

Early transcriptional pattern of angiogenesis induced by EGCG treatment in cervical tumour cells

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Abstract

The major green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) has been shown to exhibit antitumour activities in several tumour models. One of the possible mechanisms by which EGCG can inhibit cancer progression is through the modulation of angiogenesis signalling cascade. The tumour cells' ability to tightly adhere to endothelium is a very important process in the metastatic process, because once disseminated into the bloodstream the tumour cells must re-establish adhesive connections to endothelium in order to extravasate into the target tissues. In this study, we investigated the anti-angiogenic effects of EGCG treatment (10 μ M) on human cervical tumour cells (HeLa) by evaluating the changes in the expression pattern of 84 genes known to be involved in the angiogenesis process. Transcriptional analysis revealed 11 genes to be differentially expressed and was further validated by measuring the induced biological effects. Our results show that EGCG treatment not only leads to the down-regulation of genes involved in the stimulation of proliferation, adhesion and motility as well as invasion processes, but also to the up-regulation of several genes known to have antagonist effects. We observed reduced proliferation rates, adhesion and spreading ability as well as invasiveness of HeLa tumour cells upon treatment, which suggest that EGCG might be an important anti-angiogenic therapeutic approach in cervical cancers.

Keywords: EGCG • gene expression • angiogenesis • HeLa cells

Introduction

In 2007, cervical cancer was reported to be the second most common female cancer worldwide, being the third cause of female cancer mortality annually [1]. Although in the developed countries cervical cancer incidence and mortality have decreased, this disease still remains a serious problem with high estimated incidence and mortality [2].

Cervical cancer can be prevented and if detected early it is generally curable [3]. However, the treatments for metastatic or recurrent cervical carcinoma are poorly effective and with serious

adverse effects. There is a great need to identify and investigate new systemic acting agents for cervical cancer treatment.

Lately, the research has been focused on natural compounds; several dietary components show prevention and therapeutic properties in the pathogenesis of cancer. The use of dietary compounds for cancer prevention and therapy could be of major importance because in addition to the diverse biological effects, they have low toxicity and fewer adverse effects than traditional chemotherapeutic agents.

Green tea has received much attention, particularly its major component (-)-epigallocatechin-3-gallate (EGCG). EGCG has been shown to possess remarkable cancer chemopreventive and therapeutic potentials against various cancers, by modulating cell proliferation and apoptosis both *in vitro* and *in vivo* [4–8]. In addition, recent work revealed that EGCG could interfere with cell signalling

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pathways of angiogenesis, metastasis and migration in prostate, liver and breast cancer cells [9–11].

Angiogenesis is a central process in cervical carcinogenesis and progression [12]. Cervical tumour's ability to develop vasculature in order to respond to the metabolic needs is essential to its progression. The antiproliferative effect of EGCG in cervical cancer has been intensively investigated [13–17], but little is known about its anti-angiogenic potential [18].

With these considerations, in this study we report the effect of EGCG on the angiogenic potential of HeLa cervical adenocarcinoma cells, specifically the molecular transcription pattern induced by EGCG treatment. Tumour invasiveness properties including cell morphology change, *in vitro* invasion, spreading and adhesion to extracellular matrix (ECM) and endothelium were evaluated in order to validate our findings.

Materials and methods

Cell culture

HeLa cells (ECACC, Salisbury, UK) were cultured in DMEM growth medium with 1000 mg/l glucose and Human Umbilical Vein Endothelial Cells (HUVEC) cells were cultured in RPMI-1640 medium at 37°C in 5% carbon dioxide–humidified air. Each culture medium was supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 1% Non-essential amino acids (NEAA), 100 U/ml penicillin and 100 U/ml streptomycin. Cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

EGCG treatment

A total of 5×10^5 HeLa cells per well were seeded in 6-well plates in Opti-Mem medium (Gibco, Invitrogen, Carlsbad, CA) and treated with EGCG (Sigma-Aldrich) to a final concentration of 10 μ M for 24 and 48 hrs. Control cells (UT) were treated with phosphate-buffered saline (PBS) instead of EGCG. All our experiments were conducted under serum starvation conditions in order to synchronize the cells and stimulate growth factor production.

Total RNA isolation

Total RNA was isolated with TriReagent (Sigma-Aldrich), purified with RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) and further analysed for quantity and quality with NanoDrop ND-1000 and Agilent Lab-on-a-chip Bioanalyzer 2100 (Agilent Technology Inc., Santa Clara, CA, USA). All the RNAs presented a RNA Integrity Number (RIN) between 9 and 10.

PCR array

Eighty-four genes involved in angiogenesis modulation were simultaneously evaluated using the Human Angiogenesis RT² Profiler™ PCR Array

(SABiosciences, Frederick, MD, USA). One microgram of total RNA isolated after 24 hrs of treatment was reverse-transcribed with RT2 First Strand kit (SABiosciences), and subsequently amplified in a 96-well plate (The Human Angiogenesis RT² Profiler™ PCR Array) with RT2 Real-Time SyBR Green (SABiosciences) using LightCycler 480 Detector System (Roche Diagnostics, Bucharest, Romania). The mRNA levels were analysed with The PCR Array Data Analysis Software using the $\Delta\Delta C_t$ method.

ELISA

Three of the differentially expressed genes were randomly selected and their protein levels were measured by standard sandwich ELISA method. Cell media were collected at 24 and 48 hrs after treatment and quantified for the expression levels of interleukin 1 β (IL-1 β , DLB50; R&D Systems, Inc., Minneapolis, MN, USA), platelet-derived growth factor α (PDGFA) (R&D, MAB1739, BAF221) and transforming growth factor- β_2 (TGF- β_2 ; R&D, MAB612, BAT302) proteins according to the manufacturer instructions and ELISA protocol. For IL-1 β quantification the media were concentrated five times under speed vacuum.

Proliferation assay

A total of 2×10^4 HeLa cells were treated with EGCG (10 μ M final concentration) in a 96-well plate and incubated for 24 and 48 hrs, respectively. After incubation, the cells were treated with 0.1% 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and incubated 1 hr for dye incorporation. Blue formazans were eluted in Dimethyl sulfoxide (DMSO) and quantified with Tecan Sunrise (Tecan Group Ltd., Männedorf, Switzerland) plate reader.

Attachment assay

Twenty-four and –48 hours after treatment, HeLa cells were harvested with 0.25% trypsin-ethylenediaminetetraacetic acid, followed by centrifugation at 1000 rpm for 5 min. at room temperature (RT). The cell pellets were suspended in serum-free DMEM medium at a density of 2.5×10^5 cells/ml. A total of 100 μ l of cell suspension (25,000 cells/well) were seeded in a 96-well plate which was pre-treated with 15 μ g/ml type IV collagen (Sigma-Aldrich) or laminin (Sigma-Aldrich) overnight at 4°C, rinsed with PBS, followed by blocking with 0.2% bovine serum albumin (BSA) at 37°C for 45 min. Cells were allowed to adhere for 20–30 min. in a cell culture incubator and non-attached cells were removed by gently washing with PBS. Attached cells were fixed with 4% formaldehyde for 30 min. at RT, followed by staining in 0.1% (w/v) crystal violet for 30 min. Stained cells were lysed in 1% SDS and the intensity of stain, which is proportional to the number of adherent cells, was quantitated by using a microtitre plate reader at the absorbance of 570 nm.

Spreading assay

HeLa cells were harvested with 0.25% trypsin at 24 and 48 hrs after treatment, centrifuged at 1000 rpm for 5 min., and resuspended at a population density of 5×10^4 cells/ml in warm DMEM pre-equilibrated with 5% CO₂. A total of 100 μ l of cell suspension were plated onto

a 96-well pre-treated plate coated with 15 µg/ml type IV collagen (Sigma-Aldrich) or laminin (Sigma-Aldrich) overnight at 4°C, washed with PBS and blocked with 0.2% BSA for 45 min. at 37°C. Cells were allowed to spread for 20 hrs at 37°C in a cell culture incubator. Cells were washed with PBS and fixed in 3% glutaraldehyde for 30 min. at RT. Cell images were taken by an Axiovert Zeiss (Carl Zeiss Instruments, Bucharest, Romania) microscope with 100× magnification. Spreading cells were defined as cells with extended processes, and non-spreading cells were defined as round cells. After image acquisition, the cells were stained for 30 min. with Giemsa solution, extensively washed and lysed in 1% SDS. The intensity of stain was quantified at 630 nm with a microtitre plate reader.

Adhesion to endothelium assay

Confluent monolayers of HUVEC cells were cultured on plastic cover slips which were pre-treated with 1% gelatine for 30 min. After 24 hrs of culture the medium was removed and 5×10^5 HeLa cells treated as previously described were added to each well and incubated for 50 min. at 37°C. After incubation the non-adherent HeLa cells were thoroughly washed with PBS–Alb, fixed in 4% paraformaldehyde for 15 min. at RT and stained with Giemsa solution for 5 min. The cover slips were washed with PBS, allowed to air dry and mounted on a microscope slide. Using light microscopy the number of cells adherent to the monolayer were counted in five fields of view per well selected at random.

In vitro invasion assay

The effect of EGCG treatment on the invasive properties of cervical cancer cells was determined using the matrigel transwell migration assay in co-culturing conditions. HeLa and HUVEC cells were trypsinized and resuspended in Opti-Mem and RPMI-1640 medium, respectively. Both cell types were treated with EGCG as described. A total of 1×10^5 HeLa cells were plated in the top chamber of the cell culture inserts (6.5 mm diameter insert, 3.0 µm pore size; Millipore, Milford, MA, USA) pre-treated with 1:10 diluted Matrigel BD (BD Biosciences, Franklin Lakes, NJ, USA) and 3×10^5 HUVEC cells were plated onto the bottom well of the chamber also pre-treated with matrigel. A total of 10% FBS was added in the bottom chamber as a chemoattractant. After incubation for 24 and 48 hrs, the cell inserts were removed from the plate and cells that did not migrate were mechanically removed with a cotton swab. Cells on the lower surface of the membrane were fixed in ice cold methanol; the membranes were cut from the housing insert and mounted on microscope slide with 4',6-diamidino-2-phenylindole (DAPI) mounting medium. The migrated cells were examined using inverted phase fluorescence Zeiss Axiovert microscope and counted from six randomly selected fields in a blind way.

Statistical analysis

All experiments were performed in triplicates. All the data are presented as mean ± standard error of mean (S.E.M.). Differences were assessed by parametric and nonparametric tests according to data distribution using the statistical program SPSS 9.0 for Windows. $P < 0.05$ was considered to be statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 1 Genes differentially expressed after 24 hrs of EGCG treatment versus control cells (UT)

Gene	Fold regulation	P value
Angiopietin-like 4 (ANGPTL4)	2.22	0.0009
Inhibitor of DNA binding 1 (ID1)	1.98	0.001
Interferon beta 1 (IFN-β ₁)	2.49	0.01
Interleukin 1 beta (IL-1β)	1.77	0.0001
Monocyte chemoattractant protein 1 (CCL2)	-2.117	0.0007
Granulocyte chemotactic protein 2 (CXCL6)	-1.6958	0.001
Ephrin A1 (EFNA1)	-1.7116	0.01
Platelet-derived growth factor alpha (PDGFA)	-1.6958	0.003
Transforming growth factor beta 2 (TGF-β ₂)	-1.6764	0.008
Thrombospondin 1 (THBS-1)	-3.2013	0.0006
Tumour necrosis factor alpha-induced protein 2 (TNFAIP2)	-1.5213	0.02

Results

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 factors 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 anti-angiogenic process. We found significant expression differences ($P < 0.05$) for 11 out of the 84 investigated genes. Four genes were up-regulated and seven down-regulated (Table 1).

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 down-regulated levels of PDGFA and TGF-β₂ proteins and up-regulated levels of IL-1β (Fig. 1).

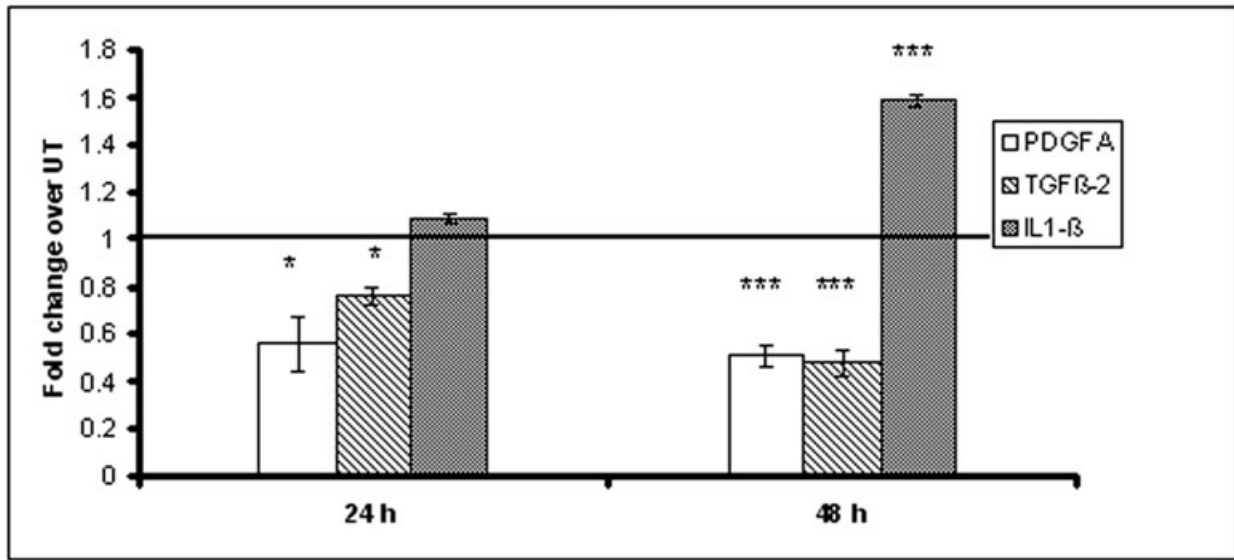


Fig. 1 Fold change in protein expression upon EGCG treatment over untreated group. HeLa cells were incubated with 10 μM EGCG final concentration and the media were collected after 24 and 48 hrs and analysed by ELISA for PDGFA, TGF-β₂ and IL-1β protein levels.

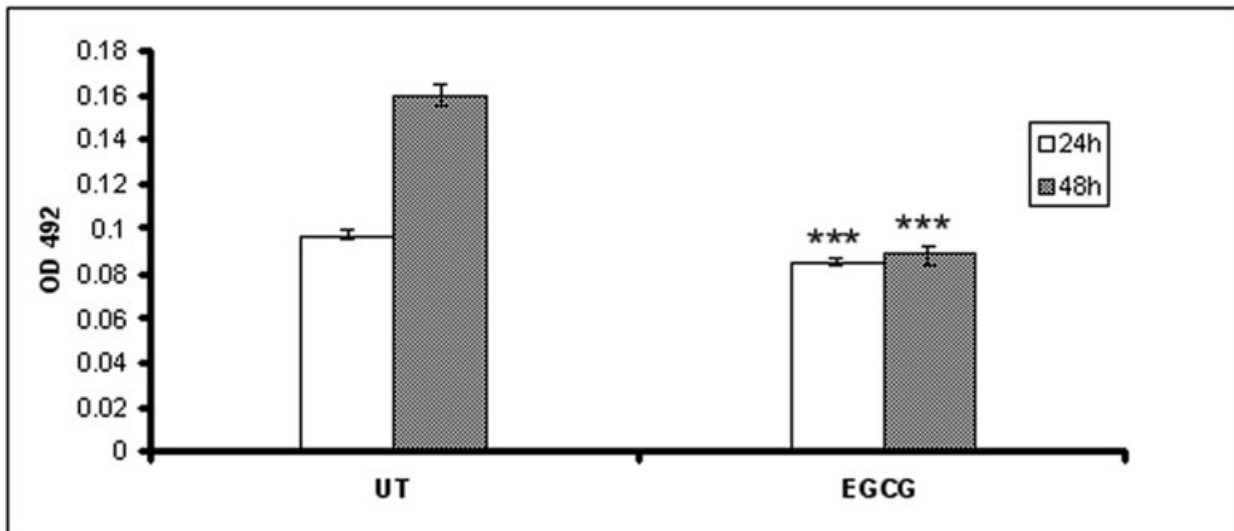


Fig. 2 The effect of EGCG on HeLa cell proliferation. The HeLa cells were seeded in 96-well plates and treated with 10 μM EGCG final concentration in serum-free medium. After 24 and 48 hrs, the cells were treated with MTT and analysed for the number of live cells.

Effect of EGCG on HeLa cells proliferation

We examined the effect of EGCG on HeLa cells proliferation 24 and 48 hrs after treatment by MTT test. At both time-points, the cell proliferation was found to be significantly inhibited (Fig. 2). EGCG induced a 45% inhibition effect on the proliferation rate measured after 48 hrs of treatment compared to untreated cells; moreover, there was no significant difference between the number of cells evaluated at 24 hrs compared to cells treated for 48 hrs.

Effect of EGCG on cell attachment and spreading

We evaluated the influence of EGCG on cellular adhesion and motility on type IV collagen and laminin coated plates (Figs 3 and 5), two major components of ECM. Our results show (Fig. 3) that after 48 hrs 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 hrs after treatment (data not shown). In contrast, if the cells

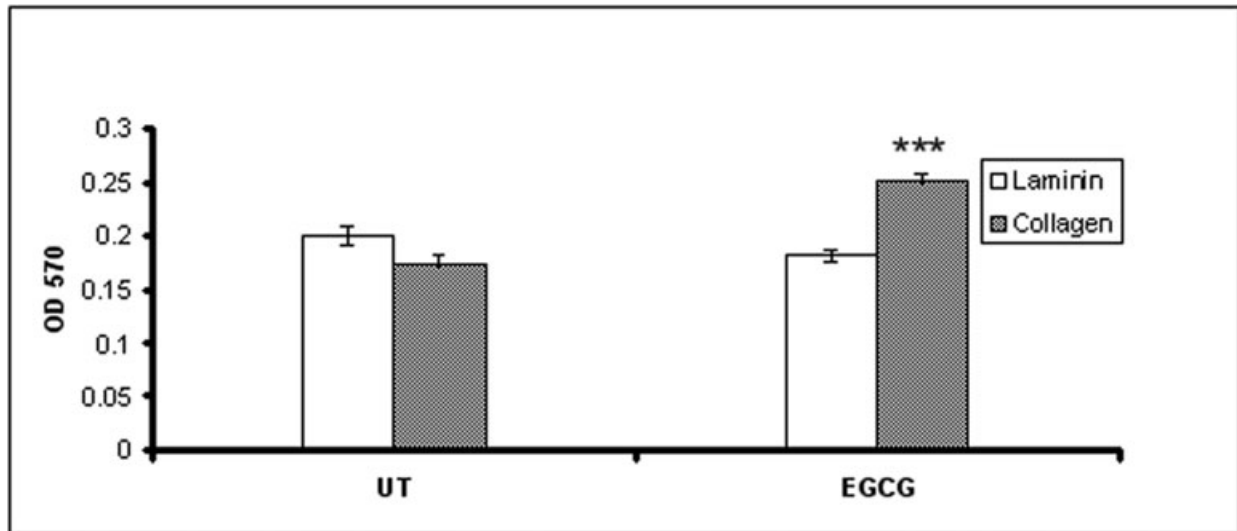


Fig. 3 Adhesion of HeLa cells to collagen and laminin after EGCG treatment. Forty-eight hours after treatment, cells were harvested, resuspended and seeded in a 96-well plate which was pre-treated with 15 $\mu\text{g/ml}$ type IV collagen or laminin. Cells were allowed to adhere for 20 min. and non-attached cells were removed by washing. Attached cells were fixed, stained with crystal violet, followed by measuring the absorbance at 570 nm.

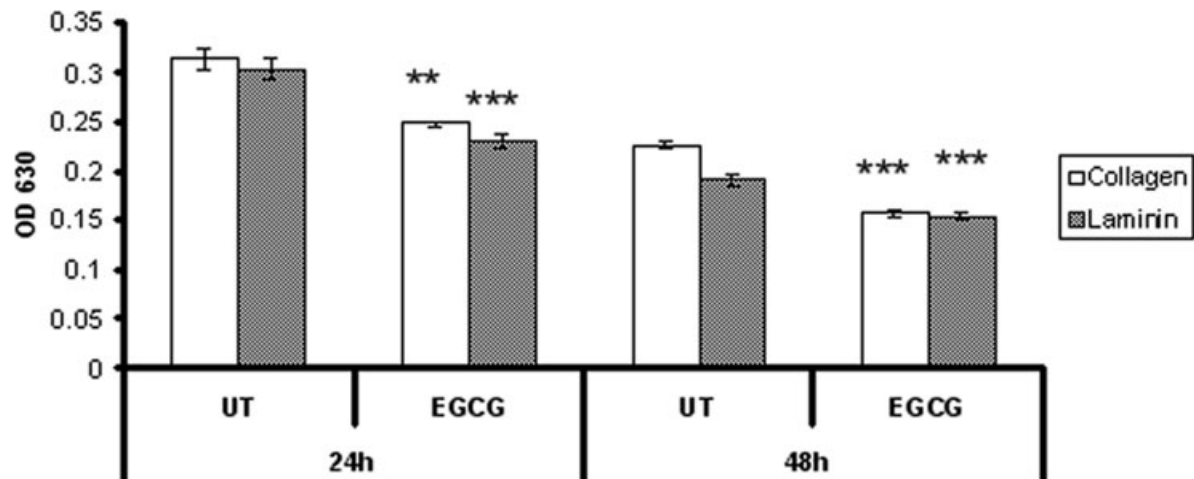


Fig. 4 Adherent HeLa cells to collagen and laminin after 20 hrs of incubation. Twenty-four and forty-eight hours after treatment, cells were harvested, resuspended and seeded in a 96-well plate which was pre-treated with 15 $\mu\text{g/ml}$ type IV collagen or laminin. Cells were allowed to adhere for 20 hrs and non-attached cells were removed by washing. Attached cells were fixed, stained with Giemsa solution, followed by measuring the absorbance at 630 nm.

were allowed to attach for 20 hrs, the adhesion was dramatically inhibited (Fig. 4). Untreated cells presented multiple filopodia and lamellipodia, characteristic for spreading cells, whereas most of the EGCG treated cells maintained their round shape even after 20 hrs, indicating reduced spreading ability (Fig. 5).

24 and 48 hrs after 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 stained and counted. As shown in Figure 6, the EGCG treatment reduced the adherence of HeLa cells to endothelial cells by 40%.

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

Inhibition of cell migration by EGCG

Migration towards a chemoattractant is a distinct cellular phenotype of metastatic tumour cells, and it is an essential step for tumour

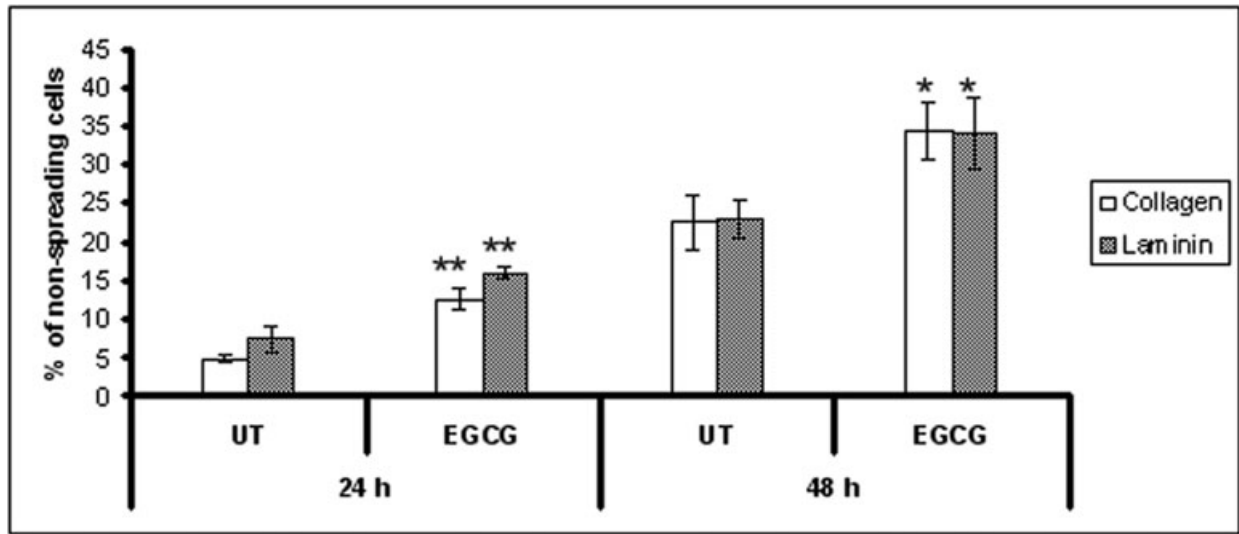
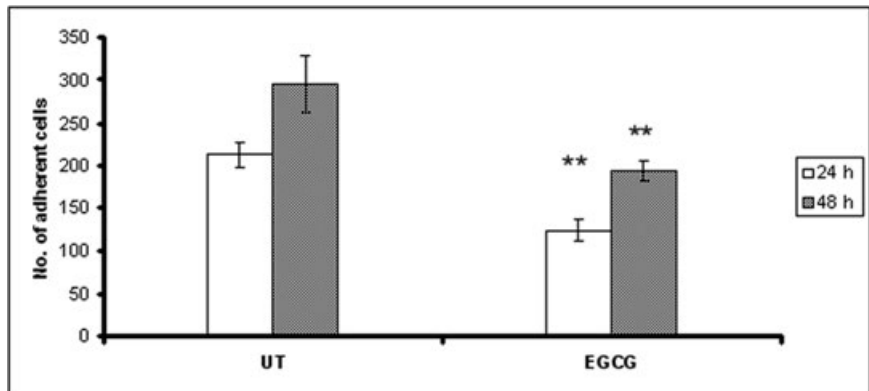


Fig. 5 Effect of EGCG treatment on HeLa cells spreading. HeLa cells were treated with EGCG, and harvested at 24 and 48 hrs after treatment. Harvested cells were plated onto a 96-well plate which was coated with type IV collagen or laminin. Cells were allowed to spread for 20 hrs at 37°C. Spreading cells were defined as cells with extended processes, and un-spreading cells were defined as round cells. The cells were counted in nine different views.

Fig. 6 Adhesion of HeLa cells to endothelial cells monolayer after EGCG treatment. Twenty-four and 48 hours after treatment, cells were harvested, resuspended and seeded onto cover slips containing a HUVEC monolayer. Cells were allowed to adhere for 50 min. and non-attached cells were removed by washing. Attached cells were fixed and stained with Giemsa solution, followed by image acquisition and cell counting. Number of adherent HeLa cells were counted in five random views per well.



invasion and metastasis. Because cervical cancer is associated with more aggressive tumour phenotypes, we examined the effect of EGCG on the migration ability of HeLa cells using an *in vitro* migration assay, which simulates the *in vivo* metastatic process. As shown in Figure 7, there was a dramatic inhibition of 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 after 24 and 48 hrs, respectively.

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 [8]. In this study, we report the transcriptional mechanism that might modulate the anti-angiogenic and anti-metastatic 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 1). These genes are known to mediate multiple mechanisms like endothelial cell

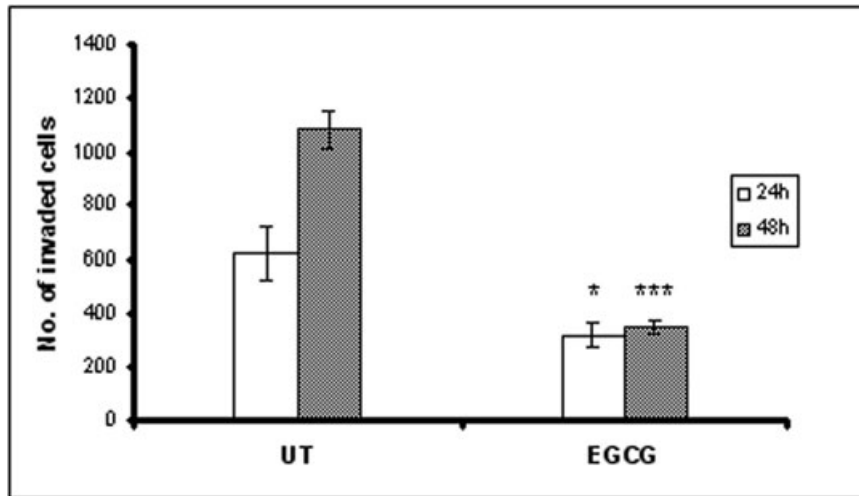


Fig. 7 Inhibition of HeLa cells migration by EGCG. HeLa cells were trypsinized and plated in the top chamber of the transwell. HUVEC cells cultured in RPMI-1640 medium with 10% FBS was added in the lower chamber as a chemoattractant. After incubation for 24 and 48 hrs, cells that did not migrate through the pores were mechanically removed and cells on the lower surface of the membrane were fixed and stained. The number of migrated cells was counted from six randomly selected fields per well in a blind way.

proliferation (tumour necrosis factor α -induced protein 2 [TNFAIP2], ephrin A1 [EFNA1], PDGFA, chemotactic protein 2 [CXCL6], interferon β_1 (IFN- β_1), inhibitor of DNA binding 1 [ID1]), adhesion (thrombospondin 1 [THBS-1], CXCL6), migration (EFNA1, THBS-1, TGF- β_2 , chemoattractant protein 1 [CCL2]) and invasion (angiopoietin-like 4 [ANGPTL4], THBS-1, TGF- β_2 , IL-1 β , ID1). The literature analysis relating the genes of interest showed that most of them act synergistically, on both vascular and tumour compartment. On the premises that every gene expression is associated with a biological effect, we further validated our findings by measuring the effects of EGCG treatment on tumour cell proliferation, adhesion, spreading and invasiveness.

The anti-proliferative effect of EGCG has been reported by several groups [13–17]. Cell proliferation and growth is promoted through various signalling pathways and EGCG seems to interfere with these pathways and lead to decreased proliferation rates in cervical cancer cells (Fig. 2).

PDGFA is a growth factor that upon binding to their receptors activates a variety of intracellular signalling molecules that control cell growth, proliferation and differentiation, leading to cancer progression. PDGF isoforms act mainly through a paracrine mechanism, their expression promoting the establishment of a well-vascularized and prominent stroma, which in turn provides growth factors and anti-apoptotic signals for tumour cells, support for tumour angiogenesis and facilitate invasion [19, 20]. Pietras *et al.* [21] have recently showed in a mouse model of cervical carcinoma that PDGFA stimulates the stromal components and impairment of the PDGF/PDGFR signalling with imatinib affected the angiogenic phenotype of both premalignant cervical neoplasias and invasive carcinomas; the treated lesions exhibited diminished blood vessel density and reduced pericyte coverage.

A considerable number of solid tumours [22, 23] including cervical cancer [24] have been showed to express PDGF receptors which suggest that these molecules also act by an autocrine mechanism leading to increased proliferation rates. We found that

EGCG down-regulates 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.

IFN- β_1 antitumour activity has been intensively investigated in human cancers. Studies have shown that its expression leads to immune stimulation [25, 26], tumour regression [27, 28] and angiogenesis inhibition by down-regulation of proangiogenic factors like bFGF *in vivo* [29–31] and blocking endothelial cell migration *in vitro*. Vannucchi *et al.* [32] reported that IFN- β_1 induces a direct anti-proliferative effect on cervical tumour cells by inhibiting the cell cycle progression. This report seems to be in concordance with our study. We observed that the number of cells after 24 and 48 hrs of treatment remained the same and we assume that EGCG treatment decreases the proliferation rate by stopping the cell cycle progression through induced IFN- β_1 expression.

ID genes are regulators of transcription being responsible for changes in gene expression that lead to increased growth, invasion and metastasis in several human carcinomas [33]. The precise role of ID1 in cervical cancer is little investigated; however, several studies correlated increased expression of this protein with higher tumour cell aggressiveness, poor prognosis and survival in early stages [34, 35]. Lyden *et al.* showed that ID proteins are required for the proliferative and invasive phenotype of endothelial cells during tumour-associated angiogenesis [33]; however, there was no association between ID1 expression and angiogenesis in cervical cancer, which suggest that ID1 expression on prognosis cannot be attributed to its proangiogenic effect alone [34]. Another study reported that ID genes are strongly responsive to signalling pathways coupled to growth factors [36]. ID proteins enhance cell cycle progression and their overexpression induces apoptosis in serum-deprived fibroblasts [37]. The exact mechanism of action of EGCG through ID1 gene up-regulation in HeLa cervical cells is still to be determined, ID1 overexpression might be induced as a resistance mechanism against EGCG

treatment in order to progress the cell cycle or the serum withdrawal coupled with ID1 expression might lead to cell apoptosis as reported for fibroblasts.

IL-1 β seems to engage different cellular signalling pathways depending on the cell type in a dose-dependent manner, leading to genotoxic damage, cell apoptosis or cell growth [38]. Breast cancer cells under genotoxic stress secreting moderate levels of IL-1 β stimulated clonal expansion, whereas high levels of IL-1 β engaged the apoptotic pathway. In angiogenesis, TNF- α and IL-1 β induce increased TNFAIP2 mRNA levels [39, 40] during capillary tube formation *in vitro* [39]. In small cell lung cancer IL-1 β up-regulates CXCL6 production and increases tumour cell proliferation [41]. CXCL6 overexpression has been associated with increased metastatic phenotype in small cell lung [41] and prostate [42] cancers. In prostate cancer, CXCL6 overexpression was also associated with increased cell adhesion to ECM and endothelium [42]. Roomi *et al.* reported recently that EGCG and IL-1 β stimulation on HeLa cells leads to decreased matrix metalloproteinase 2 (MMP-2) secretion [43], a crucial matrix metalloprotease in cell invasion. Our results show that EGCG treatment seems to stimulate the transcription of IL-1 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 hrs of treatment (Fig. 3). Cell motility is the key step in organ invasion by tumour cells and increased adherence to the ECM is the first process that indicates increased spreading ability and subsequently metastatic potential of a tumour cell. However, if the time of incubation was extended, the cells lost both their adherence as well as spreading potential (Fig. 4). We observed significant morphology change of the cervical cancer cells after the treatment with EGCG, indicating reduced motility (Fig. 5). 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, a 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 tumour metastasis [44]. Our results show that EGCG treatment reduces the adherence of HeLa cells to endothelial cells (Fig. 6).

Adherence of tumour 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 tumour cell adhesion [45], migration and invasion [46]. THBS-1 has been proposed to have both pro- and anti-metastatic properties [46, 47] by regulating epithelial cell growth, motility [46], stromal/epithelial interactions and angiogenesis. THBS-1 has been shown to be a potent inhibitor of angiogenesis by multiple mechanisms, including direct interaction with vascular endothelial growth factor (VEGF) and inhibition of MMP-9 activation [47, 48]. However, recent studies reported different biological action in tumour progression and metastasis [46, 47]. Overall, the

effects of THBS-1 on any given tumour's metastatic potential probably depends on genetic and/or epigenetic changes in the tumour cells themselves, as well as its anti-angiogenic effects on endothelial cells [49].

The TGF- β pathway is known to be one of THBS-1's targets. High levels of TGF- β have been associated with various tumours of epithelial origin [50, 51] including cervical tumours [52, 53]. Many of these tumours show increased invasiveness [54], particularly those that overexpress TGF- β 2 [55] and are correlated with CIN progression [56–58], higher metastatic phenotype and/or poor patient outcome [52].

Although generally known for its anti-proliferative effects, TGF- β is a well-characterized inducer of epithelial–mesenchymal transformation in tumour cells, resulting in enhanced cell migration and invasion. TGF- β controls the transcription of numerous integrins and although the down-regulation of integrin expression has been reported, in most cases TGF- β stimulates integrin expression in several cell types and tissues, as well as in various human cancers (rev. in [59]). We assume that EGCG treatment down-regulates the THBS-1 expression (fold regulation –3.2), which in turn down-regulates its downstream target, TGF- β 2 (fold regulation –1.67) and subsequently integrin secretion leading to decreased tumour cell adhesion, motility and invasion (Fig. 7).

It is well known that VEGF can regulate the proliferation and migration of both endothelial and tumour cells. EGCG has been shown to induce VEGF down-regulation in different cancers [60–63], including in HeLa cervical cancer cells [18]. Although we did not find decreased VEGF mRNA levels upon EGCG treatment (probably due to the different experimental conditions) we found decreased levels of EFNA1. EFNA1 is a prototypic ligand which binds EphA2 receptor tyrosine kinase and triggers downstream signals that regulate cell growth [64, 65] and migration [66, 67]. EFNA1 and its receptor are known to be involved in the VEGF pathway by up-regulating VEGF expression in tumour cells and subsequently activate endothelial cells [68]. It seems that EGCG treatment down-regulates EFNA1 expression which might lead to VEGF inhibition as reported by Zhang *et al.* [18]. Concomitant increased expression of VEGF with EFNA1 was observed in patients with cervical cancer; following radiation therapy [69] and blocking EFNA1 signalling cascade decreased tumour-associated angiogenesis and consequently, tumour progression [70, 71].

Recently, Galaup *et al.* [72] showed that ANGPTL4 has an important role in the metastatic process; increased levels of ANGPTL4 inhibited both vascular activity and tumour cell motility and invasiveness. ANGPTL4's role in angiogenesis is controversial suggesting a tissue specific activity, being induced in both endothelial cells [73, 74] and tumour cells [75]. Depending on the context, ANGPTL4 has been reported to exert complex biological function, acting as a proangiogenic factor [74, 76] by mediating endothelial cell survival [77] or having anti-angiogenic properties [78] by modulating endothelial cell adhesion [79]. It seems that in HeLa cells the ANGPTL4 up-regulation by EGCG treatment induce an anti-angiogenic and anti-metastatic effect.

EGCG treatment seems to mediate the angiogenic phenotype in HeLa cervical cells also through CCL2 inhibition. In human

cervical cancer, CCL2 is abundantly expressed in the stroma and associated with macrophage infiltration [80]. CCL2 induces the migration and activation of monocytes, and it has been implicated in the recruitment of tumour-infiltrating macrophages (TIMs) in several tumour types [81, 82]. Previous studies showed that TIMs are a major source of MMP-9 [83] as well as a broad range of tumorigenic factors [84] promoting angiogenesis, tumour progression and metastasis. Zijlmans *et al.* [85] showed that cervical tumours that do not express CCL2 are characterized by a decreased number of TIMs, smaller tumour size and reduced vascular invasion as well as increased overall survival.

Conclusions

In this study, we explored the early transcriptional pattern of angiogenesis induced by EGCG treatment in cervical cancer cells. Our data show that EGCG treatment modulates the angiogenic pathways by interfering with the transcription pattern of several genes that act both on endothelial cells and tumour cells. EGCG treatment decreases the tumour cell proliferation rate, inhibits cell adhesion, motility and invasion potential of HeLa cells. Our results

show that EGCG could be used as an anti-angiogenic agent in cervical adenocarcinomas treatment [86].

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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