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SiRNA-Mediated Down-Regulation of Livin Expression in Breast Cancer Cells

[1]Hussein Sabit, [1]Mohamed M.M. Ibrahim and [2]Nabil S. Awad

[1]College of Biotechnology, Misr University for Science and Technology, Egypt
[2]Biotechnology and Genetic Engineering Unit,
Scientific Research Deanship, Taif University, KSA

Abstract: Livin, also called melanoma inhibitor of apoptosis protein (IAP) or kidney IAP, is an anti-apoptotic protein belonging to the IAP family which consists of eight members. The genes of this family render cancer cells insensitive to apoptotic stimulation. The aim of the present study was to investigate and assess the role of siRNA in the regulation of livin gene expression in two breast cancer cell lines (4Ti and MCF-7). Lipofection was carried out to introduce the livin-specific small interference RNA (siRNA) segment (19 mer) into the cancerous cells and the livin expression was determined using RT-PCR. Trypan blue assay was conducted to assess the integrity of the cell membranes after being transfected. 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay was also implemented to assess the cell viability through the mitochondrial reductase enzymes activity. The obtained results concluded that transfecting the cancerous cells with livin-specific siRNA have led to the down regulation of livin expression. RNA interference (RNAi) targeting the anti-apoptotic genes such as livin is a promising approach and may help as a future therapeutic tool for breast cancer.

Key words: SiRNA · Livin · Down-regulation · Breast cancer

INTRODUCTION

Cancer cells are by nature apoptosis-resistant [1] and the abnormalities in the control of programmed cell death play an important role in tumor genesis [2]. Breast cancer is the second most common type of cancer in the world after lung cancer and the fifth most common cause of cancer death [3]. There are approximately 1000000 new cases worldwide per year, causing 370000 deaths affecting most frequently women [4].

Programmed cell death or apoptosis is critical for development and homeostasis in metazoans [5]. IAP proteins antagonize cell death by suppressing active caspases.

It is well-established that two processes, proliferation and apoptosis have a great impact on tumor progression and aggressiveness. Apoptosis is a form of physiological cell death driven by an intrinsic cellular suicide program aimed at excluding unwanted, infected or transformed cells without inflammation [2].

The classical apoptotic pathway consists of activation of the caspase family cascade. Chemotherapeutic drugs induce caspase activation mainly via the mitochondrial pathway. The activity of caspase is negatively regulated by their interaction with the IAP family [6].

RNAi is a fundamental protective process in eukaryotic cells including invertebrates and vertebrates, which is able to block harmful signal by targeting complementary mRNA and cleaving thereof [7].

RNAi therapy is a novel way to treat malignant tumor because it has many advantages such as high degree of specificity, high performance and hypotoxicity. It is one of the most important members in the AIPs family and it can suppress apoptosis [8].

Due to the potential therapeutic value, several strategies have been used to overcome the apoptotic resistance of neoplastic populations [9]. One of the most promising strategies is the use of RNAi technology to suppress the expression of IAPs [10].The antiapoptotic

Corresponding Author: Hussein Sabit, College of Biotechnology, Misr University for Science and Technology, Egypt.

factor Livin has been considered critical for tumor progression and poor prognosis for variant types of tumors [8].

IAPs were first identified in baculoviral genomes by serendipitously [11]. During a screen for baculovirus genes that mimicked the actions of p35 -induced apoptosis a novel 1.6-kb gene encoding a 31-kDa anti-apoptotic protein with a zinc finger-like motif was identified. The gene functions as suppressors of the host-cell death response during viral infection [11].

Additional IAPs were discovered subsequently in both invertebrates and vertebrates, which inhibit apoptosis by blocking caspases activation [12]. To date, eight human IAP family members have been identified including: cIAP1 and cIAP2 (cellular inhibitors of apoptosis 1 and 2), XIAP (X-chromosome binding IAP), ILP-2 (IAP–like protein 2), NAIP (neuronal apoptosis inhibitory protein), BRUCE (Apollon), Survivin and Livin (ML-IAP, BIRC7 or KIAP) [13-17].

Livin, also known as melanoma inhibitor of apoptosis protein (ML-IAP) and kidney inhibitor of apoptosis protein (KIAP) have been identified as a new member of the IAP family proteins with one baculovirus IAP repeat domain and COOH-terminal RING finger domain [18, 19]. Like other IAP family proteins, Livin/ML-IAP interacts with downstream caspases, such as caspase-3, caspase-7 and caspase-9, leading to their inactivation and degradation. It's over expression can protect cells from several pro-apoptotic stimuli (*i.e.*, tumor necrosis factor, Fas, DR4 and DR5 stimuli). Very importantly, treatment of cancer cells with Livin/ML-IAP antisense oligo DNA causes apoptotic cell death, indicating that Livin/ML-IAP expression may be essential for survival of certain cancer cells [20].

However, the aim of the present work was to study the role of siRNA transfection in the down regulation of livin gene expression as a potential therapeutic approach for breast cancer.

MATERIALS AND METHODS

Cell Culture: MCF-7 and 4Ti breast cancer cell lines were kindly provided by the Holding Company for Biological Products & Vaccines, Egypt (VACSERA). Cells were cultured in RPMI 1640 medium (Gibico, USA) supplemented with 10% fetal bovine serum (Sijixin Inc., China) and 1% penicillin–streptomycin (Invitrogen, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay: Cell viability was assessed using the MTT assay (Chemicon, Temecula, CA) as a colorimetric method. The procedures were as follows: the medium was removed and replaced with 100 µl of fresh culture medium (RPMI 1620). Transfected cells were treated with 20 µl of 5 mg/ml MTT. Four wells were used to represent each cell line. Each well of the 96-well microplate contained $5x10^3$cells. A negative control was included by adding 20 µl of the MTT stock solution to 100 µl of cells-free RPMI 1620 medium. The microplate was incubated at 37°C for 3, 4, 5 and 6 hours in a humidified chamber. The formed formazan crystals were solubilized with 150 µl/well dimethylsulfoxide for 10 minutes and then the absorbance values of the solution in each well were measured at 570 nm at the indicated intervals using microplate reader (BioTek Instruments,Winooski, VT).

Trypan Blue Staining: Cells were incubated with trypan blue stain (Invitrogen, 15250–061) after transfection with siRNA. Cells that excluded the dye (viable) and cells that retained the dye (dead) were counted. Samples were diluted in Trypan blue dye of an acid azo exclusion medium by preparing a 1:1 dilution of the cell suspension using a 0.4% trypan blue solution.

SiRNA Transfection: MCF-7 and 4Ti cells were transfected with livin-targeting siRNA at a final concentration of 50 nmol/l (Santa Cruz Biotechnology, USA) and were plated in 6-well plates (2×10^5 cells per well). Prior to transfection, the medium was replaced by serum-free RPMI-1640 (2000µl/well, without antibiotics) and 250 µl serum-free RPMI-1640 was mixed with 5 µl lipofection reagents. The resultant mixtures were kept at room temperature for 30 min and then seeded again into the plates (500 µl each well). The plates were incubated at 37°C for 4 h in CO_2 incubator. Cells were then cultured for an additional 48 h before further analysis. The sequences of livin-targeting siRNA were; Livin siRNA-1: 5-GGAAGAGACTTTGTCCACA-3 and Livin siRNA-2: 5-AGAGGTCCAGTCTGAAAG-3.

Reverse Transcriptase PCR: Total RNA was isolated by RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. RT-PCR was carried out with a RT-PCR Kit (Takara, Japan) according to the kit's instructions. Livin specific primers were: Livin F: 5'-GTCCCTGCCTCTGGGTAC-3' and Livin R: 5'-CAGGGAGCCCACTCTGCA-3'. The PCR conditions were:

one cycle at 95°C for 2 min. Then followed by 38 cycles at 94°C for 30 seconds, 64°C for 45 seconds and 72°C for 30 seconds. Five µl of RT-PCR product was resolved on 1.5% agarose gel after being stained with ethidium bromide.

Real Time PCR Analysis: Livin gene expression has been measured by real time PCR. 5 µl of RNA was subjected to reverse transcription via (High Capacity C-DNA kit, Applied Biosystem, USA). Generated C-DNA has been subjected to Real Time PCR against livin specific primer. Gene expression was calculated according to the cycle threshold in both siRNA-transfected and non-transfected cells. 18SrRNA gene specific primers were used as an endogenous control gene (normalization gene). The 18S rRNA primer sequences were as follows: 18s F: TCA AGA ACG AAA GTC GGA GG and 18s R: GGA CAT CTA AGG GCA TCA CA. PCR amplification was carried out in triplicate for each sample and performed in a total volume of 20 µl containing 400 ng of cDNA, 300 nM each primer and 6 µl of SYBR Green Master Mix. Thermal cycler program was 95°C for 15 sec. and 40 cycles of 94°C for 3 sec., 60°C for 1 sec. and 72°C for 30 sec. Melting point analysis was carried out for the identification of target PCR fragment from 95°C-65°C in 3°C intervals.

RESULTS AND DISCUSSION

Cell Viability Assay: 6 hours after transfection the cell viability was 60 and 49% in 4Ti and MCF-7, respectively (Figure 1).

Trypan Blue Test: Trypan blue test was employed to assess the cell membrane integrity. The results showed that the cell membrane was disintegrated due to the apoptotic pressure occurred by the suppression of the anti-apoptotic protein, livin. Due to the disintegration in the cell membrane, the trypan blue stain could enter the cell through the pores created de novo (Figure 2).

Reverse Transcriptase PCR: Livin gene was detected at a molecular weight of 191 bp (Figure 3). Other molecular weights for livin such as 368 bp (livin α) and 314 bp (livin β) could be detected [21]. These minor differences may be due to the design of primers used.

Real Time PCR: Comparative gene expression analysis carried out by RT-PCR showed that the livin gene expression has been down-regulated in all tested cell lines: MCF-7 and 4Ti, with average Ct values of 30.39 and 26.82 respectively. The untreated sample produced an

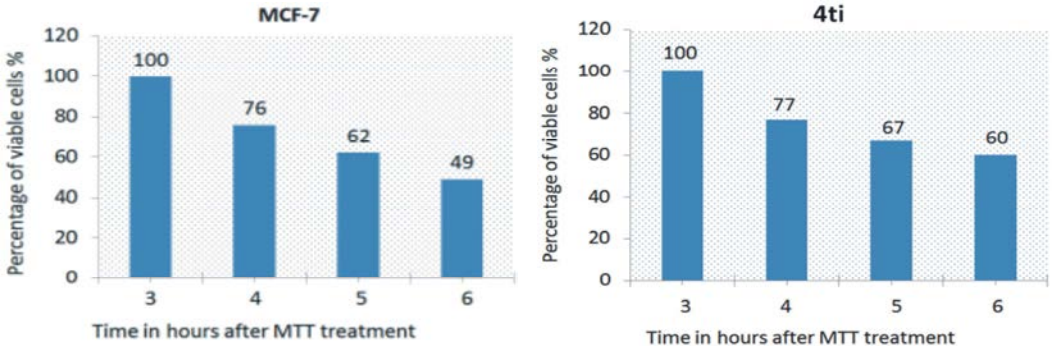

Fig. 1: Percentages of viable cells after being treated with MTT at different time intervals

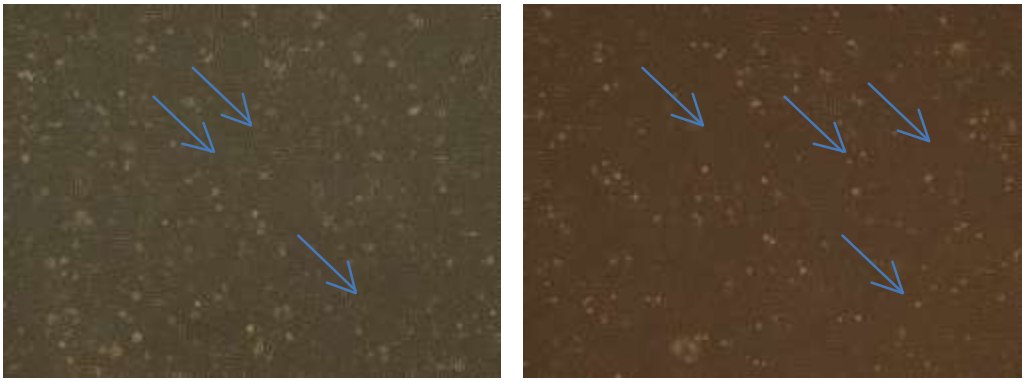

Fig. 2: Trypan blue test; dead cells are arrowed

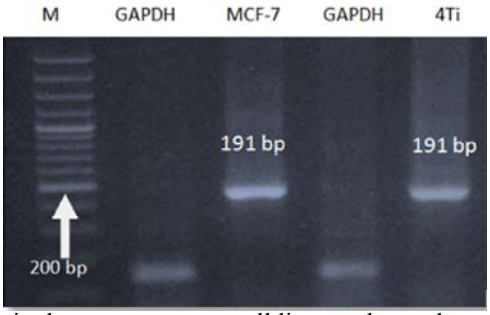

Fig. 3: Detection of the livin gene in the two cancerous cell lines under study

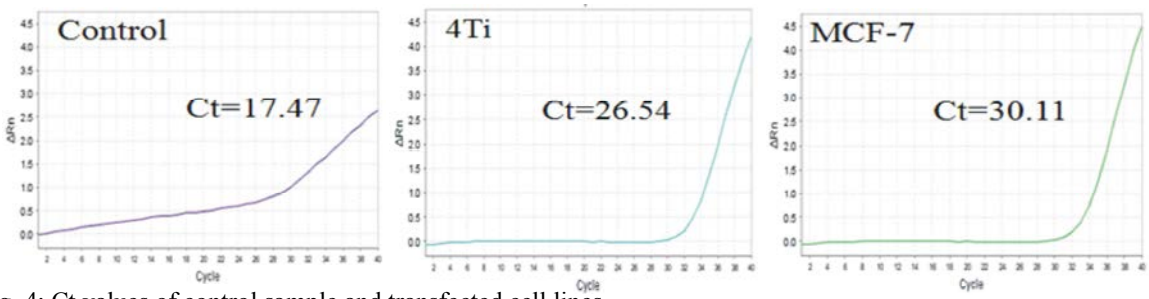

Fig. 4: Ct values of control sample and transfected cell lines

Table 1: Fold differences between treated and untreated cell lines

Sample	Livin Average Ct	Average Ct of treated cell lines – Untreated ones)	Fold Differences
Untreated	17.75	0.00	0.00
MCF-7	30.39	-12.64	3.8x10=38
4Ti	26.82	-9.07	2.73x10=27.3

average Ct value of 15.75 (Table 1 & Figure 4). However, due to an unexpected failure of the amplification of the indigenous control gene (18SrRNA gene) an alternative formula to $\Delta\Delta C_T$ method has been used. This formula assumed that the amplification efficiency of the target gene is 100%. In this case, the 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 fold differences. Therefore, the fold differences between the untreated sample and the transfected cell lines will be 38, 27.3 and 35.4 respectively. Because, there is an adverse relationship between Ct values and m-RNA quantity, these data indicated that the livin expression has been responded negatively (down-regulated) to the anti-apoptotic siRNA lipofection treatment. These data accept the hypotheses of this study which was assuming that siRNA- transfection treatment might down regulates the gene expression of livin gene.

CONCLUSION

Over the last decade it has become increasingly clear that IAPs play an integral role in maintaining cellular homeostasis. In particular, one of these proteins, livin serves many functions involved in cell survival including complex intracellular signaling, stabilizing mitosis and facilitating cellular adaptation.

Livin gene expression has been down-regulated in response to the treatment with siRNA targeting livin mRNA in the cell lines under study. These data confirmed the important role of livin protein as a member of the anti-apoptotic machinery. Much remains to be learned regarding the biology of livin and further larger studies are needed to evaluate the role of all the eight IAP family members as potential therapeutic agents for different types of cancers.

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