2/3 phosphorylation.

Interestingly, we found that depletion of SMAD2, but not SMAD3, significantly abolished the inhibitory effects of TGF β 1 on the growth of GBM cells, possibly through pSMAD2-mediated increases in cell-cycle inhibitor, p27. The control of p27 by pSMAD2 has been well-established [12, 20, 47-49]. Since pSMAD2 forms a complex with SMAD4 and then

translocates into the nucleus to allow its direct binding to DNA to modulate gene expression, e.g. p27, our data suggest that pSMAD2, or nuclear SMAD2, has a potential effect in restraining the growth of GBM. Since p21 is also known to be regulated by SMAD signaling [13-18], we also examined p21 in our study but got negative results. Thus, p27, but p21 is the direct target of SMAD2 signaling in this model. Thus, biological or chemical induction of SMAD2 phosphorylation and nuclear translocation and/or retention may substantially inhibit the growth of GBM, which sheds light on a promising novel therapy.

Our innovative findings that differentiate pSMAD2 from pSMAD3 in the growth of GBM are critical, since SMAD3 signaling has been extensively studied and found mainly related to cell differentiation and transition [6-12, 22-31]. Thus, the effect of TGF β receptor signaling may have its effects on cell growth through pSMAD2-mediated regulation of cell-cycle controllers, and on cell migration and invasion through pSMAD3-mediated EMT-related genes. These hypothesis needs to be confirmed in different cancers, and in different models. If proved, it may provide substantially critical information for delicate control of the complex TGF β receptor signaling pathway, not only in cancer, but also in other biological researches.

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Disclosure Statement

The authors have declared that no competing interests exist.

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