TARGETING ANGIOGENESIS WITH siRNA IN CANCER

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Summary

Synthetic siRNA is used in mammalian cells for gene silencing. siRNA-induced RNAi is a key strategy for investigating gene function, a method that reduces the expression of individual genes in order to establish a link between gene identity and gene function. In our experiments we used siRNA VEGF to inhibit the main factor of angiogenesis that is considered to play an important role in tumor growth and metastasis. In this study a VEGF small interfering RNA (siRNA) was synthesized and through reverse-transfection technology was introduced in hepatocellular carcinoma cell line HepG2, using siPORT™ NeoFX Transfection Agent. VEGF expression was analysed by real time PCR, and the results were confirmed at protein levels by ELISA assay. MTT assay was used to detect cell viability. Apoptosis was analyzed using DAPI staining. The evaluation of real-time PCR gene expression showed a downregulation of VEGF at mRNA level in transfected cells compared to control group. These results were confirmed also at a protein level. VEGF-siRNA cell growth inhibition assessed by the MTT assay, showed that the transfection agent had no cytotoxic effect at 24 and 48 hours. An increased level of apoptotic processes was observed especially at 48 hours. MTT analysis shows that the transfection agent has no cytotoxic effect; the low cellular proliferation rate may be caused by the inhibition of VEGF gene and protein expression leading to the activation of apoptotic pathways.

Key Words: siRNA, hepatic cancer, angiogenesis, VEGF, culture cell lines

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent type of cancer (Lovet et al., 2003) and the third most common cause of death from cancer in the world (Tang et al., 2003, Parkin et al 2005) and it's incidence is still increasing in developed countries because of the hepatitis B and C viral infections and increasing cirrhosis incidence (Bolog et al., 2011). Considerable progress has been made in diagnostic and therapeutic modalities (Rampone et al., 2009). The main cause of HCC development, growth and metastasis is caused by angiogenesis. The new blood vessel formation is critical for tumor formation, providing it with the necessary amount of nutrients and oxygen. Tumor blood vessels are generated by various mechanisms involving numerous proangiogenic factors. These specific factors which are normally involved in liver regeneration are believed to be able to influence the growth of residual or dormant micrometastases after PH and modulating tumor angiogenesis (Chen et al., 2010).

The most important factor of tumor angiogenesis is vascular endothelial growth factor (VEGF). The increased expression and secretion of vascular endothelial growth factor leads to the activation of endothelial cells resulting in the formation of new capillaries (Raskopf et al., 2011, Pircher et al., 2011). Members of the VEGF family are VEGF-A, -B, -C, -D, -E, and placenta growth factor (PIGF).VEGF-A gene is alternatively spliced to form several isoforms of which VEGF-A165 is considered to be the most predominant isoform (Yoo et al., 2006, Furuya et al .,2005). VEGF and its receptors VEGFR-1
(flt-1) and VEGFR-2 (flk-1/KDR) are key regulators of tumoral angiogenesis (Madeddu 2005) and are co-expressed in pancreatic cancer suggesting that VEGF could have autocrine effects on pancreatic cancer cells that express VEGF receptors and paracrine effects on microvascular endothelial cells (Mulder 2010).

The discovery of RNA interference heralded a revolution in RNA biology (Dykxhoor and Lieberman 2005). In eukaryotic cells, the RNAi pathways comprise different mechanisms involved in regulation of gene expression and posttranscriptional gene silencing (Dykxhoor and Lieberman 2005, Gartel and Kandel 2006). RNAi silencing pathway begins with the insertion of small RNAs that include siRNAs in the cell. One strand of the 19-21 bp siRNA, called the guide strand is inserted in the protein effector complex RISC and induces gene silencing in several ways: they direct sequence-specific cleavage of perfectly complementary mRNAs and translational repression and transcript degradation for imperfectly complementary targets (Grimm and Kay 2007, Tomari and Zamore 2011, Kim and Rossi 2007). Therefore siRNA is a key strategy for investigating gene function, a method that reduces the expression of individual genes in order to establish a link between gene identity and gene function.

**Material and methods**

**Cell culture.** HepG2 cell line is a hepatocellular carcinoma derived from the liver tissue of fifteen year old male, with a model chromosome number of 55. Cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C in humidified atmosphere with 5% CO2.

**Cell treatment.** Cells were treated using a technique called reverse transfection that involves simultaneously transfecting and plating the cells. $3 \times 10^5$ cells were seeded into six-well plates, and treated with siRNA-VEGF from Silencer® siRNA Transfection II Kit (Ambion, USA). For each well, 5 µl NeoFX transfection agent was mixed with 95 µl Opti-MEM I. After 10 minutes of incubation at RT the mixture was combined with 2.5 µl siRNA in 97.5 µl Opti-MEM I. Cells were cultured in RPMI-1640 medium antibiotic-free for 24 respectively 48 h at 37 °C, 5% CO2 before analysis.

**Cell viability assay** was performed in 96-well plates using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide (MTT) assay. After 24 and 48 h following siRNA transfection, 50 µl of MTT was added to each well. After 1 h of incubation in cell culture conditions mentioned above, 50 µl DMSO was assessed to each well. Absorbance was measured at 490 nm using a Biotek Synergy HT Microplate Plate Reader.

**Protein analysis.** VEGF immunoassay analysis was performed according to the protocol recommended by the manufacturer (VEGF Quantikine kit, R&D Systems, Romania). Immunoassay for VEGF was performed after 24 and 48 hours of treatment.

**Cell staining.** Cells were plated on a 24-well plate and treated according to the protocol mentioned above, then the cells were washed with PBS (Phosphate Buffered Saline) and were incubated with 0.5µg/ml FDA (fluorescein diacetate) for staining alive cells. For DAPI staining cells were fixed with 4% PFA (Paraformaldehyde) for 20 minutes room temp, washed with PBS then were added DAPI solution (1mg/ml) 1:5000 dilution, incubated 10 minutes at room temp and finally washed with PBS.

**Quantitative RT-PCR analysis.** Total RNA was isolated using TriReagent (Sigma, Bucharest, Romania) according to the manufacturer’s protocol. The reverse transcription was performed by Gradient Palm-Cycler™ RT-PCR device with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany). For quantitative mRNA expression analysis real-time PCR was carried out with 150 ng
of cDNA using TaqMan® Master kit (Roche Diagnostics, Germany) on LightCycler®2.0 Real-Time PCR System. To normalize VEGF mRNA levels between different samples, gene expression of 18S reference gene was also evaluated. The primers were purchased from TIB MolBiol (Germany), and the specific probes from Roche Diagnostics (Denmark). Real time PCR analysis was performed according to the manufacturer’s recommended protocol. RNA quantity was evaluated by NanoDrop ND-1000 Spectrophotometer.

**Data Analysis.** The fold change of VEGF gene expression was calculated using ∆∆Ct method.

**Results**

**Effect of VEGFsiRNA on measuring cell proliferation.** We determined cell viability by quantifying the formazan produced by the mitochondria of the living cells. Preliminary studies were made to determine the optimal concentration of siPORT NeoFX, siRNA and culture condition for a reduced cell toxicity with an increased level of gene silencing. No cytotoxic effect was observed in the case of transfection agent siPORT NeoFX. The treatment with a single dose of 50 nM VEGF siRNA on HepG2 cell line at 24 hours has registered no statistically differences compared to control (untreated cells), meanwhile at 48 hours cell proliferation was significantly lower than control, as shown in Figure 1.

We found differences in cell behavior in the case of VEGF-siRNA as shown in Figure 2. These data suggest the induction of apoptosis in the case of VEGF-siRNA transfection is 60-80%.

![Figure 1](image1.png)

**Figure 1.** Cell survival in function of cell culture medium after transfecting the HepG2 cells using siPORT NeoFX transfection agent

![Figure 2](image2.png)

**Figure 2.** Microscopy on phase contrast, HepG2 cell line, in subconfluent culture after 24 hours after VEGF-siRNA transfection, (A) DAPI staining, (B) FDA staining
Evaluation mRNA gene expression and protein expression after VEGF siRNA transfection. Relative mRNA expression of VEGF in the presence of 40 nM VEGF at 24 hours was analyzed using \( \Delta \Delta C_t \) method. Relative mRNA levels for transfected cells with VEGF siRNA and for transfection agent are presented in Table 1. As shown in Figure 2 the VEGF transcript expression level has significantly decreased after 24 hours meanwhile for the transfection agent we have an increased level of gene expression compared to the control.

The inhibition of gene expression was confirmed at protein levels at 48 h after transfection, meanwhile at 24 h were recorded lower levels of gene inhibition (Figure 4). The VEGF protein levels, in the group treated only with the transfection agent, are higher than the control group because it creates a certain level of toxicity in the medium inducing hypoxia to the cells that stimulate VEGF secretion. Therefore, as in Chengcheng et al. we can use as control HepG2 cells that were either untreated or treated treated only with siPORT NeoFX.

Table 1. Results for analysis of relative gene expression of VEGF in cells transfected with 40 nM VEGF siARN using for transfection 5 \( \square \) l siPORT NeoFX compared with control group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fold</th>
<th>Error (+), (-)</th>
</tr>
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<tbody>
<tr>
<td>siPORT NeoFX</td>
<td>1.401</td>
<td>0.389 0.304</td>
</tr>
<tr>
<td>VEGF-siRNA</td>
<td>0.566</td>
<td>0.120 0.099</td>
</tr>
</tbody>
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Figure 3. VEGF relative gene expression levels on Hep2G cell line.

Figure 4. VEGF relative protein expression levels on Hep2G cell line.

Discussion

Angiogenesis is necessary for tumors to grow beyond a certain size, and is a prerequisite for tumor invasion and metastasis (Yin et al., 2010). While a number of factors that induce and support angiogenesis have been identified (Liekens et al., 2001), VEGF appears to be the most dominant factor involved in tumor angiogenesis (Rini 2007). The VEGF family incorporates 5 structurally related ligands that bind differentially to 3 receptor tyrosine kinases (VEGFR-1, -2, and -3) and among the 5 VEGF family members, VEGF-A is the prototypical angiogenic factor, with a potent and universal angiogenic effect in most physiological and pathological conditions. VEGFR2 is the
most important one in tumorigenesis, it mediates microvascular permeability, endothelial cell proliferation, tumor cell migration and survival (Kaseb et al., 2009). However, detailed mechanisms underlying the VEGF-mediated tumor survival and formation and how VEGF-inhibition leads to apoptosis and decrease of tumors are not yet resolved.

VEGF by inducing undesired angiogenesis and blood vessel leakage, which is highly detrimental in most conditions, seems an important therapeutic target by specific inhibition using siRNA (Folkman 2007). RNA interference is also used in identifying gene function and revealing relationships between proteins of certain cellular mechanisms. The use of siRNA in mammalian cells involves the RNAi induced silencing complex (RISC) that cleaves the target mRNAs that share sequence identity with the siRNA, determining a subsequent 80%-90% decrease in the levels of corresponding protein (Leonard 2006). Thus, RNAi is an important tool that opened the door to the therapeutic use of siRNA in medicine. In our study we use siRNA-VEGF to target the VEGF protein in HepG2 cell line. RT-PCR and ELISA assay showed that the expression of VEGF was significantly reduced after 48 h since transfection, all in correspondence with the literature (Madeddu 2005; Yin et al., 2010). From the images obtained with DAPI staining we observed that VEGF silencing is associated with apoptosis. Gupta et al., (Gupta, 1999) showed that VEGF overexpression ameliorated the time-dependent increase in apoptosis, result supported by our study as well. In another study it was reported that VEGF-B is a potent apoptosis inhibitor by suppressing the expression of the BH3-only protein and other apoptotic/cell death–related genes via VEGFR-1. VEGF-B therefore appears to be the first member of the VEGF family that has been observed to have a potent antiapoptotic effect but lack general angiogenic activity (Li et al., 2008). Jurasz et al showed that by silencing the VEGF expression in endothelial cells exposed to hypoxia leads to apoptosis due to the expression of BNIP3 gene, whose effect in hypoxic conditions are masked by the VEGF (Jurasz et al., 2011).

**Conclusion**

siRNA transfection, presented results provide a useful template for the design for therapeutic siRNAs for efficient and specific gene silencing, that may block the production of some disease-causing proteins that/when are overexpressed. RNAi is an efficient method in silencing gene expression, after optimizing the transfection conditions. Our data shows that after determining the optimal parameters for siRNA transfection, efficient gene silencing can be achieved. siRNA could prove to be a useful approach in combined therapy of hepatocellular carcinoma but also for other types of cancer.

VEGF is associated with hepatocellular carcinoma progression and by blocking VEGF expression using RNAi technology we significantly reduced VEGF protein levels, suppressed the cell proliferation and induced apoptosis.

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