

The Role of Apollon Gene Silencing on Viability and Radiosensitivity of Cervical Cancer HeLa Cells

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ABSTRACT

Cervical cancer is the second common cause of cancer deaths in women worldwide. Radioresistance of cancer is a principal cause of treatment impairing. Inhibitor of apoptosis proteins (IAPs) widely block apoptosis against apoptotic stimuli, including current chemo- and radiation therapies. Apollon, a member of IAP, can support cells against apoptosis and is over expressed in some treatment-resistant cancer cells.

The aim of this study was to evaluate the effects of apollon knockdown on induction of apoptosis and also its potential for enhancement of radiosensitivity on HeLa cells.

plasmid encoding shRNA which has been confirmed its effect against apollon, transfected into HeLa cells. Consequent effects on the level of P53, Bax and BAK analyzed by real time PCR. Apoptotic phenotype of transfected cells was monitored by Tunnel assay. Viability of HeLa cells after radiotherapy was analyzed by MTT assay.

shRNA1 effectively increased transcription of p53, Bax and BAK and induced apoptosis phenotype of treated HeLa cells. Radiosensitivity of transfected cell was increased after knock-down of apollon obviously.

Apollon knockdown induces apoptosis in HeLa cell. Also it can be as new molecular target for radio-sensitizing strategies in these cells. So, apollon can be a potentially considerable therapeutic object for cervical cancer.

Key words; Apollon, Apoptosis, Radiosensitivity, Cervical cancer

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INTRODUCTION

The development of several cancers are tightly related to Human papillomavirus [HPVs]. This includes cervical cancer which is the second most common cancer in female population worldwide (Ma et al,2010). Apoptosis is a biological mechanism which results in programmed cell death (Sung et al,2007). Incontestable carcinogenesis and tumor progression are the results of apoptotic pathway malfeasance (Wei et al,2008).

Inappropriate apoptosis plays a significant role in the resistance of cancer cells to a wide range of contemporary anticancer therapies (Dong et al,2013). Chemo or radio resistance distinctly impairs the effectiveness of cancer therapy and includes anti-apoptotic signal transduction pathways that prevent cell death (Dai et al, 2009). One of the most apoptosis regulators which causing tumor cells resistant to apoptosis stimuli, like radiation and chemotherapeutic components, are IAPs [Inhibitors of Apoptosis] family (Hung et al,2008).

Apollon, also identified as Bruce or BIRC6, is the huge member of the IAP family which highly expressed in several types of cancer. Hu et al found that apollon knockdown in combination with chemotherapy may have therapeutic potential in the treatment of human colorectal cancer (Hu et al,2015).

In another study, combinations of anticancer drugs with apollon-targeted therapy had efficient impact on treatment of esophageal cancer (Zhang et al,2014).

E6 viral gene is essential for the constant growth of cell lines derived from cervical cancers. E6 blocks cell apoptosis through directing the p53 tumor suppressor protein to the proteasome (Lehoux et al,2009).

Down regulation of p53, leads to reduced apoptosis as well as higher tumor growth and development (Wong,2011).

A variety of treatments are recommended for cervical cancer include radiation therapy, surgery, and chemotherapy (Green et al,2007). The main target of radiation damage is DNA. After irradiation, p53 can detect DNA damage and be obviously increased. Thus, shortcoming in the p53 tumor suppressor gene have been associated

with radio-resistance of cervical cancer (Fulda,2014).

In this study, the effect of apollon knockdown on promoting apoptosis in cervical cancer cells and subsequent increasing of sensitivity to radiotherapy in these cells was examined.

MATERIAL AND METHODS

Cell lines

Hela cell with the source of human cervical adenocarcinoma were obtained from Iran Blood Bank. The cells were grown in DMEM supplemented with 100 units/ml penicillin (Sigma,USA) & 100 µg/ml streptomycin (Sigma,USA) and 10% fetal calf serum (Gibco, Germany) at 37°C in 5% CO₂ humidified atmosphere.

Transfection and real time PCR analysis

In previous study, we constructed three shRNA insert (shRNA1, 2 and 3) against apollon gene (Table 1) and transfected them into hela cell via electroporation method. In brief, at confluency of 70%, 100 µl of hela cell suspension (0.4×10^6 cells/well) were transfected with 5 µg of shRNA-plasmids. For electroporation achievement, multiporator (Eppendorf, Germany) was used. Electrical field for transfer induction of shRNA into the cells was 200 V at 500µs. Transfected cells were transferred into 1 mL of pre-warmed complete growth medium and incubated for 48 hours at 37 °C in a humidified incubator.

For doing real time PCR, total cellular RNA was extracted using Total RNA Purification Kit (Gena Bioscience, Germany) according to the manufacturer's instruction. cDNA synthesis was carried out, using Mmulv reverse transcriptase. Real time RT-PCR was performed with Accupower[®] 2X Greenstar QPCR Master Mix (Bio Neer, Korea). PCR reactions using specific primers under the following condition were designed: Initial 5-min denaturation steps at 94°C and 45 cycles at 94 °C for 5s, 50° C for 8 s and 72 °C for 10s, using Rotor Gen 6000 (Corbett Research, Germany). Amplification of β-actin was carried out as internal control. Primer sequences are listed in Table 2.

Real time PCR results showed the mostly effective shRNA on apollon mRNA expression was shRNA1 (Milani et al,2016).

Table 1- Sequence of shRNA against apollon mRNA

shRNA	sequence
Apollon1	CACCCTGCGCTCAACGCCATC
Apollon2	CATGCTGGAATGTTGACGTTA
Apollon3	TGGGAGATTGTTGCAAATG

Expression levels of p53, Bax and Bak

Relative mRNA expression of p53, bax and Bak genes between shRNA1- treated and pRNAin H1.2 NEO transfected cells [mock control],

performed with specific primers (Table 2) by the same protocol for apollon .

Table2- Primer sequences used for real-time PCR

Gene	Sequence
Apollon	5'-AGTGCAACGATGTGCCAT -3'/5'-GCTAACCAACAGAGAGTA-3'
P53	5'ACAATGGTTCACTGAAG3'/5'CTGTCCCAGAATGCAAG3'
Bax	5'TTTCTGACGGCAACTAC3'/5'ATGTCCAGCCCATGATGG3'
Bak	5'TTTACCGCCATCAGCAGG3' /5'TGCTGCAACATGGTCTGG3'
β -actin	5'-GATGAGTATGCCTGCCGTGTG-3'/5'CAATCCAAATGCGGCATCT-3'

Tunnel assay

To evaluate cell death by apoptosis, tunnel staining was performed using DeadEnd colorimetric kit (Promega) according to the instructions from the company. Cells (5×10^3) were transferred into poly-L-lysine coated coverslips. After 24 h, cells were transfected with mixture of liposome and shRNA1-plasmid at a 3:1 liposome:DNA ratio in 1 ml of serum-free media per transfection and incubated at 37°C for one day. Then the coverslips were incubated in 4% paraformaldehyde, permeabilised (0.1% triton X-100 in 0.1% sodium citrate) and then incubated with terminal deoxynucleotidyl transferase mediated dUTPbiotin for 1 h at 37°C. After rinsing with PBS, horseradish peroxidase (HRP) enzyme was added to coverslips at 37°C for 30 min. After adding the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB), labeled DNA fragments were visualized and measured by fluorescence microscopy.

Radiation sensitivity determination

Cells were Grown 24 h in 8-well chamber slides seeded with 10^5 cells per well. Then, sh-RNA plasmid was transfected to hela cells using electroporation. mock control group was transfected with pRNAin H1.2neo vector alone. 24

h after transfection, Cells were irradiated with gamma-rays from a 60Co source under the sterile conditions at 37°C. The irradiation dose was 4 Gy , a dose before revealed to be favorite for apoptosis induction in HeLa cells, and the dose rate was 2.92 Gy/min. The effects of irradiation on cell viability, was measured by MTT assay.

In brief, cells were covered with 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) and incubated for an further 4 h. For solubilizing the formazam crystals, 100 μ l of DMSO was added. The absorbance measurement was performed at 490 nm wavelength and reference wavelength of 620 nm in a microplate reader (Tecan, Sweden).

Statistical analysis

All statistical analyses were performed by using SPSS16. Each experiment was carried out in triplicate for all data (n=3). Differences between control and shRNA-transfected cells in terms of growth and viability of the cells were analyzed using one-way analysis of variance (ANOVA) and independent samples t-test. Results were considered statistically significant at $P < 0.05$. Also, an average expression value (*E* value) indicating gene regulation was calculated using REST

software, and 95% confidence intervals was used for expression ratios.

RESULTS

We used pRNAin H1.2 Neo vector system which is designed to facilitate the cloning of the double-strand DNA oligonucleotide which is transcribed into shRNA by RNA polymerase III promoter once expressed in cells.

Quantitative analysis of *p53*, *bax* and *bak*

In previous study, we primarily have seen that expression of shRNAs for apollon markedly reduces the mRNA levels of this gene by mean factor of 0.02 in transfected hela Cell (Milani et al,2016). In this study, we evaluated the effects of apollon gene silencing on expression level of bax,bak and P53 using real time PCR . The analysis of RT-PCR using REST software showed that the expression of bax,bak and P53 mRNA in hela cells transfected with the apollon shRNA1, was increased significantly by mean factor of 18.5,52.3 and 35 (1.164,7.67 and 1.166 fold) respectively compared to the mock control group.(Figure1).

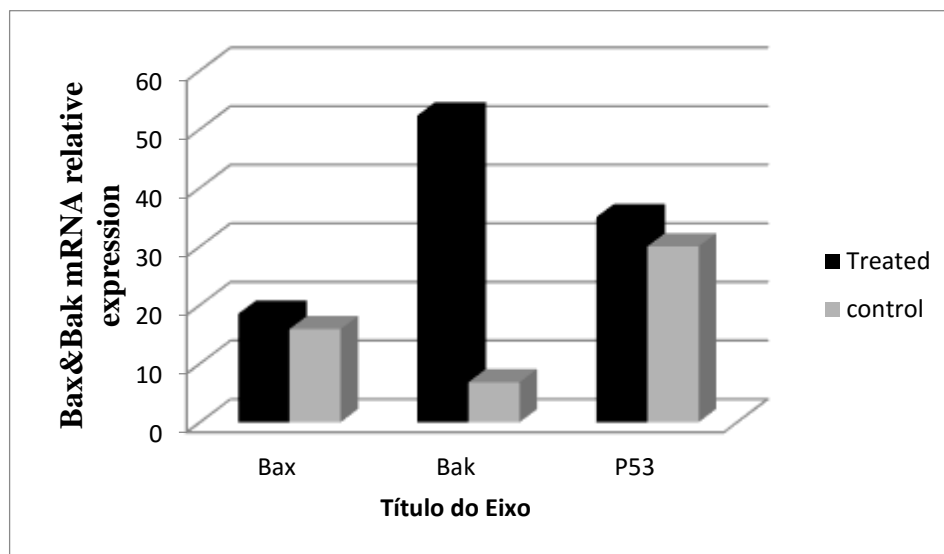


Figure1 - Up regulation of P53,Bax and Bak after knock down of apollon was shown after transfection the hela cell with shRNA1-plasmid. The mRNA expression of above genes was normalized with β -actin. An average expression value (*E* value) indicating gene regulation was calculated using REST software, and 95% confidence intervals were used for expression ratios.

Fluorescence microscopy and TUNEL assays

To affirm the apoptosis induction after knock down of apollon gene, TUNEL assay was carried out. Nuclear DNA fragmentation, a significant biochemical indicator of apoptosis was calculated. The fragmented DNA of apoptotic cells is end-labeled with TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. Biotinylated nucleotide is entered at the 3'-OH DNA ends with Terminal Deoxynucleotidyl Transferase (TdT). Horseradish-

peroxidase-labeled streptavidin (Streptavidin HRP) is then linked to these biotinylated nucleotides, and then manifested using the peroxidase substrate, H₂O₂, and the stable chromogen, diaminobenzidine (DAB). So apoptotic nuclei were stained dark brown. TUNEL staining revealed that, apoptotic nuclei and apoptotic positive cells were prevalent in transfected samples (Figure 2).

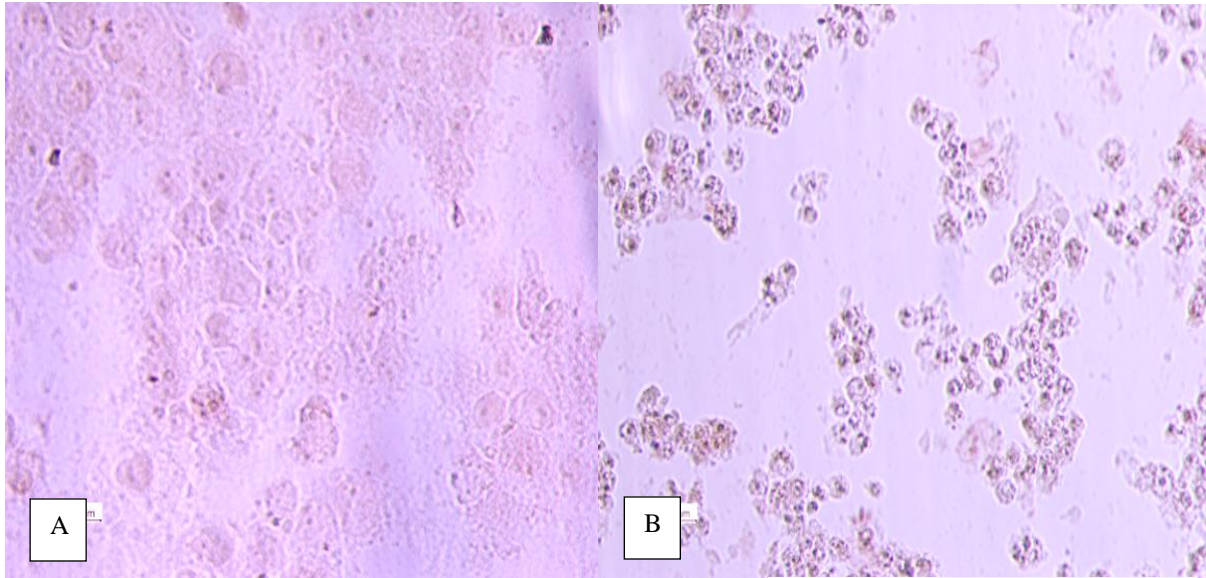


Figure2 - Tunnell staining for detection of apoptosis and apoptotic nuclei. apoptotic cells was stained dark brown. A) Control cells .B) shRNA1 transfected cells

Sensitivity to radiotherapy

More importantly, we examined the effect of combined treatment of IR with knock down of apollon in hela cancer cell lines for the first time. In the MTT assay, the cell growth inhibition

of cervical hela cancer cells treated with both IR and shRNA against apollon was considerably greater than untransfected and shRNA treated alone (Figure3).

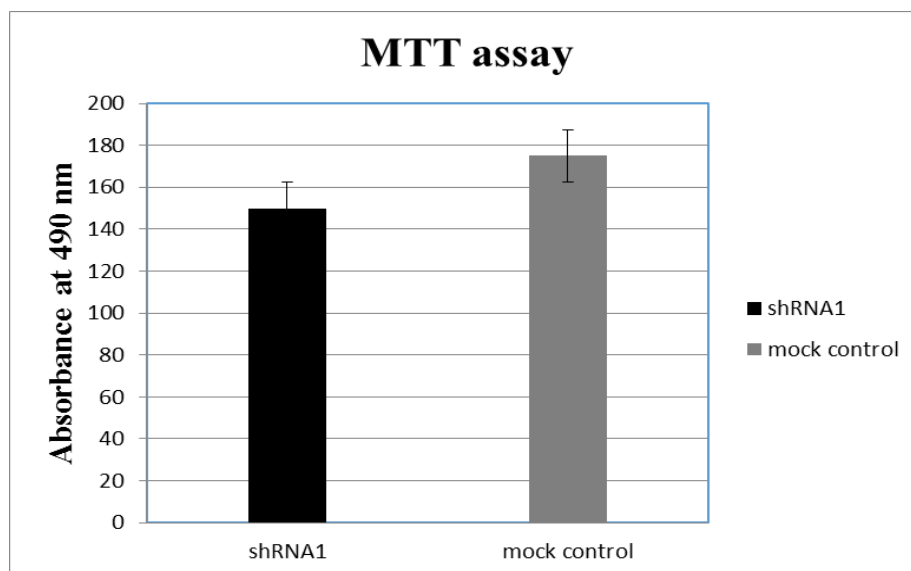


Figure3 - Apollon down-regulation on viability of hela cell after radiotherapy. cell viability was measured using MTT assays. Each bar represents the mean value \pm standard deviation (SD) of triplicate. $P < 0.05$ was compared to the control cell group.

DISSCUSION

HPVs plays a significant role in the development of cervical cancers which is the second most common cancer in females worldwide (Hung et al, 2008). There are no treatments that focus on cells containing HPV DNA, as a result, HPV-induced tumors are basically treated by surgery along with radiotherapy or nonspecific chemotherapy (Green et al 2007).

The resistance of tumor cells to apoptosis may trigger drastic clinical challenges. High-risk features at diagnosis and a poor response to various treatments, such as chemotherapy and radiotherapy can be associated with such situations (Wang et al, 2009). Radiotherapy as a treatment option has been suggested for patients with locally advanced cervical carcinoma leads to progression-free survival rates of 70% (Wang et al, 2009; Saxena et al, 2005). A wide range of stimuli can activate the process of apoptosis. For example, radiation has been shown to be one such initiator. (Saxena et al, 2005)

IAPs crucially contribute to the regulation of apoptosis. Several antiapoptotic proteins are expressed in different tumors, and their expression levels may be varied depend on the negative features at diagnosis and/or a poor response to treatment (Sung et al, 2007).

Apollon, as a member of IAP, has a significant role in apoptosis inhibition and is up regulated in specific tumors, so its protein may be a favorable molecular target for treatment of cancers (Chu, 2008). The goals for RNAi views for cancer therapy are ultimately to stop the expression of a cell cycle gene and/or an anti-apoptotic gene in the cancer cells leads to ending tumor growth and destroying the cancer cells (Wang et al, 2008).

Effective RNAi can be accomplished not only by transfection of siRNAs, but also by presentation of shRNA (short hairpin RNA) expressing plasmid into human Cells (McIntyre et al, 2006).

In previous study, we primarily have seen that expression of shRNAs for Apollon markedly reduces the mRNA levels of this gene in transfected HeLa cells (Milani et al, 2016). In this study, we evaluated the effects of Apollon gene silencing in this cell line.

Results of TUNEL assay showed that the loss of Apollon increased the apoptotic phenotype in transfected cells. Using real time PCR revealed that the decrease in the levels of Apollon caused an

increase in the levels of p53 and proapoptotic genes Bax and Bak in transfected cells.

Expression of E6 protein from HeLa cells containing human papilloma virus leads to p53 degradation (25) (Niiforovi et al, 2004). Our observation may be explained by the fact that Apollon knock down impairs the growth of cells markedly. This finding is parallel by upregulation of p53 and activation of the mitochondrial apoptotic pathway including Bax and Bak upregulation. More importantly, we examined the effect of combined treatment of IR with knock down of Apollon in HeLa cancer cell lines for the first time.

In the MTT assay, the cell growth inhibition of cervical HeLa cancer cells treated with both IR and shRNA against Apollon was considerably greater than untransfected and shRNA treated alone.

In addition, High-level expression of the HPV16 E6 oncoprotein in tumors derived from transplanted human cervical carcinoma cells confers an aggressive radiation-resistant phenotype confirmed by former studies (Jung et al, 2015). So, it is fair to assume that the observed reduction in the growth capability of tumor cells resulted from the knock down of Apollon, upregulation of p53 and following induction of apoptosis.

The results of one study showed the important role of Apollon in prostate cancer progression and treatment resistance, is and indicated for the first time that the *BIRC6* gene and its product are potentially valuable targets for treatment of prostate cancers (Low et al, 2013).

In another study, elevated Apollon protein expression confirmed as predictive marker for chemoresistance and poor prognostic factor for lung cancer patients (Dong et al, 2013).

In clinical perspective, continuous treatment with effective IAP-inhibitors throughout the cycles of chemo/radiotherapy may reduce the risk of tumor local recurrence as well as metastasis. Therefore, a promising novel strategy to enhance the efficacy of present cancer treatments might be the combination of IAP-targeting molecular therapy and conventional chemo/ radiotherapy which ultimately improve the survival of cancer patients (Dai et al, 2009).

Zhang et al found an inverse relationship between survivin- a member of IAP family- mRNA abundance and in vitro sensitivity to irradiation in pancreatic cancer cells (Zhang et al, 2006). Wang

showed that RNAi-mediated downregulation of XIAP [another member of IAP] expression can inhibit proliferation, induce apoptosis and moderate the radio-resistance of laryngeal carcinoma cells (Wang et al,2009).

Previous studies revolved around the impacts of apollon down regulation on sensitization of cancer cells to chemotherapy agents. Chu applied [ZD55-siApollon] which is directed against apollon in hela, HT-1080 and MCF-7 cells. He found that ZD55-siApollon increased the antitumor effect of 5-fluorouracil, a chemotherapeutic agent (Chu,2008). Another study determined that apollon siRNA enhanced the chemosensitivity of HCC cells to the adriamycin and 5-fluorouracil (Chen et al,2011).

Given the fact that apoptosis is the main type of cell death induced by a variety of anticancer agents and IR. our study showed a potential and critical role of apollon as a factor of the radio-sensitivity in cervical cancer cells. This study indicated that apollon knock down can induce apoptosis in hela cell line. Additionally, our results indicated a significant radiosensitivity in hela cell with down regulated expression of apollon up to now (as recorded in literature).

CONCLUSION

Results of this study showed the effectiveness of combination treatment and subsequent enhancement of radio-sensitivity after apollon silencing in cervical cancer hela cell. Additional studies needed to validate apollon as a target of radiation sensitization in animal models of cervical cancer in a greater extent.

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