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P53 siRNA - A THERAPEUTIC TOOL WITH SIGNIFICANT IMPLICATION IN THE MODULATION OF APOPTOSIS AND ANGIOGENIC PATHWAYS

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Abstract

Background and aims. *siRNAs represent an encouraging novel alternative in cancer therapy as a result of targeting the mutated tumour suppressor genes or activated oncogenes. Targeting oncogenic signals, as the mutated p53 gene that gains oncogenic role, we observed inhibition of migration, a downregulation of specific genes involved in apoptosis but also in angiogenesis, connected with a reduction in invasion rate in the case of p53siRNA therapy.*

Methods. *The study was designed to assess the role of p53 by using RNAi (RNA interference) in Hela in vitro cell culture model. Therefore cell migration rate was assessed by using xCELLigence Systems, gene expression for a panel of genes involved in apoptosis and angiogenesis, and validation of gene expression data at protein level.*

Results. *On the selected in vitro model p53 siRNA therapy was correlated with the reduction of cell migration. The downregulation of p53, PTEN, TNFα, NFkB, BCL-2, ICAM-2, VEGF, and FGFb was evidenced as response to p53 inhibition.*

Conclusion. *RNAi may be a valuable technology in order to restore the normal cellular phenotype. The results in the current research may also have an important significance outside the context of cervical cancer, by using specific inhibitors for p53 for increasing the therapeutic response in a wide range of tumoral pathology.*

Keywords: cancer, p53 siRNA, apoptosis, angiogenesis

Background and aims

Cancer is one of the leading causes of death worldwide and accounted for 7.6 million cases (13% of all deaths) in 2008 [1]; in 2013 in the United States there are 12,340 estimated new cases and 4,030 deaths by cervical cancer [American Cancer Society: Cancer Facts and Figures 2013]. Meanwhile in Romania, based on statistical data from 2012, cervical cancer is ranked third place taking in account the women cancer incidence, after the breast and colorectal cancer [2].

Cervical cancer develops in a multi-stage process, successively passing from a cervix with a normal epithelium to cervical intraepithelial neoplasia and

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finally leading to invasive cervical cancer. A relevant epidemiologic risk circumstance for the progress of the cervical cancer is represented by the presence of High Risk Human Papillomavirus infections [3]. Nevertheless, HPV infection only is not sufficient to cause cervical cancer. Consequently, considerable importance has been given to the molecular premise which leads to the progression of cervical cancer [4].

RNAi (RNA interference) is a natural mechanism able to specific inhibit a target gene [5,6]. The prospective exploitation as a therapeutic has lately been strengthened by the small interfering RNA (siRNA) delivery to human tumours [7,8].

RNAi has been demonstrated as a relevant investigation device for particular target genes by using exogenous siRNAs. siRNAs are single-stranded non-coding RNAs with 20–23 nucleotides length, being able to negatively modulate gene expression profile at post-transcriptional level. siRNAs represent an encouraging novel type of medicaments for cancer therapy, as a result of targeting the mutated tumour suppressor genes or activated oncogenes. Many recent investigations have shown that siRNA can efficiently inhibit oncogene expression in cancer cells [9,10].

Various investigations demonstrated clearly that the deactivation of p53 constitutes a crucial step in carcinogenesis [11]. The present study was designed to investigate the potential use of p53 RNAi to block p53 expression, as well as the subsequent effect on cell invasion and gene expression on the human cervical cancer cell.

Materials and methods

Cell culture and treatment. HeLa is a human cervical carcinoma cell line; this was incubated in a 5% CO_2 incubator at 37°C. For maintenance, cells were cultured in DMEM medium, 10% FCS (foetal bovine serum), 100 U/mL penicillin and 100 mg/mL streptomycin, 2 mM L-glutamine, 1% nonessential amino acids, all purchased from Sigma-Aldrich, Bucharest, Romania. For this investigations 5×10^5 HeLa cells/well were used for reverse-transfection, based on a preliminary optimised protocol for p53siRNA, using siPort NeoFx as transfection agent (Ambion, USA). A validated p53siRNA pool (sc-29435, Santa Cruz) was used at a concentration of 50nM in serum starvation condition using OptiMem medium (Gibco, Invitrogen, Romania), in accordance with the producer recommendations. SiPort NeoFx 0.25% concentration was able to achieve the maximum inhibition of target gene with no effects on cell viability. All tests were carried out in triplicate.

Dynamic monitoring of invasion of HeLa cells using the xCELLigence System. We performed the invasion assays on HeLa cells to evaluate the impact of p53siRNA treatment using the CIM-Plate 16 with the xCELLigence RTCA DP Instrument (Roche Applied Science) according to the described protocol by [12].

RNA extraction quality control and qRT-PCR. The total RNA for the cells treated with 50 nM p53siRNA andthe control group respectively at 24 hours post treatment was extracted using TriReagent (Sigma-Aldrich, Bucharest Romania) based on the producer recommended protocol for each triplicate specimen. The RNA concentrations and quality were assessed by the NanoDrop-1100 Agilent 2100 Bioanalyzer spectrophotometer. All of the samples had a RIN (RNA integrity number) higher than 7.5. The 8 genes selected were assessed using specific primer and UPL based on *in silico* design. The total RNA (500 ng) from all the samples was reverse transcribed using the First Strand cDNA Synthesis Kit for RT-PCR (Roche, Bucharest Romania). For the gene amplification we used TaqMan Universal PCR Master Mix, in a 20 µl volume in a 96-well plate using the Roche LightCycler® 480 System. The qRT-PCR reaction amplification program was as follows: 10 minutes at 95°C for enzyme activation followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C for the amplification step.

qRT-PCR data analysis. The data analysis was carried out to compare the gene expression values for the treated and untreated groups using $\Delta\Delta C_t$ method. *As* housekeeping gene was used b-active. All the results were presented as the average ± standard deviation (SD).

VEGF protein quantification. The evaluation of the VEGF protein expression at 48 hours post treatment was done using Human VEGF Quantikine ELISA Kit (R&D, catalog no. DVE00) using the producer recommended protocol.

Results

Inhibition of HeLa cell migration after p53 gene knockdown. xCELLigence System is an innovative device that allows the scanning of cellular response via an impedance-based technology in real time, lacking any exogenous labels. The CIM-Plate 16 furnishes a kinetic cell-response profile to p53siRNA throughout an investigation, specifying the commencement and ratio of invasion and migration of HeLa cells. This data can facilitate to comprehend the response to treatment in dynamic. In Figure 1 we can observe a delay and a reduction of the cell migration after the p53siRNA treatment.

qRT-PCR results for main genes involved in apoptosis and angiogenesis. TaqMan qRT-PCR assay was used to examine the effect of p53siRNA on a panel of 8 genes related to apoptosis and angiogenesis. Relative gene expression quantification using ΔΔct method leads to the downregulation of the selected gene, presented in the Figure 2.

VEGF protein expression. After 48h post transfection with p53 siRNA inhibition in HeLa cell line, VEGF protein was found dowregulated in the culture medium than in the control group (Figure 3).

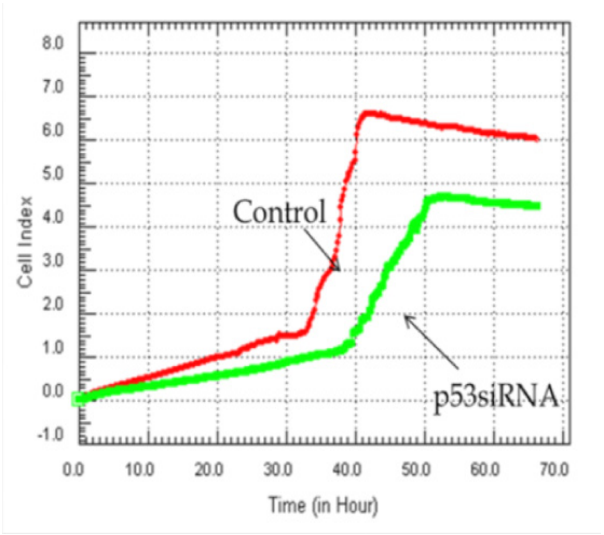

Figure 1. Evaluation of HeLa cell migration after p53 gene knockdown using the xCELLigence System.

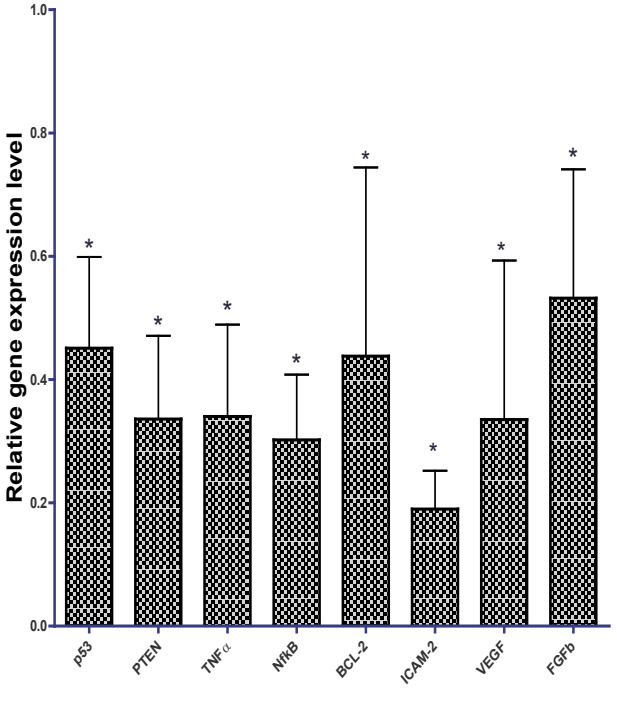

Figure 2. Relative gene expression profile assessed using ΔΔCt method and β-actin as housekeeping gene; determination performed at 24 hours transfection with p53siRNA.

Discussion

Cervical cancer remains an important cause of death worldwide [13], and particularly in Romania. Although at this moment cervical cancer is considered as a preventable disorder there is a significant risk of disease recurrence causing a persuasive necessity to research new therapeutic

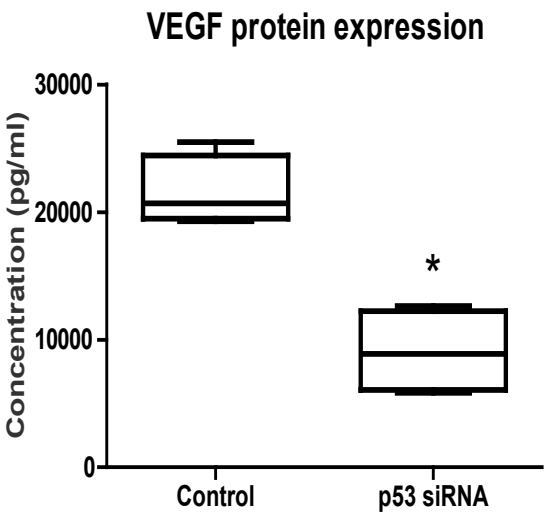

Figure 3. Alteration of VEGF protein expression determined using ELISA from cell culture medium, after 48 hours transfection with p53siRNA. VEGF concentration being expressed as pg/ml.

targets for this disease management [14]. It is now well recognized that the tumour progression of all cancers is characterized by intensified proliferation and invasion rate and diminished in apoptosis. At the same time the apoptosis and angiogenesis are interconnected as can be observed from Figure 4, using STRING.9 database.

The idea of this study is in agreement with the previous studies which are based on the hypothesis that once mutated, p53 exercised oncogenic role [15]. By using siRNA we intended to disarm the oncogenic role of p53. The role of the present study is to emphasize the cooperation between oncogenic mechanisms, confirming the crosstalk between apoptotic and angiogenic mechanisms [16]. This has a significant therapeutic relevance based on the fact that mutated p53 is related to cancer aggressiveness [17] or to promoting metastasis [18]. In a similar study was observed that, by using siRNA targeting p53/p73, tumoral cells were sensitized to chemotherapy [19].

In a recent study, PinX1 was displayed as a novel target gene of p53, proposing the suppression of p53/PinX1 pathway as a novel mechanism to enhance the telomerase activity in cervical cancer cells, and leading to reduction of cell proliferation [20]. Targeting oncogenic signals such as the mutated p53 gene that gains oncogenic role, we observed a downregulation of specific genes involved in apoptosis but also in angiogenesis, connected with a reduction of the invasion rate in the case of p53siRNA therapy [21]. After 24 hours of treatment with p53siRNA we can observe downregulations of important genes involved in apoptosis and angiogenesis, as displayed in Figure 2.

This study confirmed our hypothesis that p53 plays a central role in carcinogenesis as displayed in Figure 3, as

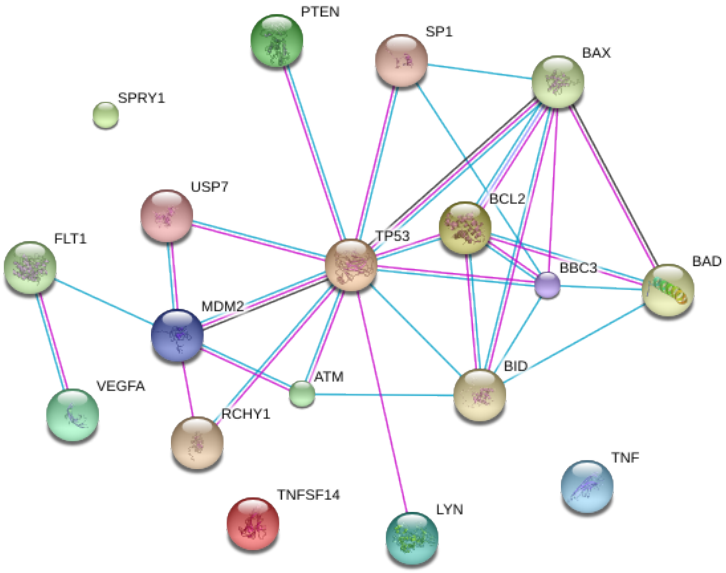

Figure 4. p53 and its relation with apoptosis and angiogenesis proteins, network generated using STRING.9 [22].

important therapeutic target [23]. P53 could promote the convergence of the apoptotic and angiogenic pathways in cervical cancer cells. It has been proved that p53 is able to indirectly downregulate *VEGF* gene expression, via the retinoblastoma (Rb), in a P21-dependent mode, confirming its function in cell-cycle regulation as displayed by recent studies [24,25].

The complete comprehension of the cross-talk between p53 and angiogenic pathways represents a challenge in cancer therapy [24-26]. Another study reveals that mutant p53 correlates with overexpression of VEGF [27]. In a similar study it has been demonstrated that p53 negatively regulates tumoral cells growth and migration [28]. Clearly the inhibition of mutant p53 represents a significant therapeutic target, a fact emphasized by Walerych as 'rebel angel' [16].

Conclusions

p53 gene knockdown in HeLa cells plays an important role not only in apoptosis but also in angiogenesis through various mechanisms that regulate tumour cell development and migration. The angiogenic phenotype related to p53 targeted by siRNA in human cancers is frequently associated with resistance to conventional genotoxic anticancer agents. Therefore, p53 inhibitions become dependent and rely on angiogenesis for growth and metastasis. The results in the current research may also have an important significance outside the context of cervical cancer, by using specific inhibitors for p53 for increasing therapeutic response in a wide range of tumoral pathology.

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