Overexpression of Biglycan is Associated with Resistance to Rapamycin in Human WERI-Rb-1 Retinoblastoma Cells by Inducing the Activation of the Phosphatidylinositol 3-Kinases (PI3K)/Akt/Nuclear Factor kappa B (NF-κB) Signaling Pathway

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Background:  
Biglycan (BGN) is an extracellular matrix (ECM) protein that regulates the growth of epithelial cells. The mammalian target of rapamycin (mTOR) inhibitor, rapamycin, is a treatment for advanced retinoblastoma. This study aimed to investigate the effects of expression of BGN on the response of human WERI-Rb-1 retinoblastoma cells to rapamycin and to investigate the associated signaling pathways.

Material/Methods:  
BGN gene expression was induced in human WERI-Rb-1 retinoblastoma cells, which were incubated with rapamycin at doses of 0, 5, 10, 20, 30, and 50 μg/ml. Cells were treated with the PI3K/Akt pathway inhibitor, LY294002. The MTT assay determined the rate of cell inhibition. Real-time polymerase chain reaction (RT-PCR) was performed to measure BGN gene expression using RT²-PCR. Western blot detected the protein levels of BGN, p-PI3K, p-Akt, nuclear NF-κB, and p65.

Results:  
Rapamycin impaired cell growth, induced cell apoptosis, and suppressed the expression levels of p-PI3K, p-Akt, nuclear NF-κB, and p65. Overexpression of the BGN gene restored growth potential and inhibited apoptosis and was associated with the activation of the PI3K/Akt-mediated NF-κB pathway. In cells that overexpressed BGN, inhibition of the PI3K/Akt pathway by LY294002 increased the sensitivity of human WERI-Rb-1 retinoblastoma cells to rapamycin.

Conclusions:  
Overexpression of BGN induced rapamycin resistance in WERI-Rb-1 retinoblastoma cells by activating PI3K/Akt/NF-κB signaling.

MeSH Keywords:  
Biglycan • Drug Resistance, Neoplasm • Phosphatidylinositol 3-Kinases • Proto-Oncogene Proteins c-akt • Retinoblastoma

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Background

As the most common intraocular malignancy type of childhood, retinoblastoma (RB) can arise from the retina of one eye (unilateral RB) or both eyes (bilateral RB) [1]. According to the findings from a study published in 2009, the incidence of RB is higher in developing countries [2,3]. Surgical ablation of the affected eye is currently the first-line treatment approach for RB. With the rapid development of novel techniques for local treatment, some more conservative methods are becoming more commonly used [4]. However, the treatment outcomes for RB depend on the stage of the tumor and can be significantly affected by the availability of medical cares, with increasing attention now being given to the early diagnosis of RB. The most common presenting sign of RB is an abnormal appearance of the pupil [5–7]. According to the 2007 study from Chakraborty et al., genes including PIK3CA, AKT1, FRAP1, and RPS6KB1 are dysregulated in RB tumor tissue samples [8], which supported the possible role of the PI3K/Akt pathway in the progression of RB. Signaling transduction is closely associated with oncogenesis in several types of malignancy and is crucial in maintaining the growth, metastasis, and chemoresistance of cancer cells [9]. Cao et al. studied the function of the PI3K/Akt pathway in RB and showed that knockdown of miR-93-5p could inhibit the growth of RB cells by suppressing the activation of the PI3K/Akt pathway [10]. Also, Huang et al. reported that down-regulation of miR-182 impaired cell viability, invasion, and angiogenesis RB through inhibition of the PI3K/Akt pathway [11]. Apart for the regulatory effect on RB cell proliferation and invasion, PI3K/Akt might also be involved in the development of RB chemoresistance, as activation of PI3K/Akt/NF-κB signaling transduction is associated with chemoresistance in several types of cancer cells [12–14]. Although the role of the PI3K/Akt/NF-κB pathway in the development of chemoresistance of RB remains unknown, this association deserves further investigation. The role of NF-κB signaling in the induction of cancer chemoresistance has been previously studied [15,16]. The identification of the upstream regulators of NF-κB has provided treatment strategies for multiple diseases [15]. In the study reported by Song et al., treatment of aortic valve interstitial cells with biglycan (BGN) inhibition of ERK1/2 attenuated NF-κB activation [17]. More recently, the activation of NF-κB by BGN overexpression was shown to induce resistance to chemotherapy in colon cancer [18]. BGN is an extracellular matrix (ECM) protein involved in the regulation of the morphology and growth of epithelial cells [19]. BGN has been shown to have a role in the oncogenesis of multiple tumor types and has been shown to induce multiple drug resistance (MDR) in human malignancy, including osteosarcoma and ovarian cancer [20,21]. In RB, BGN is involved in the regulation of cell division and cell survival [22]. Therefore, the targeted modulation of BGN gene expression levels might represent a novel approach to influence the NF-κB-mediated development of chemoresistance in cancer cells. Therefore, this study aimed to investigate the effects of expression of BGN on the response of human WERI-Rb-1 retinoblastoma cells to rapamycin treatment and to investigate the associated signaling pathways.

Material and Methods

Cell culture

The human retinoblastoma (RB) cell line, WERI-Rb-1 (ZQ0415, Zhongqiaoxinzhou Cell Research, China) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO₂. Cells from the third to fifth passage were used for subsequent assays.

Overexpression of the biglycan (BGN) gene in WERI-Rb-1 human RB cells

The coding sequence of the human BGN gene (NM_001711.4) was amplified and ligated using the pcDNA3.1 plasmid (BGN-OE vector), and the empty plasmid was used as a negative control (NC). Transfections were performed using transfection agents (c1507) (Applygen Technologies Inc., Shanghai, China) according to the manufacturer’s instructions. WERI-Rb-1 RB cells were transfected with the NC vector or BGN-OE vector to determine the effect of BGN gene overexpression on cell growth, apoptosis, and drug sensitivity. Stably transfected cells were assessed in medium containing G418 (geneticin) (0.5 μg/μl). The transfection efficiency was determined using real-time reverse transcription polymerase chain reaction (RT²-PCR) and Western blot. To confirm the role of BGN in the formation of chemoresistance in WERI-Rb-1 RB cells, the expression of BGN was knocked down using short-hairpin RNA (shRNA) (shRNA: 5’-GCCAACACUGCCGCAUC-3’) and the effect on cell proliferation, colony formation, and apoptosis was assessed. The results are included as supplementary file data (Supplementary Figures 1 and 2).

Treatment with rapamycin

To determine the concentrations of rapamycin, WERI-Rb-1 RB cells were incubated at 37°C for 48 h with rapamycin at concentrations of 0, 5, 10, 20, 30, and 50 μg/ml. The IC50 concentrations were calculated using MTT assays. To further explore the role of PI3K/Akt signaling, WERI-Rb-1 cells transfected with the BGN gene expression vector were treated with the PI3K/Akt blocker...
Table 1. Primers used in the polymerase chain reaction (PCR).

| Direction | Sequence (5’-3’) |
|-----------|------------------|
| **BGN**  |                  |
| Forward   | GGGTCTCCAGCACCTTACGCC |
| Reverse   | TGAAACATCCCCTTGGGCACCT |
| **β-actin** |                  |
| Forward   | CTTAGTGGGTTAACCCTTCTTTG |
| Reverse   | CTGCACCTTCCAGTTCCAGTTT |

BGN – biglycan.

pathway inhibitor, LY294002 (R&D Systems, Minneapolis, MN, USA) (100 nM) for 1 h before rapamycin treatment.

**Real-time polymerase chain reaction (RT-PCR) using RT–PCR**

The level of BGN gene expression after transfection of the BGN-OE vector was determined using RT–PCR. Briefly, total RNA was extracted using the RP1201 extraction kit (BioTeke, Beijing, China) and reversely transcribed into cDNA. The real-time PCR mixture contained 10 μl SYBR Green Mastermix (SY1020) (Solarbio Science & Technology Co., Ltd., Beijing, China), 0.5 μl of each primer (Table 1), 1 μl of cDNA template, and 8 μl of double-distilled water (ddH2O). The amplification was performed using Euxicler™ 96 (Bioneer Co., Daejeon, South Korea) and the relative level of BGN to internal reference gene was calculated according to the 2^−ΔΔCt method.

**Western blot**

Total protein was extracted using RIPA lysis buffer (P0013B) (Beyotime Biotechnology, Shanghai, China) and nuclear protein was extracted using a Nuclear Protein Extraction kit (P0027) (Beyotime Biotechnology, Shanghai, China). Subsequently, 40 μg of protein underwent 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 80 V for 1.5 h. After membrane transfer, primary antibodies against BGN (1: 1000) (D221985) (Sangon Biotech, Shanghai, China), cleaved caspase 3 (1: 1000) (ab2302) (Abcam, Cambridge, UK), Bcl-2 (1: 400) (ab32124) (Abcam, Cambridge, UK), Bax (1: 1000) (ab32503) (Abcam, Cambridge, UK), cleaved PARP (1: 1000) (ab32561) (Abcam, Cambridge, UK), IkBa (1: 500) (bs-1287R) (Bioss, Woburn, MA, USA), p-PI3K (bs-32089) (Abcam, Cambridge, UK), PI3K (sc-376412) (Santa Cruz Biotechnology Inc., Dallas TX, USA), p-Akt (ab38449) (Abcam, Cambridge, UK), Akt (ab8805) (Abcam, Cambridge, UK), p-IkBα (1: 500) (bs-2513R) (Bioss, Woburn, MA, USA), p65 (1: 1000) (D155097) (Sangon Biotech, Shanghai, China), β-actin (1: 500) (KGAA001-1) (KeyGen Biotech Co. Ltd., Nanjing, China), and Histone H3 (1: 1000) (bs-17422R) (Bioss, Woburn, MA, USA) were then incubated with membranes for 45 min at 37°C. The protein bands were developed using the Beyo ECL Plus reagent (P0018) (Beyotime, Shanghai, China) and the relative levels of protein to internal reference proteins were calculated using a GelPro Analyzer (Media Cybernetics, Rockville, MD, USA).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test

Cell viability of WERI-Rb-1 cells was measured using the MTT assay. Briefly, cells (4×10^4 cells/well) were cultured for 120 h and the cell viabilities at 24 h, 48 h, 72 h, 96 h, and 120 h were detected by incubation with 5 μg/ml of MTT solution for four hours at 37°C. Cell viability was detected using an ELX-800 microplate reader (BioTeke, Beijing, China) and was represented by the optical density (OD) value at 490 nm.

**Colony formation assay**

Anchorage-independent growth potential was measured using the colony formation assay. There were 5×10^3 inoculated into RPMI-1640 medium containing 3% methylcellulose and the cells were grown for three weeks. The numbers of colonies were recorded and the colony formation rate was calculated according to the equation: colony number/inoculation cell number ×100%.

**Flow cytometry for cell apoptosis**

Flow cytometry was performed to determine the effect of over-expression of the BGN gene and rapamycin administration on cell apoptosis in WERI-Rb-1 cells. WERI-Rb-1 cells were stained using an Annexin V-FITC Apoptosis Detection Kit (WLA001c) (Wanleibio, Shenyang, China). After incubation with Annexin V for 10 min, cells were resuspended with 1×binding buffer and incubated with 5 μl of propidium iodide (PI). The apoptotic rates were detected and analyzed using the Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

**Hoechst staining**

A Hoechst staining kit (C0003) (Beyotime Biotechnology, Shanghai, China) was used to detect the morphological changes.
in cell nuclei. The results were visualized using an IX53 Olympus fluorescence microscope (Olympus, Tokyo, Japan) at ×400 magnification. Cell nuclei were stained bright blue (Hoechst-positive) were identified as apoptotic cells.

Statistical analysis

Statistical analysis and graph construction were performed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA). A P-value <0.05 was considered to be statistically significant, with a two-tailed P-value. Data were expressed as the mean ± standard deviation (SD). Each experiment was performed in triplicate. The effect of different treatments was analyzed using one-way analysis of variance (ANOVA) and the difference between each of the two groups was analyzed by post hoc multiple comparisons using the least significant difference (LSD) method.

Results

Determination of the IC50 of rapamycin

The IC50 of rapamycin administration for different groups of human retinoblastoma (RB) WERI-Rb-1 cells was determined using the MTT assay. As shown in Figure 1, the rate of inhibition of WERI-Rb-1 cells in different groups increased with rapamycin concentrations. The IC50 was calculated according to the inhibitory rate curves and showed that the IC50 values for the Blank group, the normal control (NC) group, and the biglycan (BGN) group were 16.83 μg/ml, 16.11 μg/ml, and 28.76 μg/ml, respectively (Figure 1, Table 2). The higher IC50 of the BGN group indicated increased resistance to rapamycin.

Overexpression of the BGN gene increased cell proliferation potential of WERI-Rb-1 cells following treatment with rapamycin

Transfection with the BGN-OE vector induced the expression of BGN both at the mRNA level (Figure 2A) and the protein level (Figure 2B). The increased expression levels of BGN were accompanied by the increased proliferation potential of WERI-Rb-1 cells (Figure 3A), which was a finding supported by previous studies on other human cancer cells. The inhibitory effect of rapamycin on the growth of WERI-Rb-1 cells was reduced by overexpression of the BGN gene (Figure 3A), indicating increased rapamycin resistance in WERI-Rb-1 cells. In colony formation assays, the colony numbers in the WERI-Rb-1 cell BGN gene overexpression groups was significantly higher than that in WERI-Rb-1 cells transfected with NC vector, even when treated with rapamycin (Figure 3B).

Overexpression of the BGN gene inhibited apoptosis in WERI-Rb-1 cells following rapamycin administration

Cell apoptosis of WERI-Rb-1 cells was detected using both flow cytometry and Hoechst staining. The administration of
rapamycin increased the numbers of apoptotic WERI-Rb-1 cells (Figure 4A, 4B). However, induced expression of the BGN gene inhibited the induction of apoptosis resulting from rapamycin treatment of WERI-Rb-1 cells. The data showed that overexpression of the BGN gene could counteract effects induced by rapamycin in WERI-Rb-1 cells. At the molecular level, the overexpression of the BGN gene reversed the expression patterns of Bcl-2, Bax, cleaved caspase 3, and cleaved PARP, which were initially influenced by rapamycin (Figure 4C).

**Overexpression of the BGN gene increased the resistance of WERI-Rb-1 cells to rapamycin by activating PI3K/Akt mediated NF-κB signaling**

The administration of rapamycin suppressed the expression of p-PI3K, p-Akt, and NF-κB subunit p65 in WERI-Rb-1 cells. BGN gene overexpression restored the level of p-PI3K, p-Akt, and p65 and inhibited the level of IκBα (Figure 5). These results supported the PI3K/Akt-mediated NF-κB pathway was involved in the functions of BGN gene overexpression.

The WERI-Rb-1 cells that underwent BGN gene overexpression and rapamycin treatment were further treated with the PI3K/Akt inhibitor, LY294002, which increased the sensitivity of human WERI-Rb-1 retinoblastoma cells to rapamycin. Inhibition of the PI3K/Akt pathway (Figure 6A) by LY294002 impaired cell growth (Figure 6B) and induced cell apoptosis (Figure 6C) in WERI-Rb-1 cells that overexpressed the BGN gene following treatment with rapamycin, which was associated with the inhibition of NF-κB signaling (Figure 6A). These results supported the role of PI3K/Akt-mediated NF-κB signaling in BGN-induced rapamycin resistance in WERI-Rb-1 human RB cells.

**Discussion**

The current study showed that the induced expression of biglycan (BGN) resulted in rapamycin resistance in the WERI-Rb-1 human retinoblastoma (RB) cell line, which was associated with the activation of the PI3K/Akt/NF-κB signaling pathway. Furthermore, based on the results following treatment of the
Figure 4. Overexpression of the BGN gene suppressed cell apoptosis in rapamycin-treated human WERI-Rb-1 retinoblastoma (RB) cells. (A) Flow cytometry shows cell apoptosis in WERI-Rb-1 retinoblastoma (RB) cells. (B) Hoechst staining shows cell apoptosis in WERI-Rb-1 RB cells. (C) Western blot detection of Bax, cleaved caspase-3, cleaved PARP, and Bcl-2 levels in WERI-Rb-1 RB cells. * P<0.05 vs. the Blank group. # P<0.05 vs. the NC+Rapamycin group. Each assay was performed in triplicate.
WERI-Rb-1 cells with the PI3K/Akt pathway inhibitor, LY294002, which increased the rapamycin sensitivity, rapamycin resistance was shown to depend on the activation of PI3K/Akt-mediated NF-κB signaling transduction.

Overexpression of BGN is a characteristic of cancer stem cells (CSCs), which are associated with the development of chemoresistance in cancers [23]. Previously published studies have shown that an increased level of BGN expression is an indicator of the formation of chemotherapy resistance in pediatric osteosarcoma [21]. Liu et al. showed that the induced expression of BGN increased resistance to 5-FU and cisplatin in colon cancer cells [18]. In most cancer cell types, BGN has been reported to be an autocrine angiogenic factor involved in the inflammatory responses and fibrosis [24–27], it remains important to investigate the association of BGN with chemoresistance in different types of human malignancy.

Rapamycin is a chemotherapy agent used in the treatment of RB [28,29]. This study aimed to investigate the effects of overexpression of the BGN gene in WERI-Rb-1 human RB cells including the response to rapamycin. Administration of rapamycin influenced RB cell growth and increased apoptosis in and overexpression of the BGN gene inhibited the cytotoxic effects of rapamycin and was associated with the activation of the PI3K/Akt/NF-κB pathway. This finding is of particular interest, as a recent report showed that the PI3K/Akt/mTOR pathway was dysregulated in human RB [8]. As an inhibitor of mTOR, rapamycin has particular cytotoxic effects on hypoxic cell populations [28,29]. The findings of the present study also showed that inhibition of the PI3K/Akt/mTOR pathway suppressed the activation of NF-κB signaling by increasing the cytoplasmic of IxBα and reducing cell nuclear expression levels of p65.

The functions of the NF-κB pathway are essential for the formation of multidrug resistance (MDR) to chemotherapy during cancer treatments [16]. However, in this study, the induced overexpression of BGN restored the activity of PI3K/Akt/NF-κB, which increased the resistance of RB cells to rapamycin. As the upstream modulator of NF-κB pathway, BGN firstly binds to TLR2/4 and then initiates the activation of the pro-inflammatory NF-κB pathway [17,30]. The findings of the present study supported this regulation sequence. However, PI3K/Akt is also an upstream modulator of NF-κB signaling, which was shown to be activated by BGN. Given the fact that rapamycin exerted its effect by inhibiting the PI3K/Akt pathway, in this study, we also explored whether the interaction between BGN and NF-κB and resistance to rapamycin was mediated via PI3K/Akt. After the WERI-Rb-1 RB cells that overexpressed BGN were treated with the PI3K/Akt inhibitor, the effect of rapamycin on the RB cells was restored. These indicated that the abnormally high level of BGN contributed to the activation of NF-κB signaling in a PI3K/Akt-dependent manner, which was related to the formation of rapamycin resistance in RB. Because the activation of NF-κB is essential for the development of MDR in different cancer cells, rapamycin resistance induced by BGN...
overexpression might be associated with resistance to other chemotherapeutic agents. Further studies are needed to investigate the effects of overexpression of the BGN gene and chemotherapy resistance in other types of malignancy.

Conclusions

This study aimed to investigate the effects of expression of biglycan (BGN) on the response of human WERI-Rb-1 retinoblastoma (RB) cells to rapamycin treatment and to investigate the associated signaling pathways. The findings of this study showed that overexpression of the BGN gene induced rapamycin resistance in WERI-Rb-1 RB cells by activating PI3K/Akt/NF-κB signaling. Based on the assays with short-hairpin RNA (shRNA), it was also found that the inhibition of BGN contributed to the increased sensitivity to rapamycin in RB cells. Although this was a preliminary in vitro study of a single human RB cell line, the findings are of interest and increase current understanding of the role of BGN and multidrug resistance (MDR) to chemotherapy in human malignancy.

Conflict of interest

None.
Supplementary Data

**Supplementary Figure 1.** Detection of the BGN gene knockdown efficiency in human WERI-Rb-1 retinoblastoma (RB) cells. (A) Real-time polymerase chain reaction (RT-PCR) using RT²-PCR detection of BGN in WERI-Rb-1 retinoblastoma (RB) cells. (B) Western blot detection of BGN protein level in WERI-Rb-1 RB cells. * P<0.05 vs. the NC+Rapamycin group. Each assay was performed in triplicate.

**Supplementary Figure 2.** Inhibition of BGN reduced cell growth and increased apoptosis in rapamycin-treated human WERI-Rb-1 retinoblastoma (RB) cells. (A) MTT detection of cell proliferation of WERI-Rb-1 retinoblastoma (RB) cells. (B) Colony formation detection of the anchorage-independent growth potential of WERI-Rb-1 RB cells. (C) Flow cytometry detected cell apoptosis in WERI-Rb-1 RB cells. * P<0.05 vs. the NC+Rapamycin group.
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