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# Antisense p53 Decreases Production of VEGF in Follicular Thyroid Cancer Cells

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**Inactivating mutations of wild-type p53 (WTp53) tumor suppressor gene are common in anaplastic thyroid cancer (ATC) and are associated with poor prognosis. Mutated p53 (MTp53) has been implicated with angiogenesis. Therefore, the potential of MTp53 knockout by oligodeoxyribonucleotide phosphorothioates (ODNs) to affect VEGF production of undifferentiated thyroid cancer cells with a recessive MTp53 mutation was evaluated. Transient transfection with 20 bp ODNs complementary to portions of exon 10 of p53 and a negative control ODN (HIV-RT) were carried out in FTC-133 cells. In vitro secretion of VEGF protein was quantified by EIA and correlated to cell numbers, which was evaluated by in vitro MTT assay. Transfection of undifferentiated thyroid cancer cells with ODN reduced VEGF secretion of FTC-133 cells following transfection by 34% as compared to the negative control (cells transfected with ODN-HIV; $p = 0.03$). These results suggest that transient MTp53 knockout with ODNs complementary to p53 nucleotide sequences impair secretion of VEGF in the undifferentiated thyroid cancer cell line FTC-133.**

**Key Words:** Undifferentiated thyroid cancer; FTC-133 cell line; p53; VEGF; ODN; angiogenesis.

## Introduction

Mutations of the p53 tumor suppressor gene are common during oncogenic transformation. The majority of these mutations are missense mutations, located between exons 5 and 8 of the p53 gene. The prevalence of p53 mutations is low in differentiated thyroid cancer, averaging some 1–5%. However, in anaplastic thyroid cancer it may be as high as 85% and associated with poor prognosis.

In the anaplastic thyroid carcinoma cell line FRO, nonmutated (wild-type, WT) p53 has been shown to affect the expression of thrombospondin-1 (TSP-1)—a 420 kDa matricellular glycoprotein that plays an important role as antiangiogenic factor—and to inhibit in vivo growth *(1)*. It has also been suggested that WTp53 inhibits angiogenesis by suppressing vascular endothelial growth factor (VEGF), although the latter was recently questioned *(2,3)*.

Oligodeoxyribonucleotide phosphorothioates (ODNs) have been used to decrease expression of specific target genes *(4)*. For instance, ODNs complementary to p53 nucleotide sequences effectively inhibit expression of MTp53. The biological effects of MTp53 knockout have been used as an antitumor strategy for several tumors, including leukemia and experimental pancreatic cancer, consistently resulting in a reduction of in vitro or in vivo proliferation *(5,6)*. Based on this evidence, the current study aimed to evaluate the effect of transient transfection with established p53 antisense ODNs in the undifferentiated follicular thyroid cancer cell line FTC-133, known to have a recessive p53 mutation *(7)*.

In particular, this study evaluated the potential of p53 antisense ODNs to affect the expression and production of VEGF, the major angiogenic factor secreted by thyroid cancer cells in vitro *(8)*.

## Results

Transfections of FTC-133 cells, known to harbor a p53 point mutation with loss of wild-type p53 and three copies of the mutant allele, were performed using the lipofectin protocol.

Effective transfections were documented by evaluation of MTp53 status by Western blotting of total cell lysates. Cell lysates of transfected cells revealed a loss of the respective MTp53 band by Western blotting, as compared to mock transfected cells and reagent controls. Control transfections with ODN-HIV were used to detect unspecific sequence effects of ODN transfection; however, ODN-HIV did not alter p53 expression, supporting the concept of a sequence specific effect of ODN-A (Fig. 1).

There was some decrease of cell numbers in vitro determined by MTT-assay. Transfection with ODN-A resulted in a decrease of cell numbers by 22.7% ($p < 0.05$) after 5 d, when compared to cells transfected with ODN-HIV (Fig. 2).

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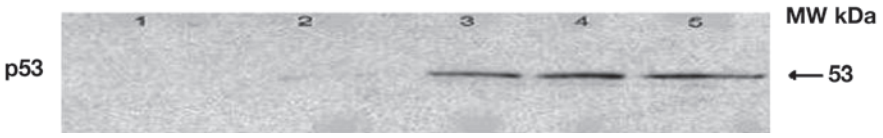


**Fig. 1.** Western blotting with a p53 antibody, documenting loss of the respective band representing p53 in FTC-133 cells, transfected with either ODN-A, ODN-HIV, or reagent control (Lane 1: mol. weight marker; 2: ODN-A; 3: ODN-HIV; 4: lipofectin; 5: nontransfected cells for positive control, all cells lysed 48 h after transfection).

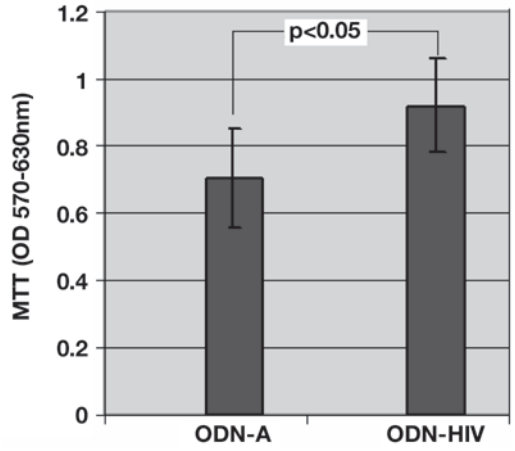


**Fig. 2.** Viability of FTC-133 cells following transfection with ODN-A and ODN-HIV as determined from the MTT assays performed on d 5. Cell numbers are expressed as a percentage of the optical densities from the MTT-assay. The difference of the cell number between ODN-A and ODN-HIV transfected cells was statistically significant ($p < 0.05$).

The ability of ODN-A to influence in vitro angiogenesis was first documented by immunostains of FTC-133 cells cultured on chamber slides, where a reduction of the intensity of VEGF stains of cells transfected with ODN-A was noted. Such changes did not occur with cells transfected with unspecific ODN-HIV, suggesting a sequence-dependent alteration of VEGF immunoreactivity. Subsequent quantitative analysis of VEGF in the 24 h conditioned medium of FTC-133 cells confirmed a reduction of VEGF secretion of cells transfected with ODN-A to as low as 34% of control samples ($p = 0.03$) (Fig. 3).

## Discussion

Anaplastic thyroid cancers (ATC) are a rare subset of thyroid cancer. They are characterized by aggressive proliferation, invasion, and enhanced angiogenesis. A widely accepted view of the pathogenesis of ATC is that the anaplastic phenotype results from a stepwise process of progressive genomic disarray, involving early tumorigenic mutations such as ras and ret and culminating in the loss of p53 function with anaplastic transformation *(9,10)*. Patients with ATC have a poor prognosis, with a median survival of only a few months *(11)*. Current treatment includes surgery to obtain local tumor control and hyperfractionated radiotherapy combined with chemotherapy *(12)*. However, mortality rates in excess of 90% within 1 yr of diagnosis underscore the desperate need for novel strategies of treatment.

This study evaluated the impact of mutated (MT) p53 on VEGF secretion in the undifferentiated human thyroid cancer cell line FTC-133. Transfection of thyroid tumor cells with antisense ODNs complementary to p53 nucleotide sequences had not previously been studied.

For any tumor cell population to grow beyond a few cubic millimeters, it is essential that the cells express the angiogenic phenotype *(13)*. It has been previously documented that VEGF and thrombospondin-1 (TSP-1)—an antiangiogenic factor—are important mediators of thyroid cancer growth in vivo. Recent studies suggest WTp53 to modulate angiogenesis through either downregulation of VEGF-expression and/or upregulation of TSP-1-expression *(2,3,14)*. These results found recent support by a report of Nagayama et al., who, after introducing WTp53 to a p53-null anaplastic thyroid carcinoma cell line observed reduced in vivo growth and angiogenesis in WTp53 tumors *(1)*.

Alternative methods to block VEGF production have been described previously. For instance, Bauer et al. showed that the use of VEGF monoclonal antibody induced angiogenesis blockade in anaplastic thyroid cancer and growth inhibition of papillary thyroid cancer xenografts in nude mice *(15,16)*.

The current study suggests an association between p53-antisense treatment and angiogenesis. Although clearly preliminary, we found a significant reduction of the amount of VEGF secreted into the conditioned medium after ODN transfection. Moreover, it was shown recently that tumor cells lacking WTp53 were less responsive to antiangiogenic therapy *(17)*, offering yet another line of evidence for a connection between MTp53 and the angiogenic phenotype of cancer cells. It should be worth while to further elaborate on the impact of MTp53 on thyroid cancer angiogenesis.

In summary, the results presented here indicate, that ODNs complementary to p53 nucleotide sequences might be responsible for a reduction of VEGF secretion by FTC-133 cells, supporting the concept that mutated p53 is involved in the production of angiogenic factors by transformed thyroid cells.

## Materials and Methods

### *FTC-133 Cell Line and Cell Culture Conditions*

The undifferentiated FTC-133 cell line was used for several reasons. FTC-133 thyroid cancer cells are well established and constitute an accepted model of undifferentiated

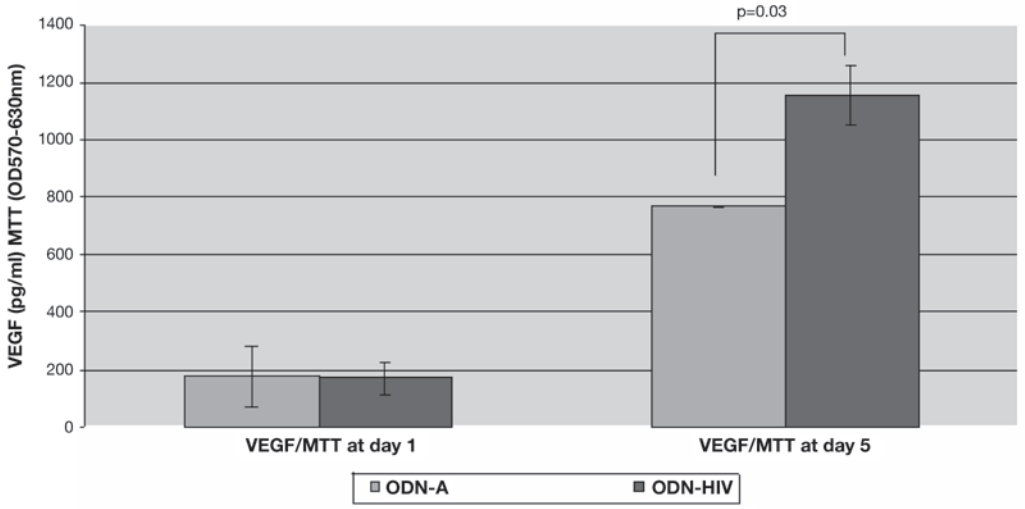


**Fig. 3.** VEGF concentration in conditioned medium of FTC-133 cells after 1 and 5 d of transfection with ODN-A compared to transfection with ODN-HIV (unspecific control ODN). VEGF quantities in conditioned medium were calculated on the basis of the number of viable cells (VEGF EIA, values are pg/mL per 100% viable cells). Differences between ODN-A and ODN-HIV transfected cells were significant on d 5 ($p < 0.03$).

thyroid cancer. The p53 status of the cell line has been repeatedly confirmed: all of the cells are MTp53 positive, have deleted wild-type and triplicated mutant p53 allele, with a known point mutation at codon 273 *(7)*. Finally, FTC cells have been shown to secrete considerable quantities of VEGF *(8,18)*. FTC-133 cells were kept in DMEM/HAM'S F-12, 10% FCS, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin. Viability was assessed by Trypan blue exclusion of cells in exponential growth harvested by brief cold trypsinization. To determine the effect of p53 knockout on the secretion of VEGF, all experiments were performed in triplicate. The following assays shows representative results.

### *ODNs and Transient Transfection Assays*

Cells were plated in 6 and 24 multiwell plates ($1–2 \times 10^5$ cells/well) and allowed to adhere for 24h before being switched to serum-free H5 medium. Transfections were performed following standard protocols using lipofectin reagent (0.5% w/v, Life Technology, Gaithersburg, MD, USA); a final ODN concentration of 5 $\mu M$ was incubated for only 20 h, and after this short incubation period the reagent was removed and replaced by serum-free medium *(5,6)*. Of several p53 antisense ODNs reported in the literature, a 20 bp Oligo directed against exon 10 (referred to as "A-ODN", 20mer: CCCTGCTCCCCCCTGGCTCC, Institute of Molecular Biology, Marburg, Germany) gave the most consistent results and was used for the experiments described here as previously described *(5,6)*. Controls were appropriately used at all times and included lipofectin, unspecific ODN directed against the reverse transcriptase gene of HIV (ODN HIV, 20mer: TCTCTC CGCTTCTTCCTGCC)—which was previously established as a negative control *(6)*—and naive FTC-133 thyroid cancer cells kept in serum-free, defined growth medium (H5).

### *p53 Western Blots*

Cells from transfection assays were kept in H5 medium for 48 h, lysed in RIPA Buffer, and total cell lysates run on 12% SDS-PAGE, transferred to nitrocellulose (Hybond-C pure, Amersham, Piscataway, NJ, USA) and detections of p53 carried out using a mouse anti-p53 Ab (Clone Pab 1801, Oncogene, Cambridge, MA, USA) and horseradish peroxidase coupled Luminol reagent, visualized on ECL-Hyperfilm (Amersham).

### *Immunostaining for VEGF*

Cells were plated on chamber slides and fixed with 4% formalin. Immunohistochemical staining for VEGF was done using a polyclonal mouse anti-VEGF antibody (1:250, sc152, Santa Cruz Biotechnology, CA). After blocking endogenous peroxidases and nonspecific binding (1:10 donkey serum), incubation with the primary antibody was carried out overnight at 4°C and further enhanced using the streptavidin-based Link and Label Detection System (BioGenex, München). Slides were stained with DAB-chromogen (Dako-Chemicals) and counterstained with hematoxylin and mounted.

### *Cell Viability*

FTC-133 cells were plated in six multiwell plates ($1 \times 10^5$ cells/well) and transfections carried out as described. Cells were continued in H5 medium for up to 5 d and cell numbers determined using the MTT vital colorimetric cell quantitation assay. Optical densities were obtained at $\lambda$ 570–630 nm (Emax-Reader, Molecular Devices, Munich, Germany) *(19)* (Fig. 2).

### *VEGF Secretion*

Quantitative analysis of VEGF secretion into the 24 h conditioned, serum-free medium on d 5 after transfection,

followed a protocol similar to the cell viability assay. Detection of VEGF was carried out using a human VEGF EIA (CYT 138, R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Because of the growth inhibitory effect of ODN transfections, VEGF values obtained during quantitative analysis were always calculated on the basis of the number of viable cells per individual well. To this end, MTT assays were performed in parallel (Fig. 3).

### *Statistical Analysis*

The results were expressed as mean ± SD and the mean values were compared by using the two-tailed *t* test (SPSS version 12.0, Chicago) to evaluate differences in variables from ODN-A transfected samples and their respective control (ODN-HIV transfected sample). The level of significance was taken to be $p < 0.05$.

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