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Small Interference RNA-Mediated Down Regulation of Survivin Expression in Breast and Hepatocellular Carcinoma Cell Lines

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Abstract: Survivin, a member of inhibitor of apoptosis protein family, has become an attractive therapeutic target in cancer due to its selective expression in different malignant tumors. It has a dual function in apoptosis suppression and in cell cycle progression. In the present study, we evaluated the role of Survivin knockdown in reactivating the apoptotic machinery and hence in reducing cell proliferation. Lipofection-based approach was used to deliver small interfering RNAs (siRNAs) to down regulate Survivin expression in two different cancerous cell line (MCF-7 for breast cancer and HebG2 for hepatocellular carcinoma). The obtained results showed that down regulation of Survivin has affected the overall cell proliferation via activating caspases-mediated apoptosis. 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was employed to assess the viability of the transfected cells. Cell toxicity was evaluated by Trypan blue test. Real-Time PCR was conducted to assess the gene expression of the cells after being transfected. The results demonstrated that Survivin mRNA translation was inhibited and this has led to attenuation of the malignant features of breast and hepatocellular carcinoma cell lines. On the light of our conclusion, Survivin-targeting siRNA may provide a novel approach to control cancer due to its specificity.

Key words: Survivin · siRNA · Cancer · MCF-7 · Apoptosis and HepG-2

INTRODUCTION

Breast cancer is the most common female neoplasm affecting around one in nine women. There is a genetic susceptibility, which accounts for up to 30% of the heritability of breast cancers [1].

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asia and Africa and its incidence is risingin Western countries [2]. HCC is the third leading cause of cancer deaths worldwide and the ninth leading cause of cancer deaths in the USA [3, 4]. Recently, large-scale studies documented the high incidence rate of HCC in uremia patients [5, 6].

Apoptosis is a process that enables multi-cellular organisms to preserve their viability by selectively inducing the death of damaged cells. The decision whether a cell divides or undergoes apoptosis is controlled by cell signals that may originate either on the cell inside (Intrinsic inducer) or outside (Extrinsic inducer) [7]. Abnormal regulation of apoptosis has been implicated in the onset of wide range of diseases including cancer. Considerable efforts have been made to develop strategies for modulating apoptosis in cells of cancer and other human diseases [8].

The process of apoptosis is tightly regulated by a number of gene products that promote or block cell death at different stages of apoptosis. The inhibitor of

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apoptosis proteins (IAP) are a family, which contains eight members of highly conserved cell death inhibitors that have been found in yeast, invertebrates and vertebrates [9]. IAPs play an important role in the negative regulation of apoptosis. Defective apoptotic signaling pathways have an important role in the initiation and progression of cancer [2].

One of the mechanisms through which tumor cells are believed to acquire resistance to apoptosis is overexpression of IAPs, which prevent apoptosis by specifically inhibiting caspases [10, 11]. Survivin is the smallest member of the IAP family. It is a 16.5 kDa intracellular protein with 142 amino acid residues [12]. Survivinis expressed during mitosis in a cell cycle-dependent manner and localized to different components of the mitotic apparatus, so it plays an important role in both cell division and inhibition of apoptosis. Survivin is expressed in a vast majority of human cancers, but not in normal adult tissues. Meanwhile, its expression is often correlated with poor prognosis in a wide variety of cancer patients [13]. Because of its differential expression in tumors versus normal tissues and its role in apoptosis for maintaining cell viability, several studies with different approaches showed that targeting of the Survivin pathway in cancer, alone or in conjunction with chemotherapeutic agents, have potential as a novel therapeutic regimen [3].

Thus therapeutic potential targeting anti-apoptotic proteins, such as Survivin, by conventional approach remained to be very difficult. Since RNA interference (RNAi) first discovered in 1998, it has been rapidly developed into one of the most widely applied technologies with therapeutic potential in molecular and cellular research [14- 17].

The use of RNA interference (RNAi) has grown at a remarkable rate since the first discovery of RNAi in *Caenorhabditi selegans* [18]. Subsequently, RNAi was shown to occur in mammalian cells in response to double stranded siRNAs that serve as the effector molecules of sequence-specific gene silencing [19]. Survivin-targeting approaches have become a promising strategy for treating HCC [20].

However, the aim of the present study was to evaluate the role of Survivin in reactivating the apoptotic machinery in human breast cancer (MCF-7) and Hepatocellular carcinoma (HepG-2) cell lines using siRNA approach as a promising future therapeutic tool for cancer treatment.

MATERIALS AND METHODS

Cell Culture: The MCF-7 and HepG-2 cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Hyclone, Utah, USA) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Hyclone) in a fully humidified incubator at 37°C with 5% CO_2. All cell lines were treated in phenol red-free RPMI-1640 medium (Gibco, Carlsbad, CA) supplemented with 10% charcoal-stripped FBS (Hyclone), 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine for 48 hours at the beginning of each experiment [18].

SiRNA transfection: The Survivin-targeting siRNA was obtained from Santa Cruz Biotechnology, USA. The protocol was as per manufacturer instructions with some modification according to Zhang *et al*. [18]. In a six well tissue culture plate, a count of 2×10^5 of each cell line was seeded in 2 ml antibiotic-free normal growth medium supplemented with FBS. The cells were then incubated at 37°C in a CO_2 incubator until they reached 60-80% confluent. Two solutions were prepared; Solution I that consists of 6µl of siRNA duplex (Equivalent to 0.5µg siRNA) diluted into 100 µl siRNA transfection medium. Solution II that consists of 6 µl of siRNA transfection reagent diluted into 100 µl siRNA transfection medium. Solution I was added to solution II and mixed gently by pipetting and incubated for 30 minutes at room temperature. Cells were then washed once with 2 ml of siRNA transfection medium. A volume of 0.8 ml siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture (Solution I + Solution II), mixed gently and then overlaid onto the washed cells. The cells were then incubated for 6 hours at 37°C in a CO_2 incubator. A volume of 1 ml of normal growth medium containing 2 times the normal serum and antibiotics concentration (2 × normal growth medium) was then added without removing the transfection mixture and the cells were subsequently incubated for an additional 24 hours. The medium was then aspirated and we proceeded immediately to the next step.

Trypan Blue Assay: Cell cytotoxity was assessed using Trypan blue assay. Samples were diluted in a 1:1 dilution of the cell suspension using a 0.4% Trypan Blue solution. Non-viable cells were stained blue; while viable cells remained unstained. The number of dead cells was

determined by using haemocytometer. The percent of viability was calculated by using the following formula: % viability = (Live cell count/total cell count) x100.

MTT Assay: Viable cells were measured by the 3-(4, 5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to MTT kit (R and D Systems) manufacturer's instructions. MCF-7 and HepG2 cells, with and without transfection, were plated in 96-well tissue culture plates at the indicated density in 100 ml of RPMI 1640 containing 10% FBS and cultured at 37°C for 3 days. Each group had three samples. The absorbance was read at 590 nm using a plate reader (TitertekMultiskan, Flow Laboratories, North Ryde, Australia). Readings were taken three times at 6, 24 and 72 hours from transfection. The OD of formazan formed in control cells was taken as 100% of viability and the positively stained cells with MTT are expressed as the percentage (percentage) compared to control cells. The data points represent the mean + Standard Deviation (SD) of a quadruplicate determination from a representative experiment that was repeated at least three times.

Detection of Survivin Gene: Total RNA was extracted from transfected and non-transfected cells using RNAiso reagent (Takara, Dalian, China) according to the manufacturer's protocol. Five µg (500 ng/µl) of total RNA was used to synthesize the first-strand of cDNA using an oligo (dT) primer and RT Superscript II, as recommended by the manufacturer (Takara). Survivin gene expression was determined by semi-quantitative reverse transcription PCR (RT-PCR) using 1µl of the first-strand cDNA as a template for the PCR.The PCR mix contained 3 µl of 10X PCR buffer, 1 µl of 10 mM dNTPs, 0.5 µl (20 pmol/ml) of each forward primer (5-AGAACTGGCCCTTCTTGGA-3) and reverse primer (5-AAGGAAAGCGCAACCGGACG-3) and 2 U of Taq DNA polymerase (Takara) in a final volume of 30 µl. The following PCR profile was used started with an initial denaturation cycle at 95oC for 3 min followed by 35 cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 30 s; and a final extension of 72°C for 5 min. To verify the reliability of the quantification, The PCR products after 35cycles amplification were visualized on 1.2% agarose gel and the sizes of the target fragments were confirmed in each experiment. The housekeeping gene GAPDH was amplified as an internal control.

Real Time PCR: Detection and quantification of Surviving gene expression has been carried out by the use Platinum Taq (5U/µl) (Invitrogen, USA). The reaction consists of Buffer (10x), MgCl2 (50mM), dNTP's (10mM), forward primer (20µM), reverse primer (20µM), SYBR Green dye 0.25 µl per reaction (Invitrogen, USA). Machine program (Miniopticon, Biorad, USA) was as follows: 5 min at 95°C for activation of Taq DNA polymerase enzyme, followed by 40 cycles for amplification with a denaturing step at 95 °C for 15 sec., gradient annealing temperatures (From 55- 65 °C) were used in order to identifying the optimal degree and for 1 min. at 72 °C for extension.

RESULTSAND DISCUSSION

Our main purpose of this research was to study the expression of Survivin gene and its role in the apoptotic pathways in breast and hepatocellular carcinoma cell lines.

We aimed to develop a cancer-specific therapeutic model, via identifying the molecular targets in the apoptotic pathway that regulate breast and HCC development/progression.

Detection of Survivin Gene: Cancerous cell lines were grown according to the ordinary laboratory procedures. Total RNA was extracted and 5 µg of the extracted RNA was subjected to RT-PCR to generate cDNA. 30 µg of cDNA was subjected to conventional PCR to Detect the Survivin Gene (Fig. 1). The obtained results indicated the presence of the Survivin gene in the two cell lines under study. PCR reactions were performed in the presence of an internal control (GAPDH). The PCR reaction resulted in a band with molecular size of 320 bp in both cell lines.

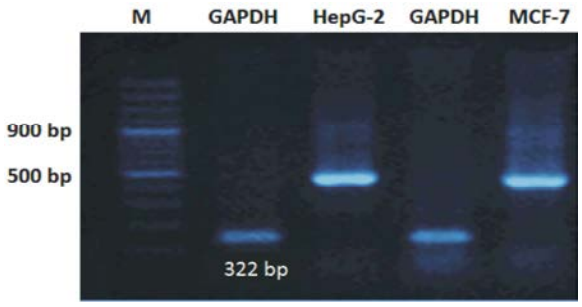


Fig 1: Reverse-transcriptase PCR profile for detecting Survivin gene in the two cell lines under study.

Table 1: Primary cell number and the percentage of cells responded to Survivin down regulation in both MCF-7 and HepG-2 cells.

	MCF-7	HepG-2
Number of cells before transfection	$2x10^4$	$1.3x10^4$
Number of cells after transfection	$1.1x10^4$	$0.7x10^3$
Percentage of cells died after transfection	55%	53.8%

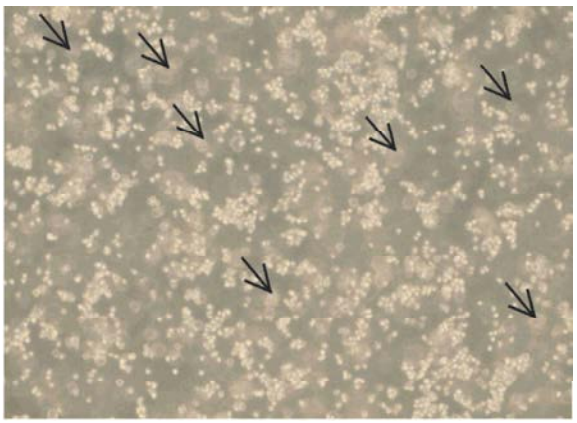


Fig 2: Trypan blue test showing dead (arrowed) and normal cells.

SiRNA Transfection and Trypan Blue Test: Transfection was performed according the manufacturer protocols. Cell viability test was carried out before and after transfection. Figure (2) represents died (Arrowed) and viable cells after being transfected. Table (1) represents the percentages of cells that died due to reactivation of apoptosis after being treated with Survivin-targeting siRNA.

MTT Assay: To investigate whether Survivin-targeting siRNA affects the viability of MCF-7 and HepG-2 cells, MTT assay was performed. Treatment with Survivin-targeting siRNA resulted in a significant decrease in the viability of MCF-7 and HepG-2 cells (Fig. 3). This test was performed to measure the activity of mitochondrial reductase enzyme which indicates the cell viability. Results show a decrease in cell viability after being transfected with Survivin-targeting siRNA. These results were in accordance with our previous study [19] and other studies [20, 21].

Figure (3) shows the decreasing number of viable cell in correlation with time. For the MCF-7 cell line, the OD readings were 0.88, 0.78 and 0.67 after 6, 24 and 72 hours respectively in comparison to 0.98 for the control. For the HepG-2 cell line, the OD readings were 0.87, 0.70 and 0.57 after 6, 24 and 72 hours respectively in comparison to 0.92 for the control.

The obtained results suggest that survivn down regulation was a key step in reactivating the apoptotic machinery, which lead eventually to the death of malignant cells.

Real Time PCR: As indicated in table (1) Survivin gene expression has been down-regulated in tested cell lines; MCF-7 and HepG-2 that generated an average Ct values of 33.39, 29.15, respectively compared to the average Ct value of the un-transfected cells, which produced an average Ct value of 18.30. However, an alternative formula to $\Delta\Delta C_T$ method has been used due to an amplification failure of the of the endogenous control gene *18S rRNA* expression. This formula depends on the assumption that amplification efficiency of the target gene is 100%. Consequently, relationship between the Ct value and the fold differences will be: each 3.32 Ct differences reflect a 10 times increase or decrease of the mRNA amount. Therefore, the fold differences between the un-transfected and the transfected cell lines will be 38, 27.3 and 35.4, respectively. Due to the adverse relationship between Ct values and mRNA quantity, this data indicated that the Survivin expression has been responded negatively (Down-regulated) to the anti-apoptotic siRNA treatment. In conclusion, this data showed that siRNA treatment might down-regulates the gene expression of Survivin gene. Finally, these data revealed that Anti-apoptotic siRNA treatment might has a positive impact on the control of cancer progression. Survivin is an anti-apoptotic protein, which is also involved in mitotic checkpoint control and apoptosis induced by growth factor in human cancer cells [22].

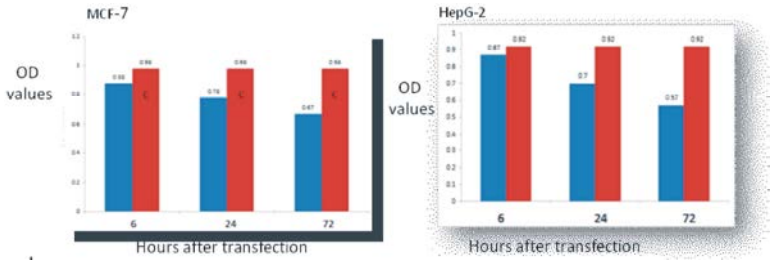
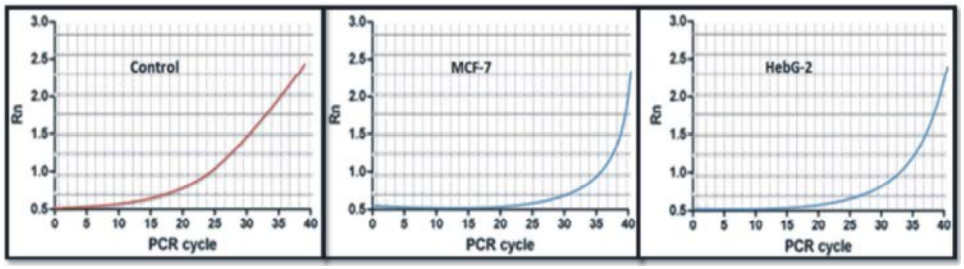


Fig 3: Cell viability as measured by MTT after transfection.



Fige 4: Real Time PCR analysis of Survivin gene expression after transfecting cells with siRNA.

Survivin is overexpressed in a variety of human neoplasms. Currently, Survivin expression has been used as a prognostic factor in several human neoplasms. High Survivin expression by neoplasms correlates with more aggressive behavior, decreased responsiveness to chemotherapeutic agents and shortened survival, when compared with cancers that are Survivin-negative [1].

In this study, real time PCR was performed to assess gene expression after transfecting the two cell lines under study with Survivin-targeting siRNA. The results obtained showed a significant decrease of the amount of RNA in both cell lines in comparison to control. This is indicated by the cycle threshold values obtained which were 0, 30 and 35 for control, MCF-7 and HepG-2, respectively.

In order to knockdown the overexpression of Survivin in cancer cells, various strategies have been investigated. Multiple hammerhead ribozymes targeting the exposed regions of Survivin mRNA synergistically produced the most potent anti-cancer effects and inhibited tumor growth in a hepatocellular carcinoma xenograft mouse model [2]. Recombinant adenoviral vectors expressing siRNA against the Survivin transcript was developed [23]. In all cell lines tested, knockdown of the Survivin expression resulted in apoptotic cell death. Cancer cells infected with these vectors almost lost their tumorigenicity, following inoculation into nude mice. Intratumoral injection with the vectors significantly suppressed tumor growth in a mouse xenograft model. This novel strategy may be a promising tool for cancer gene therapy.

In conclusion, the data obtained indicated that targeting Survivinusing siRNA resulted in a significant knockdown of its mRNA level and, in turn, led to reactivated apoptosis.

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