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Targeting Apollon *via* siRNA Induces Apoptosis in Different Human Malignancies

[1]Layaly G. Mohamed, [2]Hussein Sabit, [3]Salwa Sabet, [4]Reda El-Sayed Salem and [3]Abdel-Wahab El-Ghareeb

[1]Department of Biotechnology, Animal Production Research Institute, Agriculture Research Center, Giza, Egypt
[2]College of Biotechnology, Misr University for Science and Technology, Giza, Egypt
[3]Department of Zoology, Faculty of Science, Cairo University, Giza, Egypt
[4]Agricultural Genetic Engineering Research Institute, Agriculture Research Center, Giza Egypt

Abstract: Apollon, also called Baculoviral IAP Repeat-Containing Protein 6 (BIRC6) or Baculoviral IAP Repeat-containing Ubiquitin Conjugating Enzyme (BRUCE), 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 small interference RNA (siRNA) in the regulation of Apollon gene expression in four different human cancerous cell lines; breast cancer (MCF-7), cervical cancer (HeLa), colon cancer (CaCo-2) and hepatocellular carcinoma (HepG-2). Lipofection was carried out to introduce the Apollon-specific siRNA into the cancerous cells and the Apollon expression levels were 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- Diphenyl tetrazolium bromide (MTT) assay was also implemented to assess the cell viability through the mitochondrial reductase enzymes activity. The obtained results concluded that transfecting the malignant cells with Apollon-specific siRNA have led to the down regulation of Apollon expression compared to the control non-transfected cells. RNA interference targeting the anti-apoptotic genes such as Apollon could be considered as a promising approach and may help as a future therapeutic tool for many types of human cancers.

Key words: Apollon · SiRNA · Down-regulation · Apoptosis · MCF-7 · HeLa · CaCo-2 · HepG-2

INTRODUCTION

Cancer is a complex disease that manifests itself in a number of forms, all marked by the same uncontrolled proliferation of cells [1, 2]. Cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in the developing countries as a result of population aging and growth as well as, the, adoption of cancer-associated lifestyle choices that include smoking, physical inactivity and westernized diets [3].

Breast cancer is the most common cancer site among women in Egypt, accounting for nearly 35% of all cancers in women [4]. Although early detection of the disease plays a large role in improved prognosis, majority of breast cancers in Egypt are detected at advanced stages [5] when chances of survival are almost diminished. Among all malignancies women are facing, breast cancer comes at the top fear as it affects women self-esteem and confidence. The major concern about breast cancer is that it is the second most killer cancer worldwide [6]. Breast Cancer represents 18.9% of all cancer cases in Egypt, with a rate of 49.6 per 100.000 individuals [6, 7].

Corresponding Author: Hussein Sabit, College of Biotechnology, Misr University for Science and Technology, Giza, Egypt. Tel: +201069737737.

Cervical cancer is a malignant neoplasm arising from cells originating in the cervix uteri. One of the most common symptoms of cervical cancer is abnormal vaginal bleeding, but in some cases, there may be no obvious symptoms until the cancer has progressed to an advanced stage [8]. Cervical cancer is the third most common cancer in women worldwide [9].

Colorectal cancer is the sixth most common cancer in both males and females in Egypt [10]. It was believed that most of the colorectal cancer cases are associated with lifestyle factors and increasing age, with only a small number of cases due to known genetic disorders [11,12].

Hepatocellular carcinoma (HCC) is one of the most lethal malignancies worldwide. It is the sixth most common cancer in the world [3]. Prognosis of HCC remains poor, mainly due to the failure of early diagnosis of the disease in symptom-free patients [13, 14]. Early detection of HCC before the onset of clinical symptoms can lead to curative treatment, significantly improving prognosis [15].

Programmed cell death, particularly apoptosis, is probably one of the most widely studied subjects among cell biologists. Understanding apoptosis in disease conditions is very important as it not only gives insights into the pathogenesis of a disease but may also leave clues on how the disease can be treated. In cancer, there is a loss of balance between cell division and cell death and cells that should have died did not receive the signals to do so. The problem can arise in any step along the way of apoptosis [16]. Meanwhile, activated caspases cleave many vital cellular proteins and break up the nuclear scaffold and cytoskeleton [17].

RNAi therapy is a novel way to treat malignant IAP tumor because it has many advantages such as high degree of specificity, high performance and hypotoxicity. [18]. Due to the potential therapeutic value, several strategies have been used to overcome the apoptotic resistance of neoplastic populations [19]. One of the most promising strategies is the use of RNAi technology to suppress the expression of IAPs [20].

Inhibitors of apoptotic proteins (IAPs) can play an important role in inhibiting apoptosis by exerting their negative action on caspases (apoptotic proteins). Deregulation of these inhibitors of apoptotic proteins (IAPs) may push cells toward cancer and neurodegenerative disorders. Apollon, also known as BRUCE or BIRC6, (BIR-containing protein 6) is the largest member of the IAP family. It is a 528 kDa member of the IAP family. Apollon is a membrane-bound protein that localizes primarily to the trans-Golgi network and other vesicular structures [21]. It was first discovered in mice [22] and has been shown to have some functions outside of apoptosis inhibition [23].

However, the aim of the present study was to assess the role of siRNA transfection in the down regulation of Apollon gene expression as a potential therapeutic approach for breast, cervical, hepatic and colon cancer cells.

MATERIALS AND METHODS

Cell Culture: HeLa (Cervical Cancer), CaCo-2 (Colon Cancer), MCF-7 (Breast Cancer) and HepG-2 (Hepatocellular Carcinoma) cell lines were purchased from the Holding Company for Biological Products & Vaccines (VACSERA), Cairo, Egypt. Cells were cultured in DMEM and/or RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine and 1% of 50 U of Penicillin and 50 mg/ml of Streptomycin (Qiagen, GmbH, Germany). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Viability Assay: Cell viability was assessed by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. In a 96 well plate, each well was loaded with $5x10^3$ cells and cultured until confluence reaches 65%. After applying siRNA, MTT assay was carried out directly. Multiple wells were left as control. Both treated and control wells were loaded with media supplemented with MTT solution. Twenty µl of MTT solution (5 mg/ml) was added to each well including control wells. The cells were then incubated for 3 hours in 5% CO_2 incubator. Fifty µl media was aspirated and then 180 µl DMSO was added to each well. The plate was transferred to a shaker water bath with ambient temperature of 37°C at 250 rpm for 30 minutes to dilute the crystals of formazan. The plate was then ready for reading by ELISA reader at 545 nm.

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 on haemocytometer slide under light microscope. 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, HeLa, CaCo-2 and HepG-2 cells were transfected with Apollon-targeting siRNA at a final concentration of 50 nmol/l (Santa Cruz Biotechnology, USA) and were plated in 6-well plates (5 x 10^3 cells per well). All the media were aspirated and washed with 1 ml of transfection media. The set of transfection media was prepared from solution I and solution II. Solution I was prepared by adding 3 µl siRNA duplex in 50 µl transfection media and solution II was prepared by adding 3µl transfection reagent in 50 µl transfection media. Solution I was added to solution II and mixed well. The mix was incubated for 30 minutes at 37°C and 400 µl of transfection media was added. The media were then ready for transfection. The plates were incubated at 37°C for 6 h in CO_2 incubator. The sequences of Apollon-targeting siRNA were: Apollon-siRNA-1 5'-GGGCAUGCUGGAAUGUUGACGUUAA-3' Apollon-siRNA-2 5'-GGAGAUGAGUGGCUCUUCUUGUAAA-3'.

Reverse Transcriptase PCR: Total RNA was extracted by RNA Isolation System (Qiagen, GmbH, Germany) according to the manufacturer's protocol. RT-PCR was carried out using RT-PCR Kit (Qiagen, GmbH, Germany) according to the kit's instructions. Apollon specific primers were designed and the sequences were: Apollon-F 5'-AACCCTTACATTGGAGGTCTG-3' and Apollon-R 5' GTTCTGCTTGCTGTTCAATGC-3'. The PCR profile started with one cycle at 25°C for 10 min. then followed by 38 cycles at 37°C for 120 min. and 85°C for 5 min.

Quantitative Real Time PCR (qRT-PCR): The generated cDNA has been subjected to Real Time PCR against Apollon specific primer (1 µl for each primer). Gene expression was calculated according to the cycle threshold in both siRNA-transfected and non-transfected cells. Total reaction volume was 25 µl containing about 12.5 µl of SYBR Green Master Mix. Thermal cycler program was 95°C for 15 min. and 40 cycles of 94°C for 15 sec., 54°C for 30 sec. and 72°C for 30 sec. GAPDH was used as internal control.

Protein Extraction: Total protein was extracted from transfected and non-transfected cells by using manual method. Briefly, cells were centrifuged at 14000 rpm for 10 min. and the pellet was collected then 150 µl sample buffer was added and mixed well (by vortex) then samples were kept in ice for 3 min. followed by immediate transfer of the samples to water bath (55°C) for 2 min. and then shock again in ice.

Enzyme-Linked Immunosorbent Assay (ELISA): The cultures of the four cell lines were collected before and after transfection by centrifugation at 14,000 rpm for 5 min. The Extracted proteins were resuspended in ELISA extraction buffer, homogenized through vortex and then diluted in ELISA coating buffer. ELISA plate wells were coated by 150µl of the extracted proteins and incubated overnight at 4°C. The plate was then washed three times for 5 min each using PBS. Monoclonal antibodies (MAbs), specific to Apollon protein (Santa Cruz Biotechnology, USA), were diluted in PBS to reach final concentration of 1µg/ml and added to the ELISA plate by 150µl for each well. Plate was then incubated at 4°C overnight and washed three times for 5 min. each using PBS. For conjugation step, 150µl from the labeled universal antibody (anti-mouse) in conjugate buffer were added to the ELISA plate. Post incubation at 37°C for 3 hr, the plate was washed three times using PBS. For detection, 150µl from the substrate buffer containing 0.75 mg/ml p-nitrophinyl phosphate as a substrate (β-Neutrophenylphosfate) was added to each well. The ELISA readings were measured after 60 min. post incubation at 405 nm.

RESULTS AND DISCUSSION

The main purpose of the present study was to evaluate the expression of Apollon gene and its role in the apoptotic pathways in HeLa, CaCo-2, MCF-7 and HepG-2 cell lines.

siRNA Transfection and Trypan Blue Assay: Transfection was performed according the manufacturer protocols (Santa Cruz Biotechnology, USA). Trypan blue test was employed to assess the cell membrane integrity. The results showed that the cell membrane after transfection with Apollon siRNA was disintegrated due to the apoptotic pressure occurred by the suppression of the anti-apoptotic protein, Apollon (Figure 1).

It was well known that inhibition of a member of IAP might reactive apoptosis in malignant cells [24, 25].

MTT Assay: This test was performed to measure the activity of mitochondrial reductase enzyme, which indicates the cell viability. This enzyme was measured four times on three hours intervals after transfection (Figure 2). Results showed a decrease in cell viability after being transfected with Apollon-targeting siRNA in a positive correlation with time. The obtained results suggest that Apollon down regulation was a key step in

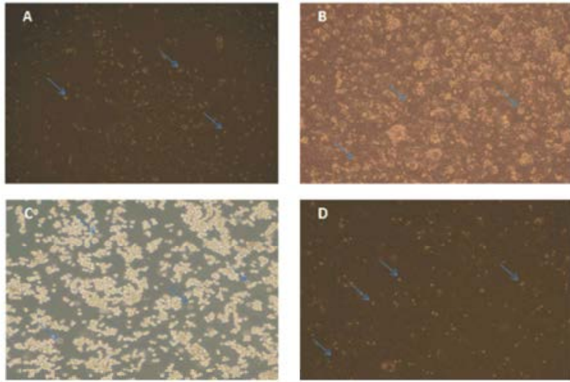

Fig. 1: Trypan blue test; dead cells are arrowed A: HeLa cells, B: CaCo-2 cells, C: MCF-7 cells, D: HepG-2 cells

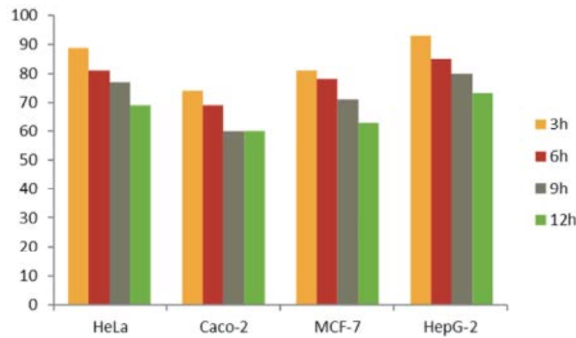

Fig. 2: Percentages of viable cells after transfection at different intervals

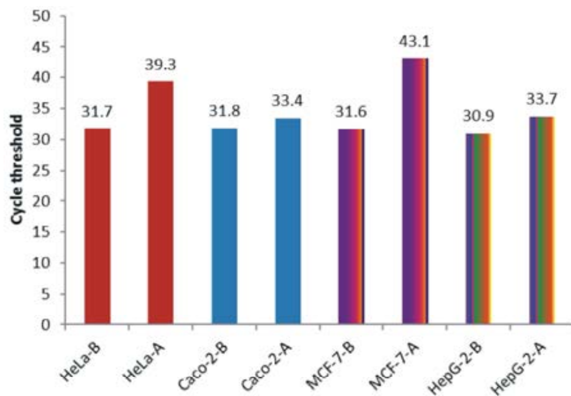

Fig. 3: Cycle thresholds (Cts) values of the four un-transfected and transfected cell lines. (B = Before and A = After)

reactivating the apoptotic machinery, which lead eventually to the death of malignant cells. The breakdown of Apollon mRNA attenuated the production of the corresponding proteins and hence BIRC domain was available to SMAC and then the rest of the apoptotic process [26-28].

Table 1: Fold differences between transfected and un-transfected cell lines

Cell Lines	Average Ct	Fold Differences (ΔΔCt)
HeLa	7.57	22.80
CaCo-2	0.87	2.62
MCF-7	11.44	34.45
HepG-2	0.94	2.83

Real Time PCR: Comparative gene expression analysis carried out by RT-PCR showed that the Apollon gene expression has been downregulated in tested cell lines; HeLa, CaCo-2, MCF-7 and HepG-2 (Figure 3). The results obtained indicated that the amount of mRNA before transfection was larger than after transfection in the four cell lines under study. Results also indicated that there is an adverse relationship between Ct values and mRNA quantity; these data might indicate that Apollon siRNA succeeded to downregulate the expression of Apollon in the studied cell lines.

The fold differences were calculated by $2^{-ÄÄct}$, where each 3.32 Ct differences reflect a 10 time increase or decrease of the mRNA amount. The fold differences between the un-transfected and the transfected cell lines are shown in Table (1) and Figure (3). Due to the reverse proportional relationship between Ct values and mRNA quantity, data indicated that the Apollon expression has responded positively (downregulated) to the anti-apoptotic siRNA treatment. These data revealed that anti-apoptotic siRNA treatment might have a positive impact on the control of cancer progression. The obtained data were in accordance with our previous work [24, 27] and other's work [7, 15, 28].

The level of Apollon expression was decreased with siRNA transfection [29]. Our data indicated that although the differences in Ct values still minor (*e.g.,* Caco-2-B and Caco-2-A, 31.8 and 33.4, respectively), some cell lines were responded positively to the treatment with siRNA (MCF-7 and HeLa). This might shed some light on the role RNAi play in targeting antiapoptotic mRNAs as tool to fight cancer [30].

Protein Extraction and ELISA: Protein extraction was performed according to the ordinary laboratory protocols. The four cell lines (HeLa, CaCo-2, MCF-7 and HepG-2) were subjected to ELISA to detect the levels of an Apollon protein in the presence of its antibody. The results are shown in Figure (4). These data indicated that Apollon protein levels have decreased after siRNA transfection. This decrease might be attributed to the cleavage of Apollon mRNA that inhibited the translation

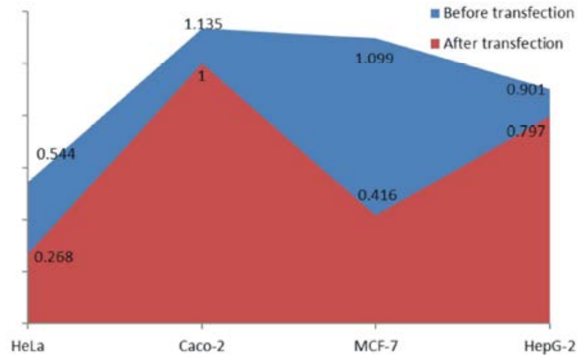

Fig. 4: Levels of Apollon protein as indicated by ELISA in the presence of Apollon antibody in the four cell lines under study

process and eventually led to decreasing the amount of protein compared to the status before transfection [31]. These differences between values for the four cell lines may indicate that the suppression of Apollon protein was successful after transfection in four cell lines but MCF-7 and HeLa cell lines were more responsive for suppression of Apollon protein than CaCo-2 and HepG-2 cell lines. The obtained results were in accordance with several literatures [29, 30], where it is indicated that the application of Apollon-targeting siRNA reduced the activity of the corresponding protein [5].

According to the overall data obtained, it was indicated that targeting Apollon with siRNA has resulted in downregulation of this gene via breakdown of its messenger RNA [31] and hence inhibiting the synthesis of the corresponding protein [32].

CONCLUSION

The data obtained indicated that targeting Apollon using siRNA resulted in a breakdown of its mRNA and, in turn, led to reactivating apoptosis. Over the last decade, it has become increasingly clear that IAPs play an integral role in maintaining cellular homeostasis. Among these proteins, comes Apollon to serves many functions related to the cell survival such as complex intracellular signaling, stabilizing mitosis and facilitating cellular adaptation. In the present study, Apollon gene expression has been downregulated in response to the treatment with siRNA targeting its mRNA in four cell lines, MCF-7, HeLa, CaCo-2 and HepG-2. These data indicated the important role of Apollon protein as a member of the anti-apoptotic proteins family. Much remains to be studied with regard the biology of Apollon and its potential role as a therapeutic agent able to decrease the malignant cell proliferation via reactivating the apoptotic machinery.

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