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# Silencing RBBP6 (Retinoblastoma Binding Protein 6) sensitises breast cancer cells MCF7 to *staurosporine* and *camptothecin*-induced cell death

Pontsho Moela, Mpho M.S. Choene, Lesetja R. Motadi *

*School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg 2050, South Africa*

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ABSTRACT

Retinoblastoma Binding Protein 6 (RBBP6) is a multi-domain protein that uses its ring finger domain to interact with p53 and pRb tumour suppressor genes. The mechanism by which RBBP6 uses to degrade p53 is still unknown; nonetheless it is well known that RBBP6 promotes cell proliferation in several cancers by negatively regulating p53 via its E3 ubiquitin ligase activity. Degradation of p53 by RBBP6 may compromise p53-mediated apoptosis in breast cancer. This study is intended to investigate, the potential applications of RNA interference (RNAi) to block RBBP6 expression, as well as its subsequent effect on cell growth and apoptosis. Our studies indicate that the knockdown of RBBP6 by siRNA modulates p53 gene expression involved in cell death pathways and apoptosis, showing statistically significant gene expression differences. RBBP6 siRNA significantly reduced cell growth compared to the control samples and inhibition of cellular proliferation was observed between 24 and 48 h, as shown in the data obtained by real time cell analysis using the *xCELLigence* system. These results were further confirmed by flow cytometer which showed some apoptotic activity. About 20.7% increase in apoptosis was observed in cells co-treated with RBBP6 siRNA and *camptothecin* when compared to *camptothecin*-only whereas in siRBBP6 and *staurosporine* treated cells there was only an 8.8% increase in apoptosis. These findings suggest that silencing RBBP6 may be a novel strategy to promote *camptothecin*-induced apoptosis in breast cancer cells.



## Introduction

Screening tests performed over a decade ago revealed Retinoblastoma Binding Protein 6 (RBBP6) as a multi-domain protein that uses its ring-finger domain to interact with the p53 and pRb tumour suppressor genes (Pugh et al. 2006; Pretorius et al. 2013). Also known as RBQ-1, PACT or P2P-R, the RBBP6 mRNA occurs in the form of three major transcripts, transcript 6.1, 6.0 and 1.1 kb as a result of alternative splicing, which code for protein isoforms 1, 2 and 3, respectively (Pugh et al. 2006).

RBBP6 is highly expressed in cancer of the oesophagus as well as other cancers and this makes it a potential target in the treatment of cancers with intact p53 (Pugh et al. 2006; Ntwasa 2008). In addition to several of its biological functions including transcription, translation and ubiquitination, it is also associated with the execution of p53 degradation following inhibition of the tumour suppressor gene by MDM2, thus facilitating cell proliferation (Ntwasa 2008; Pretorius et al. 2013). However, little is known about the mechanism with which RBBP6 uses to degrade the p53 tumour suppressor protein. Taking note of the fact that the most efficient regimens for

*Abbreviations:* Bad, Bcl-$x_L$/Bcl-2-associated death protein; Bax, Bcl-2-associated death protein; BCA, Bicinchoninic acid; BCl-2, B cell leukaemia-2; Bid, B cell leukaemia lymphoma-2; BSA, Bovine serum albumin; Caspase, Cysteine aspartic-specific proteases; CCD, Charge-coupled device; cDNA, Complementary DNA; CI, Cell index; CPT, Camptothecin; DMEM, Dulbecco's modified medium; DNA, Deoxyribonucleic acid; FBS, Foetal bovine serum; FITC, Fluoresceine-isothiocyanate; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HRP, Horseradish peroxidase; MDM2, Murine Double Minute 2; mRNA, Messenger RNA; P2P-R, Proliferation potential protein-related; P53/TP53, Protein 53/tumour protein 53; PACT, P53-associated cellular protein testisderived; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PI, Propidium Iodide; RBBP6, Retinoblastoma Binding Protein 6; RING, Really interesting new gene; RIPA, Radioimunoprecipitation assay; RNA, Ribonucleic acid; RNAi, RNA interference; RTCA, Real time cell analyser; RT-PCR, Reverse transcription PCR; SDEV, Standard deviation; SDS, Sodium dodecyl sulphate; siRNA, Short interfering RNA; STS, Staurosporine.

* Corresponding author. Tel.: +27 117176485; fax: +27 117176351.
*E-mail address:* Lesetja.motadi@wits.ac.za (L.R. Motadi).

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anticancer activity including multidrug combinations still present patients with harsh side effects, it is important to continue evaluating any possible safe therapy (Aagaard and Rossi 2007; Arakawa et al. 2009). In this study, we report on the therapeutic potential of RBBP6 gene silencing in combination with *camptothecin* and *staurosporine* in human breast cancer cells.

## Materials and methods

### *Materials*

MCF-7 human breast cancer cell line was obtained from the Japan Health Resource Centre. Silencing was achieved by the use of Ambion's *Silencer*® Select Pre-designed siRNAs supplied by Life Technologies™, which target the RBBP6, MDM2 and p53 genes. *Staurosporine* and *camptothecin* were purchased from Calbiochem®.

### *Tissue culture and treatments*

MCF-7 cell line was cultured in DMEM (Dulbecco's Modified Eagle Medium) growth medium supplemented with 1% pen/strep and 10% FBS (Foetal Bovine Serum) and incubated in a humidified atmosphere at 37 °C and 5%$CO_2$. Once they reached ~70–80% confluency, cells were trypsinized and resuspended in an antibiotic-free media. The cell suspension was then mixed with siRNA/transfection agent (Ambion®) complexes, at 100 nM concentrations, targeting RBBP6, MDM2 and p53 genes in a 24-well plate and incubated at 37 °C for 24 and 48 h. Post transfection cells were exposed to 0.25 μM *staurosporine* and 0.25 μM *camptothecin* for an additional 24 h. The cells were then harvested for subsequent analysis, i.e. RNA and protein were extracted 48 and 72 h post transfection, respectively.

### *Real time RT-PCR*

RNA was extracted using Nucleospin® RNA II total RNA isolation kit according to the manufacturer's protocol and quantified using a nanodrop (NanoDrop Technologies, USA). RNA integrity was confirmed using ethidium bromide-stained agarose gel to analyse the 18s and 28s rRNA bands. Following RNA extraction, cDNA was synthesised using ImProm-II™ Reverse Transcription system from Promega®. RT-PCR was then performed in a 20 μl reaction mixture containing 2 μg/μl cDNA, SYBR Green (SIGMA®), reverse and forward primers (GADPH: Forward – 5′-GAG TCA ACG GAT TTG GTC GT-3′, Reverse – 5′-TTG ATT TTG GAG GGA TCT CG-3′; RBBP6: Forward – 5′-CAG CGA CGA CTA AAA GAA GAG TCT-3′, Reverse – 5′-GGT AAT TGC GGC TCT TGC CT-3′ and p53: Forward – 5′-GTT CCG AGA GCT GAA TGA GG-3′, Reverse – 5′-TGA GTC AGG CCC TTC TGT CT-3′) under the following conditions: 36 cycles of 94 °C for 35 s, 59 °C for 45 s, and 72 °C for 45 s.

### *Western blot*

Whole cell protein was extracted using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 10% sodium dodecylsulfate (SDS), 3 μl/ml aprotinin and 5 μg/ml leupeptin in PBS, pH 7.4). Seventy-two hours post transfection and co-treatment with either *staurosporine* or *camptothecin*, cells were washed twice with cold PBS then resuspended in 500 μl RIPA buffer and collected by scraping. The total protein was then separated from cell debri by centrifugation at 14,000 rpm for 15 min and quantified with Pierce® BCA Protein Assay Kit. The protein was heated at 95 °C for 5 min and 30 μg was loaded per well for electrophoretic separation in 40% acrylamide gel preparation at 100 V for 1 h. The protein was transferred onto a nitrocellulose membrane using wet electro-transfer method overnight at 30 V followed by incubation with primary antibody after 1 h of blocking with 5% non-fat milk buffer. The weak light signal produced by HRP-linked secondary antibody was detected and enhanced using the Pierce® ECL Western Blotting Chemiluminescence Substrate and the blots were imaged by the CCD-based ChemiDoc™ MP system.

### *xCELLigence system*

Before cells were seeded, 16-well E-plates containing antibiotic-free medium were imposed to current flow on the xCELLigence instrument placed in a 37 °C incubator to record background readings. In each well of the E-plates, $1 \times 10^5$ cells were seeded simultaneously with 100 nM siRNA targeting RBBP6, MDM2 and p53 genes in the same antibiotic-free medium. After leaving the E-plates at room temperature for 30 min to allow for cell attachment, they were locked in the RTCA xCELLigence instrument and the experiment was allowed to run for 24 h. Twenty-four hours post transfection, cells were further treated with 0.25 μM apoptosis-inducing agents (*staurosporine* and *camptothecin*). The experiment was continued for an additional 24 h. Cell Index values were recorded at 15 min interval sweeps until the end of the experiment under the following xCELLigence parameters: [1st step: 1 sweep, 1 min interval, 00:00:39 total time; 2nd step: 100 sweeps, 15 min interval, 24:45:39 total time; 3rd step: 100 sweeps, 15 min interval, 49:30:39 total time].

### *Flow cytometry*

Cultured cells were seeded in 24-well plates and simultaneously transfected with siRNAs targeting RBBP6, MDM2, and p53 for 24 h and treated for an additional 24 h with apoptosis-inducing agents (0.25 μM *staurosporine* and *camptothecin*). The treated cells were then trypsinized, resuspended in growth medium and transferred to 15 ml tubes, pelleted for 2 min at 1500 rpm and resuspended in 100 μl 1X binding buffer (annexin V-FITC Apoptosis Detection Kit, abcam®) at a concentration of $1 \times 10^4$ cells/ml. The cell suspensions were then transferred into 1 ml tubes and 5 μl of Annexin V FITC and 5 μl of PI were added. This was followed by gentle vortexing and incubation for 15 min at room temperature in the dark. To each tube, 400 μl of 1X binding buffer was then added and the cell solutions were analysed by flow cytometry within 1 h.

### *Statistical analysis*

The results of each series of experiments (performed in duplicates) are expressed as the mean values ± standard deviation of the mean (SD). Levels of the statistical significance were calculated using the paired Student's *t*-test when comparing two groups, or by analysis of variance (ANOVA). *P*-values of $\leq 0.05$ were considered significant.

## Results

### *Gene silencing and mRNA expression analysis*

Quantitative PCR analysis was used to evaluate the transcript on the silenced RBBP6, p53, MDM2, Bax and Bcl2 genes as shown in Fig. 1. As expected, a 37% decrease in RBBP6 expression was observed following silencing with 100 nM siRBBP6 while in combination with *camptothecin* the expression was further reduced by about 49%. In cells that were treated with *Staurosporine* and siRBBP6, expression was reduced by only 22%. In siMDM2, we observed a much higher silencing following co-treatment with both *staurosporine* and *camptothecin*, at 50% and 51%, respectively (Fig. 1.1A and B). The present study emphasizes the important role