BABES – BOLYAI UNIVERSITY

FACULTY OF CHEMISTRY AND CHEMICAL ENGINEERING

Posttranscriptional control of gene expression – RNA interference in malign tumors

PhD Thesis

Summary

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1 INTRODUCTION

Worldwide, one in eight people die of cancer; this disease is the first cause of death in developed countries and the second in developing countries (after heart diseases). In 2008, there were 12.7 million new cases diagnosed with cancer, of which 7.6 million resulted in death (approximately 21000 deaths a day) and by 2030, the numbers are expected to increase to 21.4 million new cases and 13.2 million deaths [1]. The statistics are disturbing, and although a lot of effort has been put in researching this horrible disease, we are still missing the "magic bullet".

By definition, cancer is "a group of diseases characterized by uncontrolled growth and spread of abnormal cells, and if not controlled, it can result in death" [1]. Cancer develops through multiple steps, over a long period of time. Both genetic (inherited or acquired mutations, immune conditions, etc) and environmental factors (tobacco, chemicals, radiation, and infectious organisms, etc) have been shown to promote carcinogenesis, either by acting together or in sequence. To date, there is no cure to treat cancers, although certain types of cancer can be prevented by minimizing exposure to carcinogen factors, or some potential malignancies can be detected at an early stage, when the disease is most treatable.

Cancer is an ancient disease, being first described by Hippocrates. In 1971, USA declared "war" on cancer, bringing cancer research to the forefront. Since then, important progress is being made in understanding these pathologies. By comparing how normal and cancer cells function, it has been possible to elucidate, to some extent, what happens in cancer. The body is constituted from many types of cells, theoretically, each cell having the potential to develop one or more types of cancer. More than 1,000 different disorders in normal cell and tissue functions have been described, but in the end, cancers are mostly characterized by abnormal cell growth. In healthy cells, the cell division is regulated very precisely; cells have to pass several checkpoints in order to progress through cell cycle. If damaged beyond repair, the cells are marked for programmed cell death, a process known as apoptosis. Cancer cells somehow escape these checkpoints, start dividing uncontrollably, become invasive and spread throughout the body. As a consequence, normal tissue and organ functions get disrupted, leading ultimately to death.

Several cancer pathologies can be prevented and if detected early are generally curable. There are four main treatment options for cancer: surgery, chemotherapy, radiotherapy and targeted therapy. Surgery is usually performed for localized tumors with a view to remove as much cancerous tissue as possible. This was the most practiced treatment until radiotherapy was introduced. Radiotherapy involves the use of ionizing radiations that induce DNA breakpoints, therefore making the tumor cells unable to multiply. Chemotherapy uses drugs targeting cells with a high multiplication rate. These types of treatment cause serious side effects, due to the fact that cancer cells are not the only high dividing cells in the body. Targeted therapy uses drugs that target cancer cells specifically. However the success on using these therapies for early stages of cancer, the treatments for metastatic or recurrent cancers are poorly effective and with serious adverse effects. There is a great need to identify and investigate new systemic agents for cancer treatment.

2 AIMS OF THESIS

1. Evaluate the tissue and serum levels of a panel of pro and anti-neoangiogenic factors in prostate, colorectal and cervical cancers in order to assess their involvement in cancer progression.

2. Correlation of these factors with diagnosis and clinical prognostic.

3. Investigate the molecular mechanisms of VEGFA in cancer progression in order to assess the use of this molecule as a single target therapy.

4. Evaluate the molecular and cellular mechanisms induced by EGCG in order to assess the use of this natural compound as a multi targeted anti-angiogenic therapy.

3 MATERIALS AND METHODS

3.1 Human specimens

Three cancer pathologies were investigated for angiogenic factors expression: prostate, colorectal and cervical cancers. Serum samples were collected thirty-four patients with prostate affections and six patients were considered in the control group. Biopsy samples were collected from thirty-three patients with operated colorectal cancer and forty four with locally advanced squamous cell carcinoma and 10 women with normal cervix, as controls.

3.2 Cell cultures

HeLa and HUVEC cells (ECACC) were used for treatment effects investigations.

3.3 Treatments

siRNA treatment: HeLa cells were reverse-transfected in serum free Opti-Mem medium (Gibco BRL) with siPort NeoFx Transfection Agent (Ambion) and siRNA according to the manufacturer instructions. For controls we used untransfected, mock (transfection agent only) or scrambled siRNA (Silencer Negative control siRNA #1, Ambion) transfected cells.

EGCG treatment: HeLa cells were cultured in Opti-Mem medium (Gibco) and treated with EGCG (Sigma-Aldrich) for 24 and 48 hours. Control cells were treated with PBS instead of EGCG.

3.4 Molecular analysis

3.4.1 Protein analysis

Expression levels of the proteins of interest were analyzed using **ELISA**, **western blot** or **FastQuant** methods. These methods allow the quantification of proteins from any biological fluid by using antigen-antibody affinity.

3.4.2 mRNA analysis

mRNA expression levels were analyzed using **Real-Time PCR**, **PCR array** and **microarray** methods. Total RNA was isolated with TriReagent (Sigma-Aldrich), purified with RNAeasy Mini Kit (Qiagen) and further analyzed for quantity and quality with ND-1000 and Agilent Lab-on-a-chip Bioanalyzer 2100 (Agilent Technology). Only the samples that presented a RIN between 9 and 10 were used for further analysis. According to the method protocol, the

RNA was further transcribed into cDNA and amplified or labeled and hybridized onto microarray slides. Data analysis was done by comparing the group of interest to the control.

3.5 Cellular analysis

Cell proliferation was assessed using **MTT test**. Cell apoptosis was measured using **on-chip flow cytometry**. Effects on cell adhesion were quantified using **attachment**, **adhesion** and **spreading assays**. Invasiveness was measured by setting up an ***in vitro*** **invasion assay**.

3.6 Statistical analysis

All experiments were performed at least in triplicates. All the data are presented as mean ± standard error of mean (SEM). Differences were assessed by t test using the statistical program, SPSS11.0 for Windows. $P < 0.05$ was considered to be statistically significant.

4 RESULTS AND DISCUSSIONS

4.1 Expression of angiogenic factors in serum patients with prostate cancer

4.1.1 Protein levels evaluation in serum

Sera samples from all patients were analyzed for the presence of 8 factors involved in angiogenesis (PDFGBB, VEGF, bFGF, angiogenin, KGF, TIMP-1, ICAM-1, and ANG-2. Shapiro-Wilk test was applied to examine the normality of the distributions. ANG2, KGF, PDFG-BB, and TIMP-1 had normal distributions ($p > 0.05$). Angiogenin, ICAM-1, and VEGF were over-expressed, which barred performance of a test for normality. bFGF was underexpressed in most of the samples, which likewise barred performance of a test for normality. Significant differences were found for KGF: PCa vs. BPH ($p = 0.022$) and PCa vs. CP ($p = 0.050$); for ANG2: control vs. BPH ($p = 0.038$) and control vs. CP ($p = 0.037$); for PDFG-BB: control vs. BPH ($p = 0.008$) and TIMP-1: control vs. PCa ($p = 0.000$); control vs. BPH ($p = 0.001$) and control vs. CP ($p = 0.008$). Results show that there are significant serum concentration differences between the PCa, BPH, CP and control groups for the following angiogenic molecules: KGF, ANG2, PDFG-BB and TIMP-1 (Fig. 1). KGF levels were higher in BPH and CP than in PCa; ANG2 was lower in BPH and CP than in control group; PDFGBB was

higher in BPH than in control group; and TIMP- 1 was lower in PCa, BPH and CP compared to the control group.

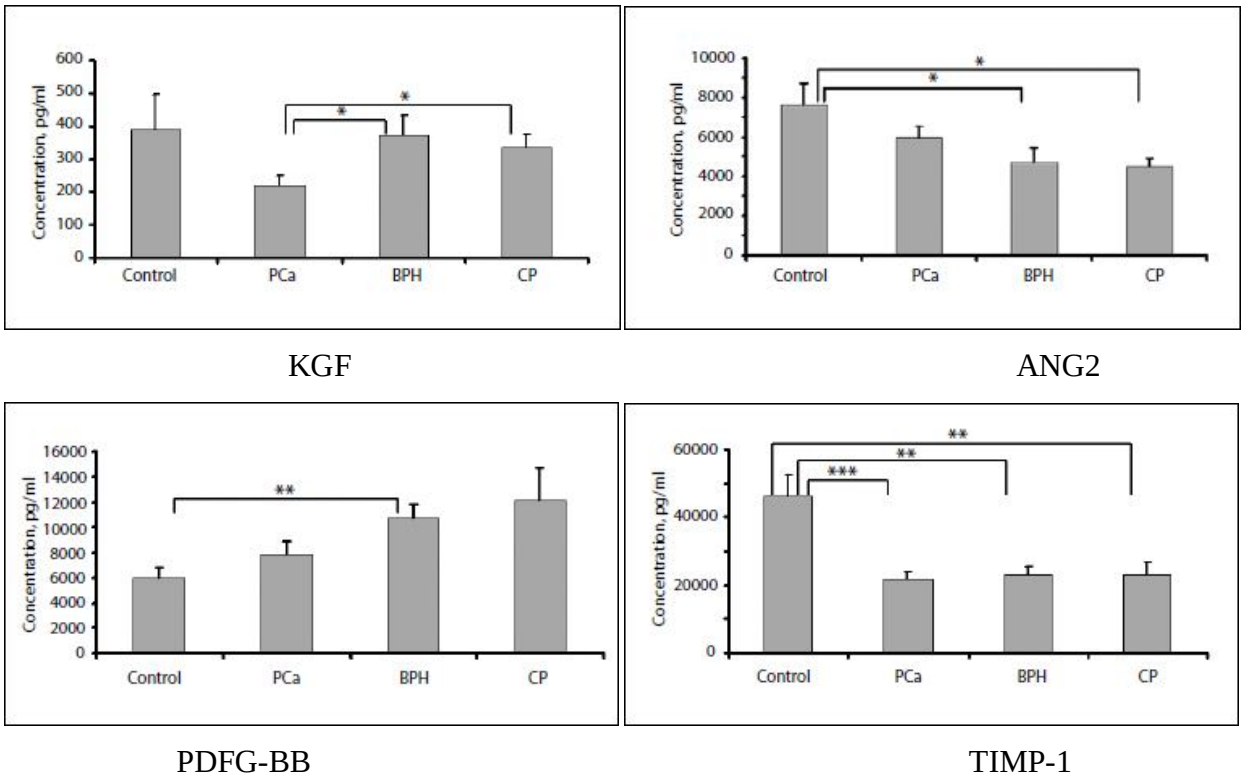

Fig. 1 Mean serum concentrations of control, PCa, BPH and CP groups (Standard Error of Mean, * $p < 0.05$).

4.1.2 Discussions

We were successful in simultaneously evaluating 4 angiogenic molecules (KGF, ANG2, PDFG-BB and TIMP-1) in the sera of 40 patients with prostate pathology (PCa, BPH, CP) using FAST Quant® multiplex technology. Our results revealed that the KGF angiogenic molecule was statistically significantly decreased in PCa (218.96 pg/ml) compared with BPH (371.28 pg/ml) and CP (334.68 pg/ml). KGF is a stromally-derived growth factor important in mediating androgen-induced activities in BPH and PCa. Our study is in concordance with Metha et al. [2], who found higher levels of KGF in BPH than in PCa. In normal prostate, KGF, also known as FGF-7, serves as a paracrine growth factor that is synthesized in stromal cells and acts on epithelial cells through its receptor. Huang et al. [3] demonstrated that KGF exhibited mitogenic and anti-apoptotic effects that correlate with the induction of cyclin-D1, Bcl-2, Bcl-xL and phospho-Akt expression in prostate cell lines. Another angiogenic molecule, ANG2, showed

statistically significantly decreased values in BPH (4,728.05 pg/ml) and CP (4,512.80 pg/ml) than in the control group (7,632.25 pg/ml). The ANG1 and ANG2 are principal regulators of vascular growth and regression; however, the role of the angiopoietins is unknown in normal prostate and prostate tumors [4]. ANG2 possesses anti-angiogenic properties [5], by disrupting the vascular remodeling ability of ANG1 and may also induce endothelial cell apoptosis [6]. Abnormal levels of ANG1, ANG2 and their receptor, Tie-2, are present in PCa and their interrelationships may be important in angiogenesis management [7, 8].

For the TIMP-1 molecule, we recorded lower values in BPH (22,984.35 pg/ml), CP (22,891.10 pg/ml) and PCa (21,832.70 pg/ml), compared to the control group (46,172.24 pg/ml). The balance between the MMPs and their natural inhibitors, TIMPs, seems to be important for progression and metastasis of different tumors. TIMPs are reported to inhibit angiogenesis directly by binding MMPs and inhibiting their activity, blocking cell proliferation and downregulating VEGF expression [9]. In PCa, there is an imbalance of MMPs and TIMPs, with a significant loss of TIMP-1 [10-12]. TIMP-1 has been shown to have a multifunctional role in PCa for tumorigenesis, including inhibition of the catalytic activity of MMPs, growth promotion, inhibition of apoptosis and regulation of angiogenesis. Several studies have shown that TIMPs and synthetic MMP inhibitors inhibit tubulogenesis and endothelial cell invasion in Matrigel and type I collagen matrices. Among the MMPs that are specifically involved in angiogenesis, 2 gelatinases, MMP-2 and -9, and the membrane type associated– MMP are inhibited by TIMP-1[13-15]. The inclusion of TIMP-1 in this panel of serum biomarkers will help to better define its role in PCa evolution. The serum concentration of PDFG-BB was doubled in BPH (10,800.66 pg/ml) relative to its concentration in the control group (5,988.72 pg/ml). Based upon our results, PDFG-BB can be used to differentiate between CP and BPH, both of which show lower levels of ANG2 and TIMP-1 than the control group. PDGF functions as a ‘competence factor’ that induces a set of early response genes expressed in the G1 phase of the cell cycle, including p21WAF1/CIP1 a functional mediator of the tumor suppressor gene p53 at the G1/S checkpoint [16].

4.2 Evaluation of angiogenic factors mRNA expression in colorectal cancer samples

4.2.1 mRNA expression in solid tumors

Our results showed increased VEGF levels in all stages, but without significant differences. The levels of bFGF and PDGF-BB were significantly higher in stage B vs. C tumors.

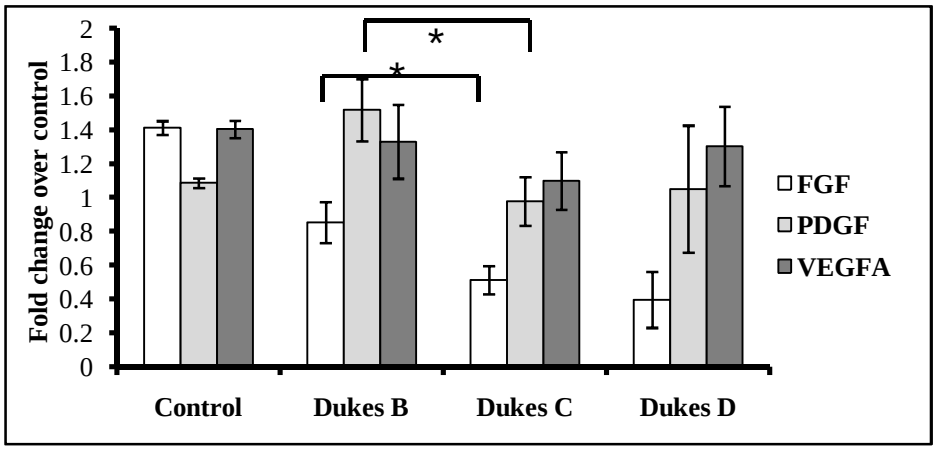

Fig. 2 Fold change in mRNA expression at different stages of tumor progression

4.2.2 Discussions

Previous studies have shown that VEGF is a key component in colon tumorigenesis, its high expression being correlated with poor prognosis [17]. In the last years, better understanding of tumor biology had led to the development of some biologic therapies, like bevacizumab which targets VEGF. This agent has been approved for the treatment of metastatic CRC since 2004 [18]. In our study the high level of VEGF even in tumors classified as stage B shows the early implication of this growth factor in CRC development and justifies the large numbers of studies with bevacizumab in the adjuvant treatment of colon cancer. bFGF and its receptors are involved in different biological functions including cell proliferation, differentiation, migration and survival. In CRC bFGF has been reported to be upregulated, thus being considered as a potential therapeutic target [19]. In our study the higher level of bFGF in Dukes B cases compared with Dukes C and D could be explained by its early implication in colorectal carcinogenesis. This result is in concordance with the results reported by Tassi et al. in 2006, who proposed the detection of bFGF in serum as a screening method for early detection of colon premalignant lesions [20].

The role of PDGF in tumor angiogenesis had been demonstrated in several studies, its overexpression being correlated with microvascular density and poor survival in many colorectal tumors [21]. Regarding the implication of PDGF in colorectal carcinogenesis the results are not very clear. The higher level of PDGF in Dukes B compared with those of Dukes C or D obtained in our study is in concordance with those reported by other authors who suggest the early implication of PDGF-BB in colon cancer development [22]. By evaluating the levels of PDGF-BB, VEGF and bFGF in different stages of CRC our study brings new information regarding the molecular pathways involved in colorectal carcinogenesis. The high level of these growth factors even in Dukes B stage could be explained by their early implication in CRC development and could justify the search for development of new biologic therapies targeting these molecules.

4.3 Evaluation of angiogenic factors mRNA expression in cervical cancer samples

4.3.1 mRNA expression in solid tumors

VEGFA and VEGFR2 mRNA expression levels were analyzed in patients tumor biopsies. The transcriptomic analysis showed increased levels of VEGFA mRNA in both stage II and stage III of tumor evolution but with no statistical significance (Fig. 3). Moreover, the VEGFA levels seem to decrease with tumor stage, indicating that once the angiogenesis is established, growth factor signaling is not necessary. For VEGFR2 we found lower levels of mRNA in tumor samples than in control group, with statistic significance for both stages. However we did not find a difference in VEGFR2 expression between the stage II and stage III, which suggest that receptor expression is not involved in tumor evolution.

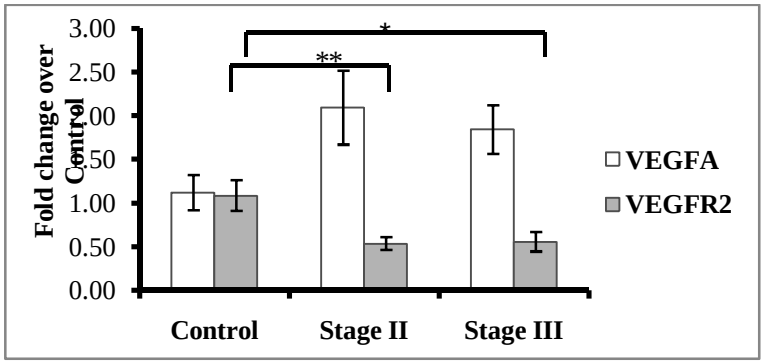

Fig. 3 mRNA expression levels according to tumor stage

Analyzing the correlation between the different clinical prognostic factors and biomarkers and the tumor response, statistically significant values were obtained for tumor size at the diagnosis (p=0.01) and VEGFR2 expression (p=0.02). Analyzing the results for angiogenesis factors, we found that a complete response was obtained in patients with higher level of VEGFR2 (Fig. 4), the cut-off value on ROC curve being 0.54 units (p=0.02) as shown in Fig. 5.

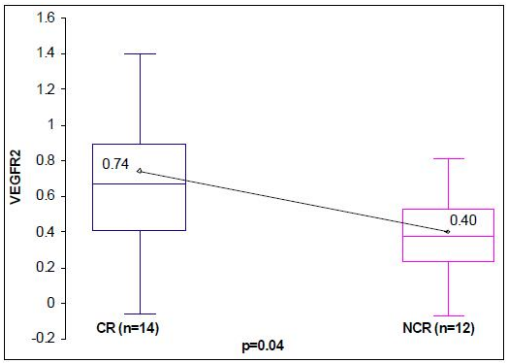

Fig. 4 Tumor response vs. VEGFR2

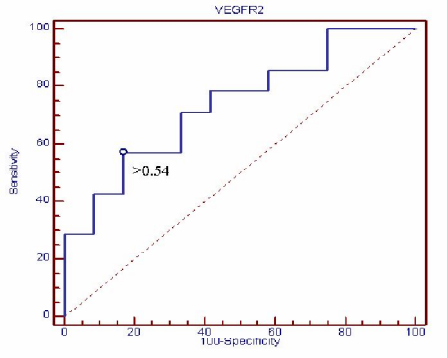

Fig. 5 ROC curve for VEGFR2

4.3.2 Discussions

Efforts to implement new therapeutic strategies in order to obtain better results in patients with cervical cancer continue to be made worldwide. One of these domains of interest is represented by targeted therapies. Thus, even in an incipient phase of the clinical research process, the combination between the anti-angiogenic aimed and current therapies seems to represent a new, feasible and promising approach in several types of cancer, including the cervical cancer. The indication of the target therapy has to be based on some predictive factors of the response to this treatment. The different new biomarkers can be prognostic factors and predictive ones, as well. Currently, the prognostic value of these biologic factors and their involvement in the treatment of cervical cancer is not confirmed; on the contrary, it is still controversial and, therefore, intensively investigated. For these reasons, this study proposes the examination of two of the main angiogenic biomarkers in the cervical cancer, for a more exact understanding of the molecular mechanisms and of the natural course of the disease; identification and validation of new prognostic factors; validation of certain prediction factors for the response to radiochemotherapy; introduction of new therapeutic strategies having a curing potential superior to that of the classic ones.

VEGFA is secreted by tumor cells and promotes tumor angiogenesis by stimulating endothelial cell proliferation, survival and their migration to tumor site. Increased levels of VEGFA were found in patients with cervical carcinomas when compared to normal tissues [23, 24] and were correlated with high stages, worst disease free survival and limited overall survival [25]. Although we did not find any correlation between VEGFA expression and tumor stage, survival or response to therapy, we observed increased levels of VEGFA in both tumor stages.

Originally it was thought that VEGFA acts only by a paracrine mechanism on the surrounding cells [26], but recent studies have showed that it can also act as autocrine stimulator promoting tumor survival, migration and invasion [27-30].

VEGFR2 receptors were originally thought to be present only in endothelial cells but recent studies showed that VEGFR2 is widely distributed in human tissues and tumors [31] suggesting an autocrine signaling VEGF/VEGFR2 loop [31-33]. To our knowledge, only one study reports VEGFR2 expression in cervical adenosquamous carcinomas and its overexpression has been associated with lack of metastases, but no association was found between VEGFR2 and overall survival and disease recurrence [34]. The authors suggest that other alternative molecules can drive the metastatic spread in these types of tumors. However, another study investigated the soluble level of VEGFR2 in patients' serum plasma and found it to be higher in recurrent compared to pre-invasive and primary invasive carcinoma, in node-positive disease and in patients with distant metastases [35]. We found that VEGFR2 is higher in patients with clinical complete response at the end of treatment; therefore, the pre-therapeutical level of VEGFR2 could be used as a predictive factor for clinical response in cervical cancer.

4.4 Genomic evaluation of the siRNA VEGFA induced molecular pattern by microarray technology and PCR validation

4.4.1 VEGF siRNA inhibits VEGFA expression in HeLa cell lines

VEGFA siRNA was transfected into HeLa cells and the supernatants were tested by FastQuant array for the secreted VEGFA protein levels. Untransfected and mock treated cells were used as controls. The VEGFA siRNA transfected cells show a 35% decrease in the secreted VEGFA protein compared to control cells (Fig. 6) after only 24 hours of treatment. These results demonstrate the knockdown of the VEGFA protein at the mRNA level.

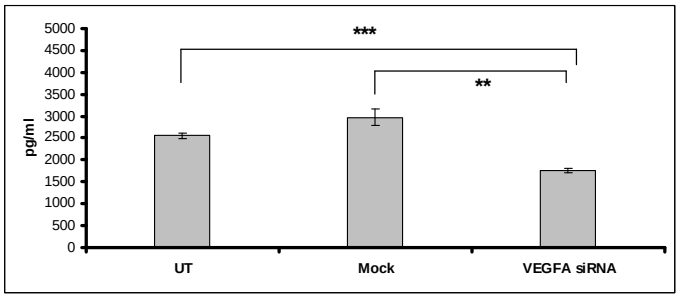

Fig. 6 VEGFA siRNA effect on secreted protein.

4.4.2 VEGFA siRNA decreases the number of live cells

We investigated the cytotoxicity of the siRNA treatment as well as the impact of VEGFA inhibition on cell proliferation rate by MTT assay. Our results show that inhibition of VEGFA induces a decrease in the number of live cells by 40% after 24 hours of treatment without observing any treatment cytotoxicity (untransfected cells compared to negative control siRNA treated cells). However, after 48 hours of treatment, we observed a decreased number of live cells in the negative siRNA treated group compared to untransfected cells indicating treatment toxicity. An increased percent of live cells was found in the VEGFA siRNA treated group unlike in the negative control group (Fig. 7).

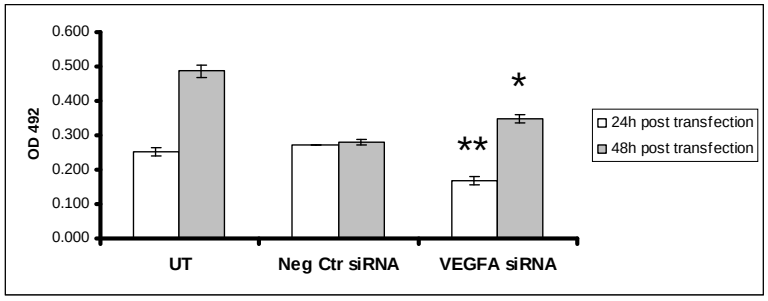

Fig. 7 The effect of VEGFA siRNA on cell proliferation

4.4.3 VEGFA siRNA induces apoptosis in HeLa cells

To assess the apoptotic effect of VEGFA inhibition on HeLa cells, we performed on-chip flow cytometry. VEGFA siRNA treatment induced significant levels of apoptosis in a time dependent manner (Fig. 8). We observed apoptotic cells in the negative control group at 48 hours after transfection, which indicates treatment toxicity.

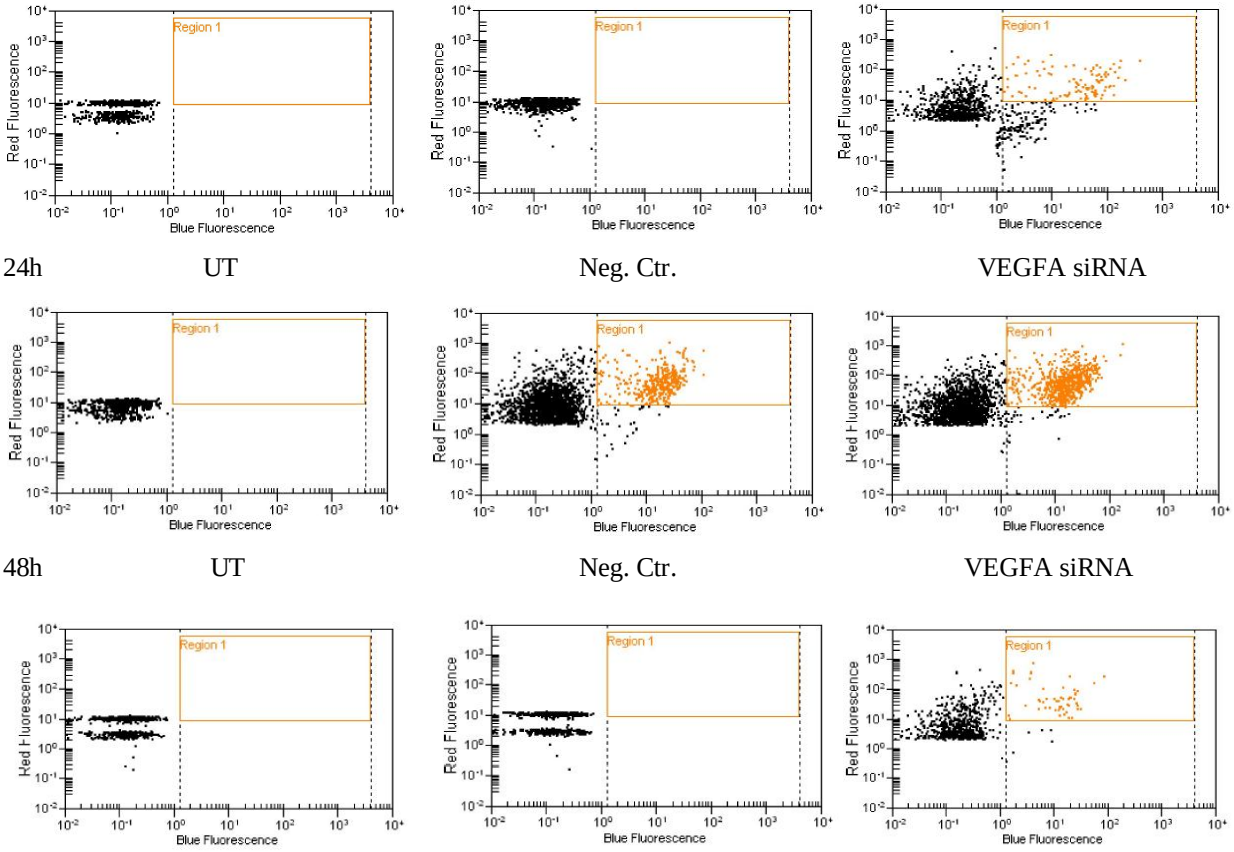

Fig. 8 On-chip flow cytometry analysis of cell apoptosis. 24, 48 and 72 h after transfection the cell were collected, stained with Anexinn V and calcein and analyzed by chip-flow cytometry with Agilent Bioanalyzer.

4.4.4 Microarray results

The microarray data were integrated in the GeneSping software, filtered by 2.0 fold change and t test and analyzed. The analysis revealed that VEGFA inhibition deregulated 480 genes of which 88 genes were upregulated and 392 genes were downregulated (Fig. 9).

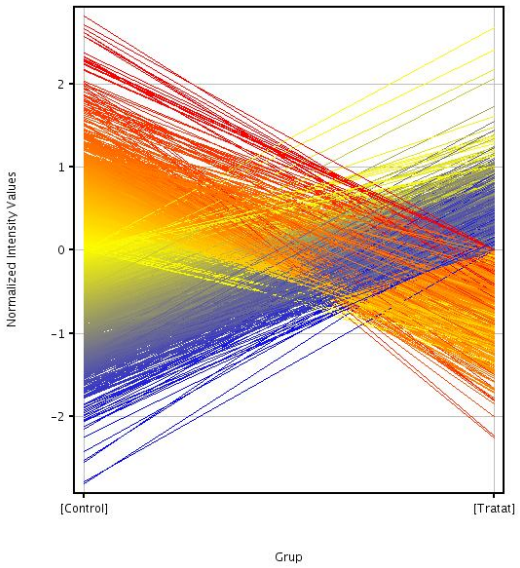

Fig. 9 Distribution of the analyzed genes (GeneSpring Sofware): red – upregulated genes, yelow- genes with the same expression levels, blue- downregulated genes

4.4.5 VEGFA inhibition modulates genes involved in the angiogenic process

All cells were cultivated in similar conditions and exposed to transfection by optimal amounts of siRNA for VEGF. The treated cells (siRNA for VEGF) were evaluated by RT-qPCR using primers and probes for all 7 transcripts of VEGF at different time moments: 24, 48 and 72 hours, respectively. The maximum inhibition of VEGF in HeLa cell line was found at 24 h, the inhibition representing 70% of expression. The results obtained at 48 h and 72 hours were lower in VEGF inhibition and had the tendency to restore the initial cellular potential.

The PCR Array analysis was done for the 24 h treatment of cells with siVEGF, regarding to qRT-PCR results. The kit design provides the simultaneous evaluation of the expression of 84 key genes involved in modulating the biological process of angiogenesis. The array includes growth factors and their receptors, chemokines and cytokines, matrix and adhesion molecules, proteases and their inhibitors, as well as transcription factors, all involved in the development of new blood vessels. Human Angiogenesis RT^2 PCR Array revealed that VEGFA inhibition deregulated 13 genes from the total number of 84 angiogenesis genes, of which 10 were up-regulated and 3 down-regulated, as indicated in

Table **1**.

Table 1. Genes differentially expressed 24 h after siRNA VEGFA treatment versus untreated cells.

Gene	Fold regulation
Angiopoietin 2 (ANGPT2)	2.4726
Hepatocyte growth factor (HGF)	2.323
Heparanase (HPSE)	2.3883
Inhibitor of DNA binding 1(ID1)	2.4217
Inhibitor of DNA binding 3 (ID3)	2.4385
Interleukin 1, beta (IL1B)	2.4385
Laminin, alpha 5 (LAMA5)	2.4897
Plasminogen (PLG)	2.0083
TEK tyrosine kinase, endothelial (TEK)	2.5245
Tumor necrosis factor, alpha-induced protein 2 (TNFAIP2)	2.2595
Thymidine phosphorylase (TYMP)	-2.45218
Sphingosine kinase 1 (SPHK1)	-2.07641
Stabilin 1 (STAB1)	-3.10366

4.4.6 VEGFA siRNA upregulates genes involved in both intrinsic and extrinsic apoptotic pathway.

The total RNA extracted from treated and untreated cells was profiled for 84 genes known to be involved in regulating the apoptotic process and programmed cell death. The array includes TNF ligands and their receptors; members of the bcl-2, caspase, IAP, TRAF, CARD, death domain, death effector domain, and CIDE families, as well as genes involved in the p53 and ATM pathways.

Table 2 Genes differentially expressed 24 h after siRNA VEGFA treatment versus untreated cells.

Gene	Fold regulation
v-akt murine thymoma viral oncogene homolog 1 (AKT1)	1.26
BCL2-antagonist/killer 1 (BAK1)	1.26
baculoviral IAP repeat-containing 3 (BIRC 3)	-1.31
tumor necrosis factor receptor superfamily, member 10a (TNFRSF10A)	1.42
tumor necrosis factor receptor superfamily, member 10b (TNFRSF10B)	1.25
Tumor protein 73 (TP73)	2.38

Five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB) were used for well to well normalization. Data analysis was done by ΔΔCt method using the Superarray Data Analysis Web Portal. We have considered of interest all the genes with -1.25 ≤ Fold regulation ≥1.25 in order to assess the genes involved in the early induced apoptosis process. We found that, there were significant expression differences ($p<0.05$) for 6 genes out of the 84 investigated genes. Five genes were up-regulated and one down-regulated (Table 2)

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4.4.7 PCR array validation of the microarray data

According to the PCR array data several genes of interest validated the microarray experiment (Table 3). Some of the genes were found to have the same regulation (VEGF, ID3, TEK, STAB1, BIRC 3) in both experiments, while some seem to be inverted (AKT1, BAK1, TP73) in the PCR array experiment.

The microarray analysis is a semi quantitative method and requires data validation by PCR. Our PCR results indicate that some genes that are involved in the apoptosis induction are overexpressed, contrary to the microarray data. Since we observed an increase in the apoptotic number of cells, our results correlate with the genes validated by PCR. There is also the need to also validate the proteins encoded by the genes of interest.

Table 3. Genes differentially expressed in the microarray experiment validated by PCR array technology

Fold change	Regulation	Gene
1.58	down	vascular endothelial growth factor B (VEGFB)
1.69	down	vascular endothelial growth factor C (VEGFC)
1.93	up	TEK tyrosine kinase (TEK)
4.01	up	inhibitor of DNA binding 3 (ID3)
1.49	down	stabilin 1 (STAB1)
1.70	down	serpin peptidase inhibitor (SERPINF1)
1.93	down	transforming growth factor (TGF)
1.77	down	fibroblast growth factor receptor 1 (FGF1)
3.70	down	baculoviral IAP repeat-containing 3 (BIRC3)

4.4.8 VEGFA siRNA induced apoptosis is p53 independent

In order to assess the tumor protein 53 involvement in the VEGFA siRNA induced apoptosis of HeLa cervical cells we quantified the levels of this protein by western blot analysis. Equal amounts of proteins from control and VEGFA siRNA treated samples were analyzed by immunoblotting with antibodies for p53, GAPDH (Fig. 10). Densitometric analysis of the immunoblots revealed that in HeLa cells the signal for p53 was unchanged in VEGF siRNA-treated samples when compared with negative controls.

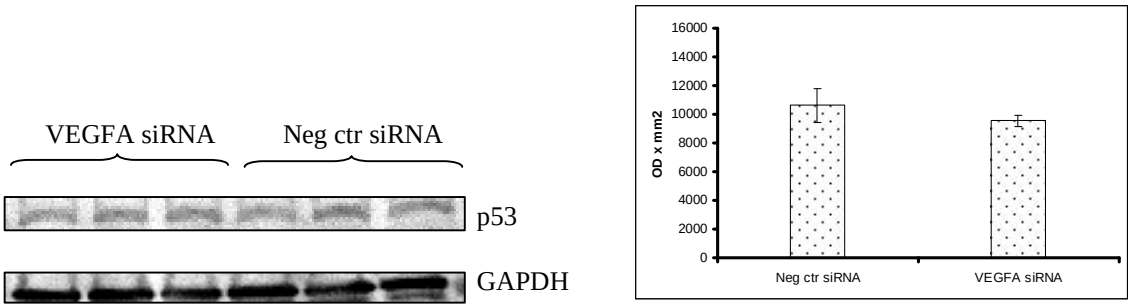


Fig. 10 Levels of p53 protein expression.

4.4.9 Discussions

This study is focused on mechanisms of action of VEGFA on cervical tumor cells in culture. We used the RNA interference mechanism as antiangiogenic therapy for its ability to target specific genes at posttranscriptional level.

VEGF is secreted by most solid tumor and, by interacting with its receptor VEGFR-2 expressed on endothelial and tumor cells; it stimulates tumor growth by either a paracrine mechanism via action on endothelial cells or by an autocrine mechanism promoting tumor growth and survival.

Using siRNA targeting the VEGFA gene to silence the expression of VEGFA transcripts at mRNA level, we obtained a decrease in the amount of secreted VEGFA protein in HeLa cervical cell line.

We obtained a decreased tumor cell proliferation rate and increased number of apoptotic cells after treatment with VEGFA siRNA, showing that VEGFA plays a critical role in cervical cancer cell survival. Our results demonstrate that VEGFA is involved in cervical tumor cells apoptosis but also an important molecule affecting tumor growth.

The results obtained in our study demonstrate that VEGFA is an important modulator of the angiogenesis mechanism. The fact that the major cell inhibition was observed after 24 hours, confirms the clinical aspects of antiangiogenic therapy administered in clinical trials, which has to be done continuously. More, the restoration of the VEGF proangiogenic potential in less than 72 hours helps understanding the multiple pathways governing the acquiring and preservation of the malignant potential. Inhibition of VEGF by RNA interference could be combined with the inhibition of other genes involved in angiogenesis in order to obtain a better reduction of their potential. $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ levels are increased in several human malignancies including breast, lung, brain, pancreatic, ovarian, kidney, and bladder carcinomas. Studies that used different agents to block the VEGF/VEGFR signaling pathways, showed reduced ECs proliferation, invasion and angiogenesis in general. To our knowledge, there are no studies concerning the effects of VEGF inhibition on cervix cancer cells. Surprisingly, our study revealed that VEGFA inhibition led to the upregulation of 10 proangiogenic genes and the inhibition of 3 (Table 2). Inhibition of VEGF activated some other important pathways for angiogenic mechanism, like the growth factor ANGPT2 which is involved in stimulation of cell invasion. ID1 and ID3 which are both transcription factors and downregulate the thrombospondin are overexpressed. HPSE (heparanase) is an endo-beta-D-glucuronidase which cleaves the HPSG (heparan sulfate proteoglycans), its expression being associated with tumor metastasis and angiogenesis, was also found to be upregulated. FGF/HPSG interaction is inhibited in our study, moreover the downregulation of FGFR3 suggest that the complex formation is inhibited as well as the presentation of FGF to the TK-receptors. Our results confirm that there are several mechanisms involving pro- and anti-angiogenic factors, as well as the necessity to search for multitargeted molecules in order to block several pathways involved in angiogenesis.

By profiling 84 genes involved in cell apoptosis and programmed death we also explored the early molecular transcriptional mechanism of apoptosis induced by VEGFA inhibition. The transcriptional analysis shows that tumor apoptosis targeted by VEGFA could be initiated by genes involved in both intrinsic and extrinsic pathways.

The apoptotic process can be induced by two molecular pathways. The extrinsic pathway operates via death receptors on external surface of the cell whereas the intrinsic pathway is triggered via mitochondria stimuli [36]. Both pathways are interconnected with other signaling

proteins, such as NK-κB and p53-MDM2, and converge at the level of the effector proteolytic enzymes, called caspases.

In this study we showed that in HeLa cervical cells VEGFA regulates the intrinsic pathway of apoptosis by a p53 independent pathway. We found no evidence for a p53 dependent or independent transcriptional activation of apoptosis, both p53 mRNA and protein levels remaining constant in VEGFA siRNA treated cells vs. negative control. The p53 gene is a well-known tumor suppressor gene with a critical role in the regulation of cell death and angiogenesis, but its molecular mechanisms are far from being fully understood. However, it is well known that p53 can down-regulate some proangiogenic proteins (VEGF, basic fibroblast growth factor (bFGF, basic fibroblast growth factor binding protein) or up-regulate antiangiogenic proteins (thrombospondin-1 (TSP1) and brain-specific angiogenesis inhibitor 1(BAI-1) [37].

In p53 deficient cells, the intrinsic apoptosis can be induced by the tumor protein 73 which apparently shares numerous regulatory principles and effector pathways with p53 [38]. p73-dependent apoptosis seems to be primarily regulated by its ability to transcriptionally activate pro-apoptotic p53 target genes [rev in [39]], being also involved in the extrinsic TRAIL induced apoptosis [40]. We found elevated levels of p73 mRNA, which suggest a VEGFA dependent transcriptional regulatory mechanism of p73 gene. The relation between p73 and VEGFA has been previously studied in human cancers with controversial results, the data showing both positive [31, 41, 42] and negative [32] regulatory functions between these proteins. Our results are in concordance with other studies which had reported the involvement of TP73 in the apoptotic process of cervical tumor cells [33-35, 43].

The extrinsic pathway is initiated by stimulation of the transmembrane death receptors belonging to the tumor necrosis factor (TNF) family by specific ligands released by other cells. The extrinsic apoptotic pathway can be triggered by two types of intracellular signaling: in type I, the direct stimulation is sufficient to activate effector caspases and to induce apoptotic death, whereas in type II cells, the signal is transmitted through activation of pro-apoptotic BCL-2 proteins which engages the intrinsic apoptotic pathways [44].When this intrinsic amplification loop is activated, the mitochondrial membrane is permeabilized with the release of mitochondrial proteins cytochrome C and DIABLO [45] through the Bax/Bak megachanels. Although both Bak and Bax are responsible for the permeabilization of the mitochondrial membrane, we found transcriptional activation only for Bak1. There could be two explanations for these findings:

either Bax is not activated through a transcriptional mechanism for these experimental conditions or its activation occurs via p53 activity. However, Bak activation has been reported to be realized in a p53 independent manner [46, 47]. Moreover a previous study showed that Bak is more important than Bax in HeLa cells apoptosis [48].

The pro-apoptotic proteins are usually blocked by inhibitor of apoptosis proteins (IAP) and anti-apoptotic Bcl-2 family proteins preventing mitochondrial damage [49]. It seems that in HeLa cervical cells, VEGFA inhibition induces a type II apoptotic signal through the transcriptional regulation of pro (Bak) and anti-apoptotic genes (c-IAP2). The exact molecular mechanism of the c-IAP2 anti-apoptotic activity is not yet fully understood. Several papers describe the involvement of this protein in the TNFα stimulated cell death [50-53], its transcription being regulated through the NF-kB signaling pathways [54]. Growth factor stimulation is known to induce NF-kB activation and the transcriptional upregulation of its target genes, including IAP proteins as part of the anti-apoptotic mechanism. It is possible that the inhibition of the VEGFA stimulation led to suppression of the NF-kB signal causing the c-IAP2 downregulation, suggesting a critical role of c-IAP2 as antiapoptotic factor in cervical cancer cells.

A change in mRNA level expression was also found for the TRIAL receptors, TNFRSF10A and TNFRSF10B known as DR4 (death receptor 4), respectively DR5 (death receptor 5). The upregulation of these receptors suggest that the VEGFA inhibition could promote TRAIL-induced apoptosis. It seems that p73, similarly to p53, has the ability to upregulate the expression of these receptors [55]. Wiley et al. [56] hypothesized that TRAIL can induce apoptosis in tumorigenic or transformed cells, but not in normal cells, later to be proved that a variety of tumor cells express both DR4 and DR5, but not normal tissues. This fact is exploited as potential cancer therapy, the stimulation of the proapoptotic receptors DR4 and DR5 showing promising safety and efficacy in several preclinical cancer models. However, not all tumor cells are sensitive to TRAIL therapy [57], studies of the intracellular mechanism of TRAIL resistance identified antiapoptotic or prosurvival molecules as being responsible for the failure of the treatment [58]. The upregulation of VEGFA could be one of the modulating factors for this resistance.

It was reported that the binding of TRAILR with its ligands can result in the activation of other signaling pathways, including the phosphoinositide 3-kinase (PI3K)-Akt, with stimulation

of cell proliferation, migration, and survival [59]. The increased cell proliferation rate observed at 48h of treatment vs. negative control cells could be explained by the Akt1 overexpression and might indicate an escape mechanism with the activation of additional pathways. Also, active Akt has been reported to impair p73 mediated apoptosis [60] and promote VEGF expression in both endothelial and tumor cells [61, 62]. These findings could explain the short term success of antiVEGF treatments.

4.5 Transcriptional mechanism of angiogenesis and apoptosis induced by EGCG on cervical tumor cells

4.5.1 Effect of EGCG on the angiogenic potential of HeLa cells

The total RNA extracted from treated and untreated cells was profiled for 84 genes known to be involved in regulating the angiogenic process. The array includes growth factor and receptors, adhesion molecules, cytokines and chemokines, proteases and matrix proteins.

Five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB) were used for well to well normalization. Data analysis was done by ΔΔCt method using the Superarray Data Analysis Web Portal. We have considered of interest all the genes with $-1.5 \leq$ Fold regulation ≥ 1.5 in order to assess the genes involved in the early antiangiogenic process. We found that, there were significant expression differences ($p<0.05$) for 11 genes out of the 84 investigated genes. Four genes were up-regulated and seven down-regulated (table 1).

Table 4. Genes differentially expressed after 24 hours of EGCG treatment versus control

Gene	Fold regulation
Angiopoietin-like 4 (ANGPTL4)	2.22
Inhibitor of DNA binding 1 (ID1)	1.98
Interferon beta 1(IFNβ1)	2.49
Interleukin 1 beta (IL1-β)	1.77
Monocyte chemoattractant protein 1 (CCL2)	-2.117
Granulocyte chemotactic protein 2 (CXCL6)	-1.6958
Ephrin A1 (EFNA1)	-1.7116
Platelet-derived growth factor alpha (PDGFA)	-1.6958
Transforming growth factor beta 2 (TGF-β2)	-1.6764
Thrombospondin 1 (THBS1)	-3.2013
Tumor necrosis factor alpha-induced protein 2 (TNFAIP2)	-1.5213

Three of these genes were validated at protein level by ELISA method. The results show the same expression pattern for the investigated proteins as their mRNA: we found downregulated levels of PDGFA and TGFβ-2 proteins and upregulated levels of IL1-β (Fig. 11).

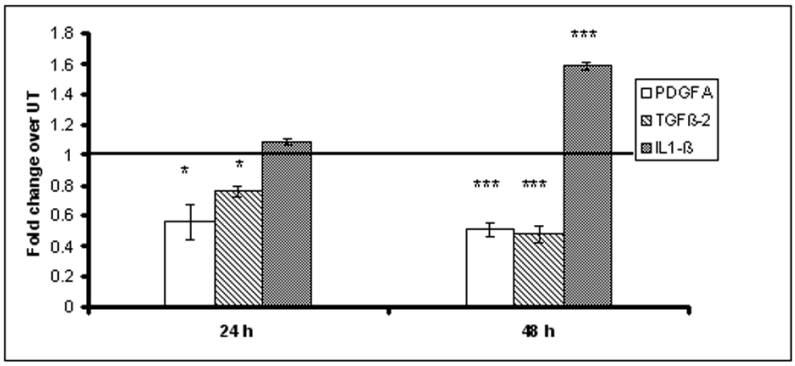
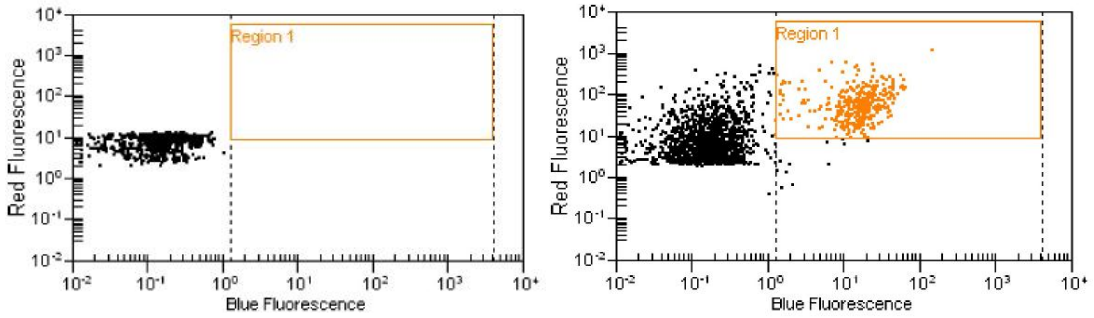


Fig. 11 Fold change in protein expression upon EGCG treatment over untreated group.

4.5.2 Effect of EGCG on the apoptotic potential of HeLa cells

The RNA from treated cells with EGCG (10μM), respectively for control group was analyzed for the profile of expression of 84 key genes involved in modulating the biological processes of apoptosis. The array includes TNF ligands and their receptors, members of bcl-2 family, caspases, IAP, TRAF, CARD, death effector domain and CIDE families, as well as genes involves in the p53 DNA damage pathways. Three housekeeping genes (B2M, HPRT1, GAPDH) were used for normalization for all the genes. $\Delta\Delta C_t$ method was used for fold-change calculation. The genes with -1.25 ≤ Fold regulation ≥1.25 were considered of interest. Our results show that there are significant gene expression differences for 11 genes, 6 being up-regulated and 5 downregulated (Table 5).



UT EGCG

Fig. 12 On-chip flow cytometry analysis of cell apoptosis.

Table 5 Genes differentially expressed after 24 hours of EGCG treatment versus control

Gene	Fold regulation
BCL2-antagonist/killer 1 (BAK1)	1.272
Lymphotoxin beta receptor (LTBR)	1.37
Tumor necrosis factor receptor superfamily, member 21 (TNFRSF21)	1.502
CD27 molecule (CD27)	1.451
Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10)	2.038
Tumor protein (TP73)	1.8319
Tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B)	-1.279
Baculoviral IAP repeat-containing 2 (BIRC2)	-1.336
BCL2/adenovirus E1B 19kDa interacting protein 1 (BNIP1)	-1.25
Caspase recruitment domain family, member 8 (CARD8)	-1.39
CASP8 and FADD-like apoptosis regulator (CFLAR)	-1.343

EGCG treatment induced significant apoptosis 48 hours after treatment as measured by on chip-flow-cytometry (Fig. 12). We did not observe any apoptotic cells after 24 hours.

4.5.3 Effect of EGCG on HeLa cells proliferation

We examined the effect of EGCG on HeLa cells proliferation 24 and 48 hours after treatment by MTT test. At both time points, the cell proliferation was found to be significantly inhibited (Fig. 13). EGCG treatment induced a 40 % inhibition effect after 48 hours; moreover EGCG treatment stopped completely the proliferation process, there was no significant difference between the number of cells evaluated at 24 or 48 hours of incubation.

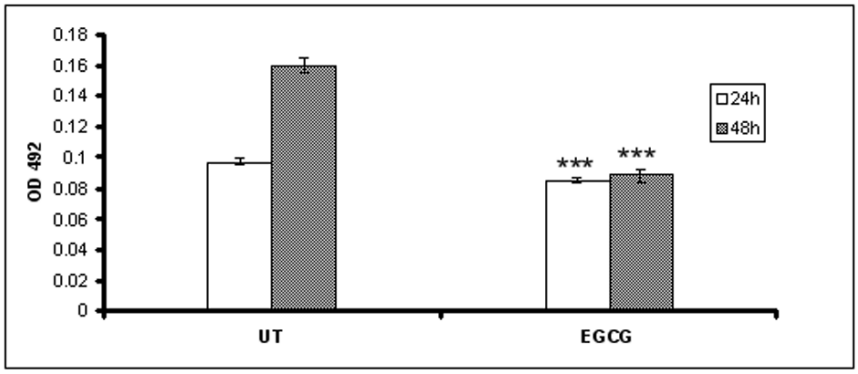


Fig. 13 The effect of EGCG on HeLa cell proliferation upon incubation with EGCG for 24 and 48 hours.

4.5.4 Effect of EGCG on cell attachment and spreading

Next, we evaluated the influence of EGCG on cellular adhesion and motility on Type IV Collagen and Laminin coated plates (Fig. 14, Fig. 16), two major components of ECM. Our results show (Fig. 14) that after 48 hours of EGCG treatment the attachment of HeLa cells to type IV collagen was stimulated, but had no effect on the adhesion to laminin. There were no differences in cells attachment observed at 24 hours after treatment (data not shown). In contrast, if the cells were allowed to attach for 20 hours, the adhesion was dramatically inhibited (Fig. 15). Untreated cells presented with multiple filopodia and lamellipodia, characteristic for spreading cells, whereas most of the EGCG treated cells remained round shape even after 20 h, indicating the inhibition of the spreading ability (Fig. 16).

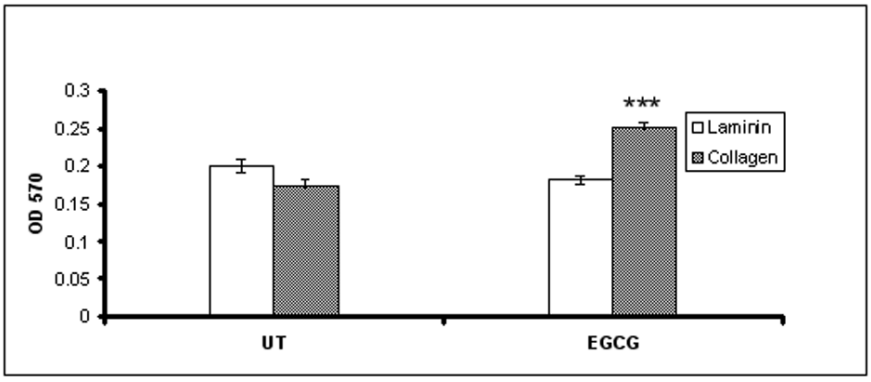


Fig. 14 Adhesion of HeLa cells to Collagen and Laminin after EGCG treatment.

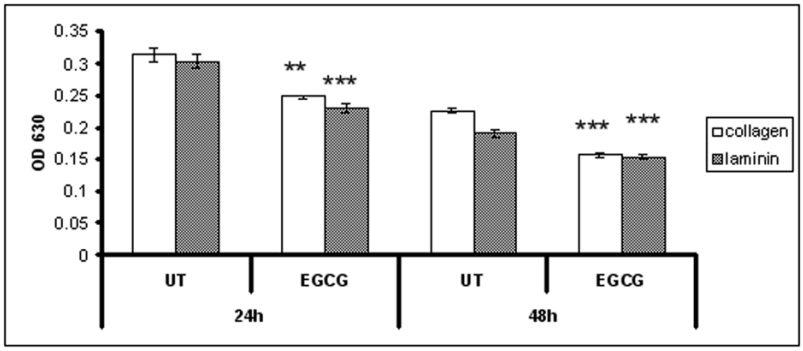


Fig. 15 Adherent HeLa cells to Collagen and Laminin after 20 hours of incubation.

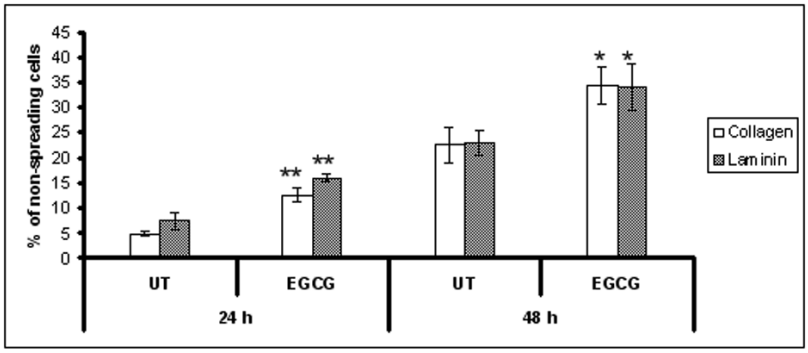


Fig. 16 Effect of EGCG treatment on HeLa cells spreading.

4.5.5 Attachment to endothelium assay

In order to quantify the adhesion of the HeLa cells to endothelial HUVEC cells, HeLa cells were treated with EGCG and harvested at 24 and 48 hours post treatment. The cells were seeded onto the HUVEC cell monolayers, and co-cultured for 50 min. After removing the non-adherent cells, the remaining adherent cells were counted. As shown in Fig. 17 the EGCG treatment reduced the adherence to endothelial cells in time dependent manner.

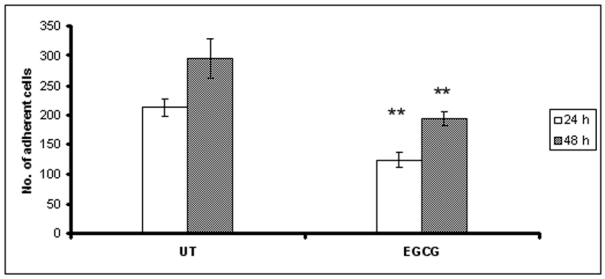


Fig. 17 Adhesion of HeLa cells to endothelial cells monolayer after EGCG treatment

4.5.6 Inhibition of cell migration by EGCG

Migration towards a chemoattractant is a distinct cellular phenotype of metastatic tumor cells, and it is an essential step for tumor invasion and metastasis. Since cervical cancer is associated with more aggressive tumor phenotypes, we examined the effect of EGCG on the migration ability of HeLa cells (Fig. 18) using an *in vitro* migration assay, which simulates the *in vivo* metastatic process. As shown in Fig. 18B, there was a dramatic inhibition on HeLa cells' migration ability after the treatment with EGCG. Compared with the untreated cells, EGCG caused an average of 48% and 68% reduction of migration ability respectively.

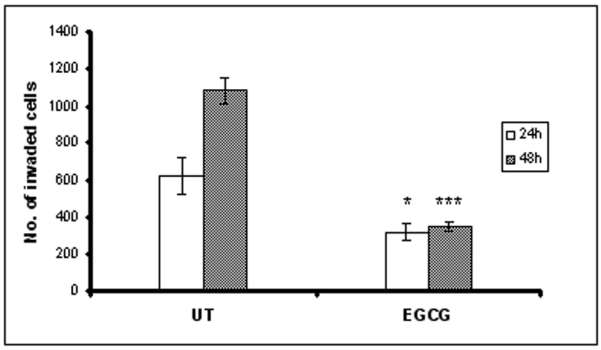


Fig. 18 Inhibition of HeLa cell migration by EGCG.

4.5.7 Discussions

Several studies describe the beneficial effects of green tea consumption, many of them pointing potential advantages compared to traditional cancer drugs like availability, and low toxicity to healthy cells. These observations led to the investigation of several compounds found in green tea in clinical trials. Currently, there are 51 ongoing clinical trials studying the effects of EGCG on different pathologies, including one on cervical cancer (information available at www.clinicaltrials.gov; accessed 12 march 2011).

To date, the precise molecular mechanism of EGCG anticancer effects remain unclear [63]. In this study we report the transcriptional mechanism that might modulate the antiangiogenic and antimetastatic effects of EGCG on HeLa cervical cells.

By profiling 84 genes involved in angiogenesis modulation we investigated the early molecular transcriptional mechanisms induced by EGCG treatment. The transcriptional analysis showed that EGCG treatment modulates the transcription of several genes involved in the angiogenic process (Table 4). These genes are known to mediate multiple mechanisms like endothelial cell proliferation (TNFAIP2, EFNA1, PDGFA, CXCL6, IFNB1, ID1), adhesion (THBS-1, CXCL6), migration (EFNA1, THBS-1, TGFβ-2, CCL-2) and invasion (ANGPTL4, THBS-1, TGFβ-2, IL1-β, ID1).

The anti-proliferative effect of EGCG has been reported by several groups [64-68]. Cell proliferation and growth is promoted through various signaling pathways and EGCG seems to interfere with these pathways and lead to decreased proliferation rates in cervical cancer cells (Fig. 13).

A considerable number of solid tumors [69, 70] including cervical cancer [71] have been showed to express PDGF receptors which suggest that this molecules also acts by an autocrine

mechanism leading to increased proliferation rates. We found that EGCG downregulates the expression of both mRNA and protein levels of PDGFA and this could be one of the mechanisms by which EGCG decreases the proliferation rate as well as the angiogenic potential of cervical cancer cells.

IL1-β seems to engage different cellular signaling pathways depending on the cell type in a dose dependent manner, leading to genotoxic damage, cell apoptosis or cell growth [72]. In angiogenesis, TNF alpha and IL1-β induce increased TNFAIP2 mRNA levels [73, 74] during capillary tube formation *in vitro* [73]. In small cell lung cancer IL1-β up-regulates CXCL6 production and increases tumor cell proliferation [75]. CXCL6 overexpression has been associated with increased metastatic phenotype in small cell lung [75] and prostate [76] cancers. In prostate cancer, CXCL6 overexpression was also associated with increased cell adhesion to ECM and endothelium [76]. Roomi, *et al.* reported recently that EGCG and IL1- β stimulation on HeLa cells leads to decreased MMP-2 secretion [77], a crucial matrix metaloprotease in cell invasion. Our results show that EGCG treatment seems to stimulate the transcription of IL1-β and decrease TNFAIP2 and CXCL6 levels.

In addition to decreased proliferation rate, EGCG treatment seems to also modulate the adhesion capability of HeLa cells to ECM and endothelium. The attachment test to ECM showed no effect on the rapid adhesion of HeLa cells to laminin at either time points, but showed increased adhesion to type IV collagen after 48 hour of treatment (Fig. 14). Cell motility is the key step in organ invasion by tumor cells and increased adherence to the ECM is the first process that indicates increased spreading ability and subsequently metastatic potential of a tumor cell. However, if the time of incubation was extended, the cells lost both their adherence as well as spreading potential (Fig. 15). We observed significant morphology change of the cervical cancer cells after the treatment with EGCG, indicating reduced motility (Fig. 16). These observations might be explained by the fact that even if cells establish initial focal complexes with the ECM, they fail to mature into stable focal adhesions, process that seems to be impaired by the EGCG treatment. Moreover, we further investigated HeLa cells adherence properties to an endothelial cell monolayer. Re-establishment of adhesive connections to endothelium after entering the bloodstream is a key step in tumor metastasis [78]. Our results show that EGCG treatment reduces the adherence of HeLa cells to endothelial cells (Fig. 17).

Adherence of tumor cells to ECM and endothelium is mainly mediated through integrin pathways. THBS-1 has been shown to be involved in modulating these pathways and affect tumor cell adhesion [79], migration and invasion [80]. THBS-1 has been proposed to have both pro- and anti-metastatic properties [80, 81] by regulating epithelial cell growth, motility [80], stromal/epithelial interactions and angiogenesis. THBS-1 has been shown to be a potent inhibitor of angiogenesis by multiple mechanisms, including direct interaction with VEGF and inhibition of MMP-9 activation [81, 82]. However, recent studies reported different biological action in tumor progression and metastasis [80, 81]. Overall, the effects of THBS-1 on any given tumor's metastatic potential probably depends on genetic and/or epigenetic changes in the tumor cells themselves, as well as its antiangiogenic effects on endothelial cells [83].

The transforming growth factor-β (TGF- β) pathway is known to be one of THBS-1's targets. High levels of TGF- β have been associated with various tumors of epithelial origin [84, 85] including cervical tumors [86, 87]. Many of these tumors show increased invasiveness [88], particularly those that overexpress TGF-β2 [89] and are correlated with CIN progression [90-92], higher metastatic phenotype and/or poor patient outcome [86].

In summary, EGCG treatment reduces the proliferation rate, adhesion to ECM and endothelium, spreading and invasiveness of the HeLa cells by modulating genes involved in the angiogenic and metastatic process.

We demonstrate that the apoptotic effects of EGCG are mediated by death domain regulation, specifically by the TNF family of receptors and ligands. The TNFSF10 ligand which is upregulated by the EGCG treatment has been found to have a profound apoptotic effect in neoplastic cells, but not in normal ones [93]. The extrinsic pathway is activated by the binding of TNFSF10 to its cell surface receptors, TNFSFR (TNSF10A/DR4 TNFSFR10A/DR5 or TNSFR11B/OPG). TNFSF10 expression can be used in cancer therapy due to the selectively induced apoptosis in a variety of cancer cells, by interacting with death receptors DR4 and DR5.

TNSFR11B is a gene with anti-apoptotic role, which was downregulated by EGCG. Recent studies suggest that TNFRSF11B plays a role in lymph-node organogenesis and vascular calcification [94]. TNFRSF21 (death receptor-6) has implications in regulating the immune response; it may also be involved in tumor cell survival and immune evasion [95]. TNFRSF21 mRNA level is up-regulated by EGCG. TNFRSF21, LTBR or CD27 have been described in

some cell lines to lead to apoptosis by activation of the MAPK (mitogen activate protein kinases) and NFkB pathways [96, 97].

As shown in the present study, an early event in EGCG mediated apoptosis is gene regulation of the death domain receptors and effectors that are linked to the formation of the "apoptosome" which recruits and activate the apoptosis initiator caspase 9 of the cell-intrinsic pathway. However, this is not observed at mRNA level. Similar to the activation of the initiator caspase of the death receptor-mediated apoptosis pathway, activation of caspase 9 also results in the activation of the downstream "executioner" or caspases effectors (caspase 3, 6 and 7), ultimately resulting in apoptosis [98, 99]. None of these caspases were proved to be statistically significant overexpressed at the mRNA level, but the present study shows down regulation of CARD8 mRNA level.

EGCG induces the cell-extrinsic or death receptor-mediated apoptosis pathway by triggering TNF receptor family and its receptors [100]. Also down-regulation of CFLAR and TNFSFR11B gene expression has pro-apoptotic activity with significant therapeutic potential.

Apoptosis is an active form of cell suicide controlled by a network of genes, in which the Bcl-2 family of proteins plays important roles in control of apoptosis via regulating mitochondrial permeability and releasing of cytochrome c, which activates the caspase cascade by EGCG treatment. From the Bcl-2 family was observed changes at the mRNA level only for BAK, BNIP1 and BIRC2. Beside their antioxidant properties, catechins have been described to display pro-oxidant activity having the potential to oxidize to quinines [100] and may explain the activation of BAK1 gene expression level. It is known that BAK1 protein, a key cell death initiator localizes to mitochondria. This protein also interacts with tumor suppressor p53 after exposure to cell stress [97]. BNIP1 gene is a member of the BCL2/adenovirus E1B 19 kd- and is responsible for the protection from virally-induced cell death; also an apoptotic protector that has been down-regulated at mRNA level by EGCG or the metabolic products. The intrinsic capacity of catechins to form quinone type metabolites upon their oxidation was previously demonstrated [101]. Also, the importance of EGCG metabolites products like: gallic acid or pyrogallol should not be underestimated. Galloil containing tea components are known as tyrosine kinase inhibitors [102]. Moreover, a pyrogallol type structure is a minimum requirement for apoptosis-induction by catechins.

Whereas apoptosis induction by the intrinsic pathway often depends on the presence of functional p53, TRAIL has been shown to induce apoptosis independently of p53 function, but possible p73 dependently [40, 103]. The p73 gene, a member of the p53 family, has both developmental and tumorigenic functions. A recent study shows that p73 is cleaved by caspase-3 and -8 both *in vitro* and *in vivo* during apoptosis, elicited by DNA-damaging drugs and TRAIL receptor ligation. Additionally, p73 protein induces cytochrome c release from isolated mitochondria providing evidence that nonnuclear p73 may have additional functions in the progression of apoptosis [40].

A recent study proved that EGCG treatment induced apoptosis in the T24 human bladder cancer cell line by inhibiting phosphatidylinositol 3'-kinase/Akt activation, which, in turn, resulted in the modulation of Bcl-2 family proteins, leading to enhanced apoptosis of T24 cells [104]. Other study find that CKI-p53, p73 play an important role for the anticancer function of EGCG. Amin *et al*. has identified a lack of functional p53 in EGCG-induced apoptosis [105], moreover, they revealed a number of known p53 targets which were expressed in the absence of functional p53. At the same time, they also identified a crucial role for p73 in EGCG-induced apoptosis and a number of previously unidentified p73 target genes.

The knowledge of the mechanisms responsible for the biological effects of EGCG are needed in order to be proposed as a chemoprevention or chemotherapeutic agent. Further questions regarding the influence of EGCG concentration or the time dependency are still to be investigated in both apoptosis and angiogenesis.

5 CONCLUSIONS

According to the first aim of this thesis, we evaluated the involvement of several pro-angiogenic factors in the progression of three different cancer pathologies: prostate, colo-rectal and cervical cancers. Our results show that in prostate pathology, four of the eight evaluated molecules were statistically significant in one or more of the developmental stages: KGF in PCa compared to BPH and CP, PDFGBB in BPH compared to CP, ANG2 in BPH and CP and TIMP-1 when used in a panel determination. The detection of other four molecules, angiogenin, ICAM-1, VEGF, and bFGF was limited by the technology used for evaluation, which can be overcome by extending the number of points included in the standard curve. Our data suggest that analysis of multiple serum molecules for angiogenesis can improve the diagnosis of prostate pathology in

a noninvasive manner. However, the implications of these molecules at tumoral level remain yet to be investigated, the values determined in this study being a sum of all the molecules secreted in the blood stream, which can include inflammatory or healing processes not directly connected to tumor development. Therefore, the investigation of these molecules at tumoral levels seems imperative.

In colo-rectal cancer, we observed higher mRNA expression in the early stages of tumor development, with statistical significant difference for FGFb and PDGF-BB when comparing the stage dukes B to dukes C. We did not see any significant difference for the VEGF expression in any of the evaluated stages, probably due to the tumor heterogeneity, which is still a problem for molecular evaluation. However, the means values suggest an overexpression of this factor when compared to normal tissues.

By evaluating the involvement of the VEGFA/ VEGFR2 signaling pathway in cervical cancer, we obtained higher levels of VEGFA in all the tumor stages, but to our surprise, we found significant decreased levels of VEGFR2 receptor expression with the tumor advancement, which suggest that in this pathology, the angiogenesis is activated through other receptors. When comparing the tumor response to radiotherapy to the receptor expression, the response was better in patients with higher levels of VEGFR2 receptor. These results indicate that angiogenesis activated through other receptors might be more aggressive and somehow develop resistance to radiotherapy. Therefore, more studies are needed in order to discover what other receptors are involved in sustaining angiogenesis in cervical cancer.

Taken together, our data suggest that, from the pathologies investigated, angiogenesis is more important in cervical cancer, therefore, further studies were concentrated on the evaluation of antiangiogenic strategies in cervical cancer cells.

Since the samples presented increased levels of VEGFA when compared to normal tissues, RNA interference against this molecule was used as a possible single targeted antiangiogenic therapy. Our results show that VEGFA inhibition lead to decreasing proliferation rates and induced apoptosis in treated cells, but only for short periods of times, the initial phenotype being restored after 72 hours. By investigating the molecular mechanisms that govern this behavior, we uncovered the fact that although the principal angiogenic signaling pathway is inhibited the angiogenic potential is sustained by the activation of other adjacent pathways. Therefore, using VEGFA as a single antiangiogenic target in cervical cancer did not have the

expected results, suggesting the need for a multitargeted approach. However, the use of RNA interference for multitargeting is in incipient stages, the technology being limited by the lack of efficient delivery agents, or the need of higher siRNA concentrations which induce cell toxicity. Therefore, upon studying the literature, we found several natural compounds to be described as multipotent inhibitors of the tumoral processes.

EGCG has been shown to have antitumoral proprieties, the existing data suggesting a multitargeting effect of this molecule on tumor cells. Indeed, we observed increased apoptosis, reduced proliferation, cell adhesion, motility and invasion of HeLa cells when treated with EGCG. Our data show that EGCG treatment modulates different angiogenic and apoptotic pathways by interfering with the transcription of several genes that act both on endothelial cells and tumor cells. We expect that EGCG is a promising starting point to derive novel anticancer drugs; our results show that EGCG could be used as a multitargeted antiangiogenic and proapoptotic agent in cervical adenocarcinomas treatment.

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