Targeting survivin suppresses proliferation and invasion of retinoblastoma cells \textit{in vitro} and \textit{in vivo}

Kuixiang Liu$^{1,2}$, Yuanyuan Liu$^1$, Guiqiu Zhao$^1$

$^1$Department of Ophthalmology, Qingdao University, Qingdao, China; $^2$Department of Ophthalmology, The Eighth People’s Hospital of Qingdao, Qingdao, China

Received May 4, 2017; Accepted July 13, 2017; Epub September 1, 2017; Published September 15, 2017

Abstract: Survivin is a member of the inhibitor of apoptosis (IAP) family and has multifunctional properties that include aspects of proliferation, invasion and cell survival control. Survivin is a promising candidate for targeted cancer therapy as its expression is associated with poor clinical outcome and more aggressive clinicopathologic features. Retinoblastoma (RB) is a highly invasive malignant tumor that often invades the brain and metastasizes to distal organs through the blood stream. However, expression of survivin in RB has not been previously characterized. In addition, whether survivin could be used for targeted RB therapy is not clear. In the present study, we demonstrated that RB tumors with invasion showed significantly higher expression of survivin compared to tumors without invasion ($P < 0.05$). High-risk tumors showed significantly increased expression of survivin compared to tumors with low risk ($P < 0.05$). Survivin inhibition by targeted siRNA suppresses the proliferation, growth, invasion, migration and induced apoptosis of retinoblastoma Y79 cells \textit{in vitro}. In addition, Survivin inhibition by targeted shRNA suppresses \textit{in vivo} orthotopic tumors and liver metastasis in BALB/c nude mice. In line with these results, surviving siRNA (shRNA) effectively induces down-regulation of target genes of surviving by western blot, RT-PCR and immunohistochemistry analysis. In conclusion, high survivin expression is associated with invasion and metastasis in RB. We suggest that survivin inhibition could be a potential therapeutic approach in retinoblastoma through suppressing tumor proliferation and invasion.

Keywords: Retinoblastoma, invasion, proliferation, survivin

Introduction

Retinoblastoma (RB), a common primary intraocular tumor, occurs in infants and children with a relative incidence of 3% of all pediatric tumors worldwide [1]. Newer molecules and pathways have to be identified for designing novel targeted therapies in managing RB to avoid enucleation and to prevent metastasis [2].

Survivin is a well-established target in experimental cancer therapy. The molecule is over-expressed in most human tumors, but hardly detectable in normal tissues [3]. Multiple functions in different subcellular compartments have been assigned. It participates in the control of cell division, apoptosis, the cellular stress response, and also in the regulation of cell migration and metastasis [4]. Survivin levels were higher in aqueous humor and serum of retinoblastoma (RB) patients than the controls. In aqueous, it was significantly correlated with the tumor stage and optic nerve affection [5]. Ferrario et al. has reported that targeted inhibition of survivin sensitizes Rb cells to chemotherapy [6]. These factors suggest that survivin is a potential therapeutic target [7].

RNA interference (RNAi) by small interfering RNA (siRNA) can be used to reduce target gene expression in a sequence specific manner by degradation of the corresponding mRNA [8]. After uptake by cells, siRNA is loaded into a RNA-induced silencing complex (RISC) [9]. The passenger strand is then degraded and the remaining strand (guide strand) binds to a complementary RNA molecule, which is then degraded. Gene silencing induced by siRNA is highly efficient and specific to the target gene and therefore has potential application in cancer treatment [10].
Our hypothesis is that survivin down-regulation, using short interfering RNAs (siRNA), will prevent or suppress retinoblastoma tumor development in vivo and in vitro.

Materials and methods

Patients and tissue samples

This study was approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University, Qingdao, China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards. 48 tumors were available from 48 eyes for the present study. Among them, there were tumors from 34 males and 14 females. The age ranged from 1 month to 7.5 years, (median =21 months). There were 36 unilateral retinoblastomas and 10 bilateral retinoblastomas.

Immunohistochemistry

For standard immunohistochemistry analysis, retinoblastoma tissue samples were fixed in 4% formalin and dehydrated. Then, the tissues were embedded in paraffin. Immunohistochemistry assays were performed on 4-μm thick sections from each paraffin-embedded retinoblastoma specimen. After dewaxing and rehydrating the sections, antigen retrieval was performed in 10 mM citrate buffer at 100°C for 5 min. Next, endogenous peroxidase activity was blocked using 3% H2O2 in methanol for 10 min. Then, the sections were incubated at 4°C overnight with mouse polyclonal anti-survivin (1:100; Hangzhou, China). After three washes with phosphate buffered saline (PBS), the sections were incubated with an anti-mouse secondary antibody for 1 h at room temperature, followed by detection using streptavidin-horse-radish-peroxidase. Finally, the sections were counterstained with hematoxylin. The survivin-positive cells showed immunoreactivity in the cytoplasm of tumor cells. Randomly 10 vital tumor fields were scanned for protein expression and percentage of positive tumor cells was noted for each field. Then finally the average expression was calculated from the 10 values for the entire slide. Depending on the percentage of positive cells, 2 categories were established: Negative, positive cells in < 10%; Positive, positive cells in ≥ 10% of total tumor cell population.

Cell lines

Y79 cells (American Type Culture Collection, Shanghai, China) were maintained in RPMI-1640 (Thermo) with 10% fetal bovine serum (FBS) at 37°C in the humidified atmosphere of 95% air and 5% CO2.

siRNA transfection

Y79 cells were transfected with survivin siRNA or control siRNA (100 nM) complexed to lipofectamine 2000 (invitrogen, Waltham, MA, USA) for 48 h as the manufacture’s instruction. survivin siRNA1 target sequence: GCACAUACCGCCUGAGUCU; survivin siRNA-2 target sequence: CCACCAAGGUUUUCGAUUG. Non-silencing siRNA (GE healthcare) was used as controls.

Lentiviral vectors for survivin downregulation and stable clones

The replication incompetent lentiviral vectors expressing short hairpin RNA (shRNA) for silencing Survivin (two shRNAs) and lentiviral vectors expressing control non-targeting shRNA were from OpenBioSystem Thermo Scientific (Pittsburgh, PA). Y79 cells were transfected with lentiviral vectors according to manufacturer’s instructions. Stable Y79 cell clones expressing the above shRNAs were selected and maintained in medium containing 9 μg/ml puromycin according to the manufacturer’s instructions, and screened by Western blot analysis (for protein level) and RT-PCR (for mRNA level).

Western blot analysis

Cells were washed with cold PBS, lysed with ice-cold lysis buffer and incubated on ice for 30 min. Lysates were centrifuged, supernatants were collected, and protein concentration was determined using Bio-Rad Protein Reagents (Bio-Rad, Hercules, CA). Protein lysates (30 μg) were separated by SDS-PAGE, blotted onto membranes, and probed with the appropriate dilution of each primary survivin antibody. Membranes were rinsed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody, rinsed again, and the bound antibodies were detected using.
Effect of targeting survivin on retinoblastoma

enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) following by autoradiography in a FluorChem™ 8900 (Alpha Innotech Corporation, San Leandro, CA).

RT-PCR analysis of survivin

Total RNAs were isolated and used for RT-PCR using the forward (5'-CAGTTCTTGAATGTAGAGATGCCGGT-3') and reverse (5'-TGCCCGCAGTGTGCC-3') primers in 35 cycles of 1 min denaturation at 95°C, 30 s annealing at 55°C, and 1 min elongation at 72°C. PCR products were analyzed by agarose gel electrophoresis.

Cell proliferation assay

BrdUrd incorporation into cells was accomplished using a BrdUrd cell proliferation assay kit from Oncogene (San Diego, CA). Cells were seeded in 96-well plates (5 × 10^3 to 1 × 10^4 cells per well) and transfected with siRNA for 72 h. BrdUrd was added to the medium 10 h before treatment termination. The levels of BrdUrd incorporated into cells were quantified by anti-BrdUrd antibody, measuring absorbance at dual wavelengths of 450/540 nm with a microplate reader.

Growth inhibition assay

Cells were cultured in 96-well plates at a density of 5000 cells/well and left to recover. The quantity of viable cells was estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT (10 μL of 5 mg/mL solution, Sigma Chemical Co., Germany) was added to each well of the titration plate and incubated for 4 h at 37°C. The cells were then treated with DMSO (40 μL/well) and incubated for 60 min at 37°C. The absorbance of each well was determined in an enzyme linked immunosorbent assay (ELISA) plate reader using an activation wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of viable cells was determined by comparison to untreated control cells.

Detection of apoptosis

Following a treatment protocol similar to the one described above, cells in early and late stages of apoptosis were detected with an annexin V-FITC apoptosis detection kit from Bio Vision (Mountain View, CA) as the manufacture’s instruction.

Wound healing assay

Y79/survivin shRNA, Y79-shNC and Y79 cells were grown to form 60% confluent monolayers, in which a wound of about 0.3 mm width was set with a sterile pipette tip. The medium was exchanged to remove non-adherent cells. The progress of wound closure was monitored daily with microphotographs of 10 × magnification taken with the Leica DM IL light microscope and a Kappa CF 15/4 MCC-MLUII Modul camera (Leica Microsystems) up to day 2. The wound healing experiment was performed three independent times. The spreading of cells across the area of the wound at day 4 was quantified with ImageJ. The background value (day 1) was subtracted and the average values were normalized to Y79 cells.

Boydchen chamber transwell invasion assay

Y79/survivin shRNA, Y79-shNC and Y79 cells were used in cell migration and invasion analyses performed with Boyden chamber assay. 2.5 × 10^5 cells were seeded into each transwell chamber with filter membranes of 12 μm pore size (Millipore, Germany). For invasion, filter membranes were coated with Matrigel (BD Biosciences; diluted 1:3 in growth medium) 4 hours before cell seeding, and fresh medium was added to the bottom chamber. After 48 hours, insets were removed and cells, which had migrated through the membrane to the lower chamber, were trypsinized and counted in a Neubauer chamber (LO-Laboroptik, Bad Homburg, Germany). Each well was counted ten times. Each invasion experiment was performed in duplicate. The average number of migrated or invaded cells was determined from at least three independent experiments.

Subcutaneous xenografted Y79 cell tumor model

Y79/survivin shRNA, Y79-shNC and Y79 clones grew to 80% confluence, and were harvested via trypsinization, washed once in PBS, and resuspended to a final concentration of 5 × 10^6 cells/ml. An s.c. xenografted model injected with Y79/survivin shRNA or Y79-shNC or Y79 cells was prepared. Body weights and tumor volume were measured twice weekly starting on the first day of treatment until 6 weeks. Tumor size was measured using Vernier calipers and tumor volume was calculated as 0.5 ×
Effect of targeting survivin on retinoblastoma

longest diameter × width². TUNEL staining for tumor tissue was based on the protocol of the Dead End Colorimetric TUNEL System (Promega). The tissue sections were viewed at ×100 magnification and images were captured with a digital camera. Four fields per section were analyzed, excluding peripheral connective tissue and necrotic regions. Percentage of apoptotic cells was defined as TUNEL-positive cells among 1000 tumor cells. Percentage of TUNEL-positive cells in each group were calculated from three tumor specimens.

In vivo antitumor activity against peritoneally disseminated Y79 cell tumors Stably transfect-ed shRNA-expressing cell lines, 3 × 10⁶ cells (Y79/survivin shRNA or Y79-shNC or Y79) were inoculated into the peritoneal cavity, respectively. Mice were sacrificed at day 28. Livers (metastasis target organ) were removed and shock-frozen in liquid nitrogen. The level of liver metastasis was evaluated by scoring. The score for each liver was calculated as the sum of the volumes V of the individual metastases, calculated by $V = L^2 \times W$ (with L as the length of the smaller axis, and W as the length of the larger axis). Luminescence signals of isolated organs were quantified with ImageJ.

Statistics

Differences in data from the various groups were tested by the two-tailed unpaired t test using the SPSS 22 program (SPSS Inc, Chicago, USA).

Results

Table 1. The expression of survivin protein in high-risk and low-risk retinoblastoma

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>Survivin immunohistochemical staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Overall cohort</td>
<td>48</td>
<td>9</td>
</tr>
<tr>
<td>High-risk tumors</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Low-risk tumors</td>
<td>35</td>
<td>7</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Immunohistochemistry analysis showed survivin immunoreactivity was observed in the cytoplasm of retinoblastoma specimens (Figure 1A and 1B). survivin immunoreactivity was observed in 81.2% (39/48) of the retinoblastoma specimens. We observed that tumors with invasion showed significantly higher expression of survivin compared to tumors without invasion (t test, $P < 0.05$). No massive invasion of choroid or optic nerve is lower risk; Tumors with invasion of choroid/optic nerve/orbit is higher risk. The immunoreactivity of survivin in high-risk and low-risk tumors is shown in Table 1. High-risk tumors showed significantly increased expression of survivin compared to tumors with low risk (t test, $P < 0.05$).

Silencing survivin using siRNA reduces Y79 cell proliferation and viability, and induces apoptosis in vitro

To examine whether survivin is a strong candidate for RNAi in RB cells, we tested the gene silencing efficacy of survivin siRNA in Y79 cells. Survivin siRNA potently silenced survivin mRNA expression in Y79 cells by > 90% (t test, $P < 0.001$) (Figure 2A), 48 h post-transfection when complexed to the commercial transfection agent Lipofectamine 2000. Next, we assessed the effect of silencing survivin expression on Y79 cell proliferation. Y79 were transfected with survivin siRNA (100 nM). Seventy-two hours post-transfection cell lysates were collected and surviving expression
Effect of targeting survivin on retinoblastoma

Figure 2. Effect of siRNA on proliferation, viability, and apoptosis in Y79 cells in vitro. Y79 were transfected with survivin siRNA (100 nM) for 0-72 h. A. Survivin mRNA was detected in Y79 cells by RT-PCR assay; B. Survivin protein was detected by western blot assay; C. Cell growth rate was measured by the MTT assay. D. Cell proliferation was detected by BrdUrd incorporation. Relative levels of BrdUrd incorporation were expressed as percentage of Silentfect control. E. Cell apoptosis was detected with Annexin V/PI staining. Representative results of three experiments with consistent results are shown. Vs control, *P < 0.001.
Effect of targeting survivin on retinoblastoma

Survivin protein expression was reduced in Y79 cell lines compared to controls (Figure 2B). Furthermore, knockdown of survivin significantly inhibited cell proliferation in Y79 cell lines (Figure 2C). Notably, cell growth was reduced by > 70% (t-test, P < 0.001) in Y79 cells when compared to controls (Figure 2D).

Figure 3. Effect of siRNA on invasion and migration in Y79 cells in vitro. A. RT-PCR analysis of survivin mRNA in Y79/survivin shRNA, Y79-shNC and Y79 cells. B. Western blot analysis of survivin protein in Y79/survivin shRNA, Y79-shNC and Y79 cells. C. Boyden Chamber assay of cell migration. D. Wound healing assay of cell migration. Data represent the number of migrated parental and shRNA transfected cell lines normalized to the parental Y79 cell lines (P < 0.05).
To confirm whether the observed decrease in cell proliferation in Y79 cells following treatment with survivin siRNA was associated with increased cell death, we treated Y79 cell lines with survivin siRNA complexed to Lipofectamine 2000, and measured apoptosis by annexin V staining and flow cytometry. Silencing survivin expression using siRNA markedly increased cell death in Y79 cells, 72 h post-transfection when compared to cells treated with control siRNA (Figure 2E). This suggests that survivin may be playing an important role in regulating RB cell survival. Collectively, these results provide strong evidence that survivin is highly expressed in Y79 cells, and that silencing its expression using siRNA strongly inhibits cell proliferation via an induction of and cell death.

**Down-regulation of survivin by stable survivin shRNA transfection inhibits cellular migration and invasion in vitro**

We began our survivin knock down studies using Y79/survivin shRNA, Y79-shNC and Y79 cells. Survivin mRNA level showed a significant reduction to 5% in Y79-survivin shRNA cells, compared to Y79-shNC (P < 0.001) (Figure 3A). Western blotting of total cell lysates and immunostaining against survivin confirmed the decrease of endogenous survivin expression level in survivin-shRNA transfected cells, compared to the respective control cells (Figure 3B). The directed cellular migration was evaluated by closing an applied scratch in a cell layer, documented daily until day 4. Y79-shSurvivin cells showed a strong delay in wound closure compared to the control cell lines (Figure 3C). The closure of the wound was quantified by image analysis, resulting in a decrease of 35% in Y79-shSurvivin (P < 0.001), compared to the control cell lines. Consistent with this result, targeting survivin led to decreased penetration of Y79 cells via the Matrigel-coated membrane compared with the control cells (Figure 3D).

**In vivo antitumor activity of surviving shRNA xenografted Y79 cell tumors**

The in vivo antitumor activity of survivin shRNA was studied using s.c. tumor-bearing mice. As shown in Figure 4A, survivin shRNA inhibited growth of s.c. xenografted tumors, compared with the Y79-shNC and Y79 cell xenografted tumors (P=0.013). survivin shRNA showed 75% inhibition of tumor growth on 6 weeks. As shown in Figure 4B, in the s.c. xenografted tumors of mice treated with survivin shRNA, the expression levels of survivin was decreased compared with the Y79-shNC and Y79 cell xenografted tumors. TUNEL staining showed that cell apoptosis was significantly increased in the Y79/survivin shRNA xenografted tumors (Figure 4C).

Scoring of the liver metastases showed a significant decrease of number and size of metastases in the animals with transplanted Y79/survivin shRNA cells (P=0.018). Liver metastases of control mice had an average score of 29.4 (± 31.6), whereas liver metastases of the mice with transplanted Y79/survivin shRNA cells had an average score of 0.8 (± 2.4) (Figure 4D). Quantification of bioluminescence signal intensities of organs confirmed the reduction of liver metastases with transplanted Y79/survivin shRNA cells (P=0.027) compared to transplanted Y79-shNC cells (Figure 4E).

**Discussion**

Retinoblastoma (RB) is the most common intracocular malignant tumor arising in the retina of children, typically diagnosed at 2-3 years of age [11]. Despite the identification of the RB1 gene and the current insight into the function of pRB, the understanding of the sequence that leads to human retinoblastoma is still incomplete. Recent development of new animal models of retinoblastoma will increase the knowledge on tumorigenesis and provide an opportunity to develop treatment strategies [12]. Although retinoblastoma has a good prognosis in industrialized countries, mortality due to development of a second tumor remains high. Development of a non-mutagenic therapy (such as photodynamic therapy) could be interesting, particularly in case of hereditary retinoblastoma [13-15]. Gene therapy for treatment of retinoblastoma is under evaluation [16].

Survivin, a member of the inhibitor of apoptosis proteins (IAP) [17], plays an important role in cell apoptosis, mitotic regulation, and in the control of cell migration as well as metastasis [4]. Survivin is expressed in fetal and cancer cells, but not in normal adult cells. Survivin is highly expressed in breast, colorectal, lung, gastric and bladder cancers, as well as in melanoma, hepatocellular carcinoma and malignant
Figure 4. Application of survivin-shRNA-plasmids inhibited growth and reduced metastasis formation of xenografted tumors in vivo. A. Tumor volumes were measured two or three times a week, and tumor volume (mm³) was calculated as 0.5 × longest diameter × width². Tumor volumes are presented as means ± SD of 6 mice for each group (vs control, P=0.013). B. Immunohistochemical staining for survivin in xenografted tumors; C. TUNEL staining for apoptosis in xenografted tumors; D. The level of metastasis was evaluated by scoring. The score for each liver was calculated as the sum of the volumes of the individual metastasis. Data represent the mean liver metastasis score (n=6) ± SD of each group. E. Quantification of ex vivo bioluminescence intensities of isolated livers. Data represent mean intensities (n=6) ± SD of each group.
Effect of targeting survivin on retinoblastoma

lymphoma, and its expression in these cancers is associated with poor clinical prognosis [18-26]. However, whether survivin is highly expressed in RB remains unclear. To obtain more insight into the expression status and clinical significance of survivin in RB, we performed immunohistochemistry staining to explore the expression level of survivin and to analyze its association with clinicopathological parameters of RB patients. We observed that tumors with invasion showed significantly higher expression of survivin compared to tumors without invasion. High-risk tumors showed significantly increased expression of survivin compared to tumors with low risk. Thus, our data suggest that high survivin levels promote RB progression, and survivin may be a well-established target in RB therapy.

The role of survivin in RB was further investigated by detecting the alterations of biological behaviors in RB cell lines after survivin knockdown. Knockdown of survivin resulted in suppressed proliferation and growth in Y79 cells, concomitant with induction of apoptosis in vitro and vivo.

Metastasis of cancer is the major cause of death among cancer patients [27, 28]. In our study, wound healing and Transwell analyses demonstrated that knockdown of survivin expression markedly weakened the migration and invasion ability of Y79 cells compared with the negative control. The reduction of cell motility in vitro also suggests a decrease in the metastatic potential of survivin knock-down cells in xenografted mice. We transplanted the Y79-shNC cells into the peritoneal cavity of NOD/SCID mice, leading to liver metastases. In contrast, transplantation of Y79-shsurvivin cells with stable down regulation of survivin expression resulted in significantly less liver metastases in mice. In our study, we observed a significant decrease of liver metastases to less than 30% of the control group by systemic application of survivin-shRNA expression plasmids. This was caused by reduced survivin expression in tumors of RB xenografted mice.

In conclusion, RB with invasion showed significantly higher expression of survivin compared to tumors without invasion. High-risk tumors showed significantly increased expression of survivin compared to tumors with low risk. Our study showed that in Y79 cells, targeting survivin suppressed proliferation and invasion, while inducing apoptosis. The systemic application of survivin-specific shRNA decreased survivin expression levels in tumors and metastases, and, most importantly, reduced formation of liver metastasis. Thus, survivin could be a promising therapeutic target and novel molecular biomarker for RB.

Acknowledgements

This study was supported by grants from National Natural Science Foundation of China (No: 81170825).

Disclosure of conflict of interest

None.

Address correspondence to: Yuanyuan Liu, Department of Ophthalmology, Qingdao University, Qingdao, China. Tel: +86-532-82916783; E-mail: wujiangqdsd@sina.com

References

Effect of targeting survivin on retinoblastoma


