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The sensitization of glioma cells to cisplatin and tamoxifen by the use of catechin

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Abstract Telomerase expression strongly correlates with the grade of malignancy in glioma with inhibition illustrating a definite increase in chemosensitivity. This study was designed to investigate the effects of a green tea derivative, epigallocatechin-3-gallate (EGCG); together with either cisplatin or tamoxifen in glioma, and to investigate whether these effects are mediated through telomerase suppression. EGCG showed a significant cytotoxic effect on 1321N1 cells after 24 h and on U87-MG cells after 72 h ($P < 0.001$) without significantly affecting the normal astrocytes. Treatment with EGCG inhibited telomerase expression significantly ($P < 0.01$) and enhanced the effect of cisplatin and tamoxifen in both 1321N1 ($P < 0.01$) and U87-MG ($P < 0.001$) cells. EGCG, as a natural product has enormous potential to be an anti-cancer agent capable of enhancing tumour cell sensitivity to therapy.

Keywords Epigallocatechin-3-gallate (EGCG) · Telomerase · Glioma · Cisplatin · Tamoxifen

Introduction

Glioblastoma multiforme (GBM), the most malignant form of glioma has a median survival of less than a year and a five year survival rate less than 3% [1]. While less than 20–30% of malignant gliomas respond to chemotherapy, surgery presents constraints due to the diffused nature and close proximity of tumours to vital anatomy. Recent studies have been aimed at identifying specific targets responsible for the non-responsiveness of glioma cells to chemotherapy. Telomerase expression has been strongly correlated to the grade of malignancy and prognosis in glioma with inhibition illustrating a definite increase in tumour cell sensitivity [1]. Thus, telomerase is a potential target for the alteration of chemoresistance in cancers.

Telomerase activity is present in 70–80% of cancers often correlated to the level of malignancy however it is absent or undetectable in normal adult somatic cells [2, 3]. *hTERT* the telomerase active subunit is found only in telomerase positive cells, thus may be a more precise measure of telomerase activity [4]. The absence of telomerase protein in normal cells, makes cancer cells specific targets for anti-*hTERT* treatment thereby reducing the side-effects. The use of natural agents or dietary factors possessing anticancer properties along with other chemotherapeutic drugs at lower doses is a novel approach to control the side-effects of anticancer agents.

Green tea is one of the most popular natural agents known for its chemo-preventive and anti-proliferative properties against cancers. Consumption of green tea has been associated with the reduced risk of lung, ovarian, breast, prostrate, skin and oral cancers [5–8]. The anticancerous properties of green tea have been attributed to its catechin, epigallocatechin-3-gallate (EGCG) [9] via telomerase inhibition, apoptosis induction and cell cycle dysregulation [10]. Although there is accumulative evidence for anti-proliferative and anti-telomerase activity of green tea on cancers, no published data is available on glioma. Glioma being one of the most hostile malignancies to tackle, this study focusses on the suppressive effects of EGCG on telomerase in glioma; either alone or in combination with other chemotherapeutic agents (cisplatin and tamoxifen).

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Materials and methods

Cell culture

Human glioma cell lines 1321N1 (astrocytoma) and U87-MG (GBM) were purchased from the European collection of cell cultures (ECACC, UK). U87-MG cells were cultured in Eagle's modified essential medium (EMEM), while the 1321N1 cells were cultured in Dulbecco's modified eagle's medium (DMEM); both supplemented with 10% (v/v) foetal bovine serum (Gibco-BRL, UK) and 2 mM L-glutamine (Sigma, UK). The normal human astrocyte cells (NHA) were cultured in astrocyte basal medium (Cambrex, UK). All the cells were seeded in either 96-well culture plates (approximately 4,000 cells/well) for chemotherapy treatment or in tissue culture treated flasks for RT-PCR.

Cell treatment

The two glioma and the normal NHA cell lines were treated for 24, 48 or 72 h with 0–100 μM of EGCG prepared according to the manufacturer instructions (Sigma, UK) in order to establish the IC_{50}. The two glioma cell lines were treated with 100 μM (IC_{50}) of EGCG before they were treated for 48 h with cisplatin (0–50 μM) or tamoxifen (0–20 μM) both purchased from Sigma and dissolved in 100% (v/v) ethanol in 96 well plates. Cell viability was measured using the manufacturer's protocol for the Cell-Titer Glo® Cell Viability Assay (Promega, UK).

mRNA, cDNA extraction and PCR

EGCG treated 1321N1 and U87-MG cells were used to isolate mRNA according to the manufacturer's protocol (mRNA isolation kit, Roche, UK). The isolated mRNA samples were converted to cDNA using the First strand cDNA synthesis kit (Roche, UK). Quantitative real time PCR was used to evaluate the expression of *hTERT* (214 bp) and *GAPDH* (238 bp, control). Primers used for *hTERT* 5′CGTGGTTTCTGTGTGGTGTC3′ (sense) and 5′CCTTGTCGCCTGAGGAGTAG3′ (antisense), and for *GAPDH* 5′GAAGGTGAAGGTCGGAGT3′ (sense) and 5′GAAGATGGTGATGGGATTTC 3′ (antisense) were designed using Primer3 software and manufactured by TIB MOLBIOL (Germany). PCR was performed using Fast Start DNA master ^{PLUS} SYBR Green 1 (Roche, UK) in a LightCycler real-time PCR detection system (Roche Diagnostics, Germany) according to the protocol described previously [11]. All PCR reactions were performed in triplicate and a negative control (no template DNA) was included. The NHA is a telomerase negative control cell line previously tested by shervington et al. [1], thus was not subjected to PCR analysis.

Telomerase immunofluorescence

Cells cultured on chamber slides were fixed using 4% paraformaldehyde (BDH, UK) for 10 min and then incubated with 0.3% Triton X-100 (BDH, UK) in PBS for 7 min after EGCG treatment. Slides were incubated in a blocking solution containing 1% BSA (Sigma, UK) and PBS for 30 min. Monoclonal primary telomerase antibody (1:2000) (Abcam, UK) was diluted in the blocking solution and applied to the cells as previously described [11].

Statistical analysis

All values were expressed as means ± SD for four independent experiments. Student's paired *t*-test was used to test the difference between the samples. Values of $P < 0.01$ and $P < 0.001$ were considered to be statistically significant.

Result

The cytotoxicity of EGCG treatment on 1321N1, U87-MG and NHA cells was determined using the IC_{50}. The expression of telomerase in both glioma cell lines was compared between the IC_{50} treated and untreated EGCG cells. While treatment with EGCG for 24 h resulted in significant reduction in cell viability of 1321N1 cells ($P < 0.001$), it required 72 h treatment with EGCG to generate a similar result in the U87-MG cells. Treatment of NHA cells with the highest concentration of EGCG (100 μM) after 72 h resulted in only a 30% reduction in cell viability compared to the untreated (Fig. 1), thus this observation led us to believe that further analysis need only be carried out on the glioma cell lines.

The expression of *hTERT* was significantly reduced ($P < 0.01$) after 24 h in EGCG IC_{50} treated 1321N1 cells and after 72 h in EGCG IC_{50} treated U87-MG cells compared to the EGCG non treated cells (Fig. 2a, b). The expression of *GAPDH*, the control gene was found to be similar in all cells irrespective of their EGCG treatment status. Telomerase antibody assay confirmed similar results with a drastic decrease in telomerase protein expression in EGCG treated cells (Fig. 2c).

EGCG IC_{50} treated 1321N1 and U87-MG cells (24 and 72 h) were subjected to treatment with cisplatin or tamoxifen for a further 48 h. After treatment with cisplatin, EGCG treated 1321N1 ($P < 0.01$) and U87-MG ($P < 0.001$) cells were found to be significantly less viable compared to those not treated with EGCG. The IC_{50} of

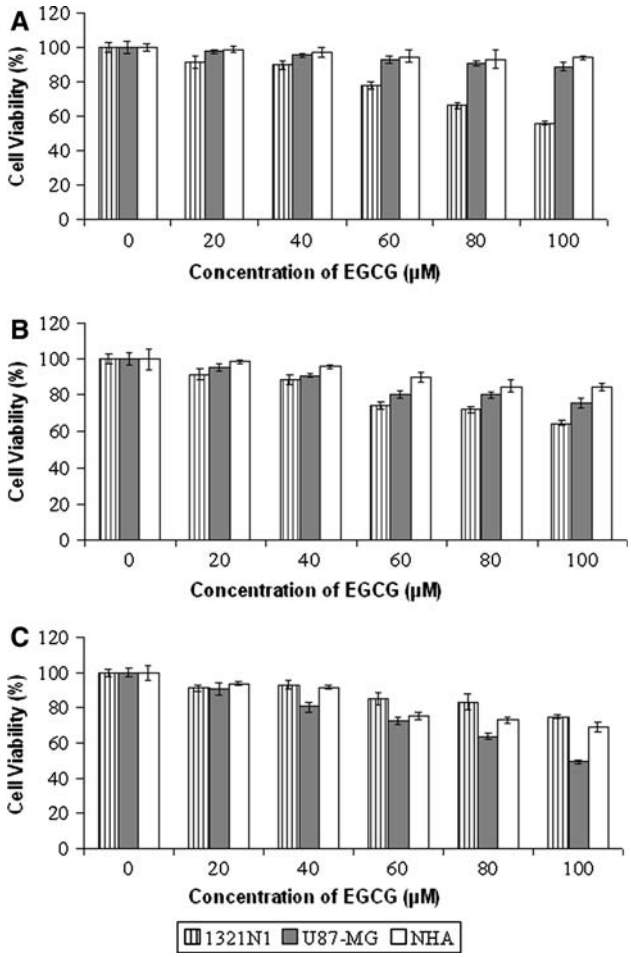

Fig. 1 Cytotoxicity of EGCG on 1321N1, U87-MG and NHA cells after 24, 48 and 72 h treatment. Data values are mean ± standard deviation of four independent experiments

cisplatin for 1321N1 and U87-MG cells was 20 μM. However, only 36% and 13% respectively of EGCG treated cells were alive at this concentration of cisplatin (Fig. 3a). Similarly, EGCG and tamoxifen combined affected the viabilities of 1321N1 ($P < 0.01$) and U87-MG ($P < 0.001$) cell lines significantly compared to tamoxifen alone. EGCG non-treated 1321N1 and U87-MG cells when

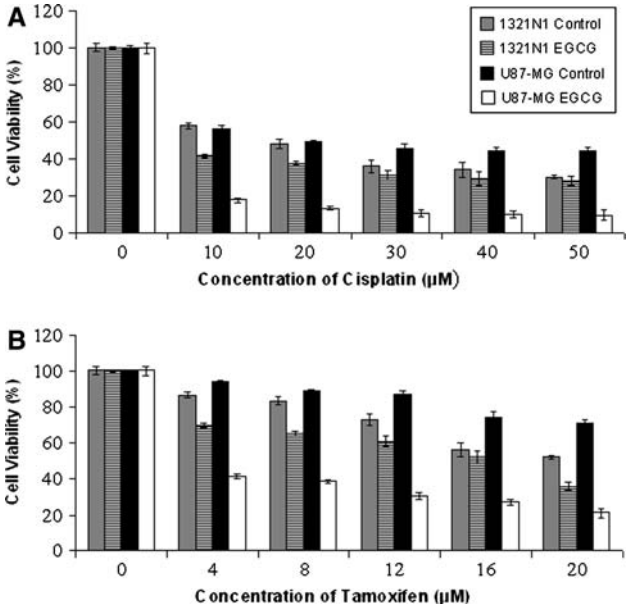

Fig. 3 Cell viability assessment of 1321N1 and U87-MG cells treated with EGCG (100 μM) for 24 h and 72 h respectively, followed with a combination of increasing concentrations of cisplatin (**a**) and tamoxifen (**b**). Data values are mean ± standard deviation of four independent experiments

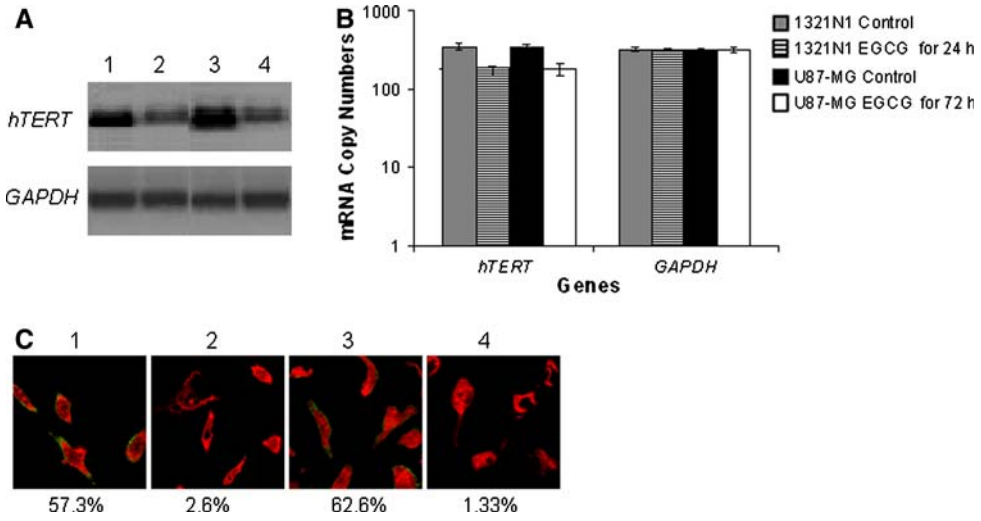

Fig. 2 Gene expression levels of *hTERT* and *GAPDH* in EGCG treated 1321N1 and U87-MG cells along with telomerase protein levels. (**a**) Agarose gel electrophoresis: Lane 1 represents untreated 1321N1 cells, and Lane 2 1321N1 24 h EGCG treated cells, Lane 3 untreated U87-MG cells, Lanes 4 U87-MG 72 h EGCG treated cells. (**b**) Copy numbers of gene expression. (**c**) Telomerase protein levels assessed using immunofluorescence in each corresponding sample. The percent represent the number of positive cells within the sample. The sample size is 150 cells (data values are mean ± standard deviation, $n = 3$)

treated with tamoxifen had an IC_{25} of 20 and 16 μM respectively. While at this concentration cell viability was only 36% and 26% in EGCG treated 1321N1 and U87-MG cells respectively (Fig. 3b). Thus in both the glioma cell lines there was a significant difference in the viability of cells treated with a combination of EGCG and tamoxifen or cisplatin in comparison to those treated with the drug alone.

Discussion

EGCG hinders tumour cell proliferation thus hampering its growth [12], however, its effect on cell viability; telomerase expression and chemosensitivity specifically in glioma are unknown, consequently making the results presented in this study novel. This study showed that EGCG causes induction of death upon treatment in both 1321N1 and U87-MG cells in a dose and time dependent manner. Yokoyama et al. [13] previously reported that EGCG significantly affects the viability of U87-MG cells after 72 h with an IC_{50} of 100 μM, which is in agreement with this study, however, no correlation was made regarding telomerase expression. EGCG treatment did not induce death of NHA cells (telomerase negative cells) suggesting that normal cells may be preserved or spared if this treatment regime is used.

U87-MG which is a high grade glioma (GBM) demonstrated a slightly elevated telomerase level compared to 1321N1 which is a grade II astrocytoma. Supporting the fact that telomerase activity is often correlated to the progression and the grade of malignancy in glioma [14]. Importantly, the addition of EGCG significantly reduced the expression of *hTERT* mRNA in both 1321N1 and U87-MG cells with a comparatively greater reduction in U87-MG cells compared to 1321N1. EGCG is known to have telomerase inhibitory effects in a variety of cancers including oral cavity, thyroid, lung and liver carcinoma, breast and colon cancers as well as in cervical tumours [10, 15, and 16]. However, in this study the inhibition of telomerase by EGCG in glioma cells is reported for the first time.

EGCG treated 1321N1 and U87-MG cells further subjected to chemotherapeutic drug treatments such as cisplatin and tamoxifen were found to be significantly more effective compared to the EGCG untreated cells. EGCG has previously been reported to act synergistically with both cisplatin and tamoxifen in breast cancer cells resulting in increased cell death and apoptosis [17, 18]. Overall, EGCG has a combinational effect thereby increasing the sensitivity of malignant cell lines towards chemotherapy probably via telomerase inhibition. Furthermore, doxorubicin resistant cancer cell lines were rendered vulnerable to the drug after EGCG treatment, possibly due to the inhibition of protein kinase C, leading to the down-regulation of drug resistance proteins [19, 20]. Given that EGCG treatment reversed P-glycoprotein mediated drug resistance in multi-drug resistant carcinoma cells [21] this may also be true for glioma cells.

The results from this study indicate that EGCG inhibits cell proliferation and telomerase, and acts in conjunction with other drugs such as cisplatin and tamoxifen both in 1321N1 and U87-MG glioma cells. In agreement with previously reported data that showed the cytotoxic effects of EGCG are exerted by the induction of apoptosis in cervical cancer cells [16]. Telomerase inhibition causes shortening of cell telomeres to a critical length leading to genome instability which activates the apoptotic pathway leading to cell death [22]. Given that telomerase has previously been associated with the regulation of cell cycle regulatory proteins, telomerase inhibition could inflict senescence by influencing the cell cycle [23].

The telomerase-positive glioma cell lines namely 1321N1 and U87-MG were more sensitive to EGCG treatment than the telomerase-negative control NHA cell line [11]. U87-MG a higher malignancy grade cell line, clearly demonstrated a greater decline in *hTERT* mRNA expression in comparison with 1321N1. Thus it can be postulated that EGCG diminishes the viability of cells by induction of apoptosis and senescence via telomerase inhibition. Another important conclusion from this study was that EGCG acts synergistically with other chemotherapeutic drugs. U87-MG cells, which showed greater telomerase suppression after EGCG treatment compared to 1321N1 and comparatively enhanced inhibition in cell viability when treated with the combination of EGCG and the drug. This effect which was significant for both cisplatin and tamoxifen treatment, upheld the expectations of the previous postulated mechanism of telomerase suppression mediated apoptotic induction by EGCG. Telomerase inhibition leads to increased sensitivity of cells to drugs such as cisplatin and tamoxifen and telomerase expression is related to chemosensitivity in various cancer cells. Furthermore coinciding with previous data that shows inhibition of telomerase activity in glioma cells was associated with increased cisplatin sensitivity [24].

EGCG has also been associated with alterations of several cell signalling pathways equilibrating tumour growth and cell death, such as the inhibition of signal transduction pathways epidermal growth factor receptors [25], and insulin like growth factors [13], vascular endothelial growth factor involved in angiogenesis [26] and cyclo-oxegenase 2 involved in tumour growth [27]. Animal experiments have shown that EGCG induces apoptosis in tumour cells by inhibiting angiogenesis and certain matrix metalloproteinases [28]. With the involvement of numerous mechanisms exerting their anti-proliferative effects,

EGCG seems to emerge as the much awaited potential therapeutic agent targeting the multifaceted machinery of cancer.

Non selectivity of chemotherapy to cancer cells, leading to toxic side-effects and multi-drug resistance are the major hurdles faced in glioma management. EGCG may be used to increase the sensitivity of tumour cells to chemotherapy via telomerase inhibition, as observed in this study thus achieving tumour suppression at lower doses resulting in overall reduced cellular toxicity. Thus, telomerase inhibition by EGCG can provide a valuable key for tackling multi-drug resistance and dow-regulating therapeutic side-effects in gliomas.

Since EGCG actively inhibits telomerase in glioma followed by induction of apoptosis and senescence, and acts in conjunction with cisplatin and tamoxifen, it is a promising natural chemical with enormous potential as an anti-cancer agent. The effect of EGCG specifically on chemoresistant glioma cells should be further studied as a means to tackle multi-drug resistance in glioma. Moreover, the effect of EGCG on primary samples obtained from glioma patients in addition to experimental animals could also be studied. Furthermore, the effect of the administration of green tea given orally to animals and human subjects suffering with glioma should also be investigated.

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References

1. Dome J, Bockhold C, Li S, Baker S, Green D, Perlman E et al (2005) High telomerase RNA expression level is an adverse prognostic factor for favorable-histology Wilms' Tumor. J Clin Oncol 23:9138–9145
2. Gertler R, Rosenberg R, Stricker D, Friederichs J, Hoos A, Werner M et al (2004) Telomere length and human telomerase reverse transcriptase expression as markers for progression and prognosis of colorectal carcinoma. J Clin Oncol 22:1807–1814
3. Takeuchi H, Kanzawa T, Kondo Y, Komata T, Hirohata S, Kyo S et al (2004) Combination of caspase transfer using the human telomerase reverse transcriptase promoter and conventional therapies for malignant glioma cells. Int J Clin Oncol 25:57–63
4. Morii K, Tanaka R, Onda K, Tsumanuma I, Yoshimura J (1997) Expression of telomerase RNA, telomerase activity, and telomere length in human gliomas. Biochem Biophys Res Commun 239:830–834
5. Seely D, Mills E, Wu P, Verma S, Guyatt G (2005) The effects of green tea consumption on incidence of breast cancer and recurrence of breast cancer: a systematic review and meta-analysis. Integr Cancer Ther 4:144–155
6. Borrelli F, Capasso R, Russo A, Ernst E (2004) Systematic review: green tea and gastrointestinal cancer risk. Aliment Pharmacol Ther 19:497–510
7. Zhang M, Binns C, Wand L (2002) Tea consumption and ovarian cancer risk a case-control study in China. Cancer Epidemiol Biomarkers Prev 11:713–718
8. Katiyar S, Mukhtar H (1996) Tea in chemoprevention of cancer: epidemiologic and experimental studies. Int J Oncol 8:221–238
9. Mittal A, Pate MS, Wylie RC, Tollefsbol TO, Katiyar SK (2004) EGCG down-regulates telomerase in human breast carcinoma MCF-7 cells, leading to suppression of cell viability and induction of apoptosis. Int J Oncol 24:703–710
10. Sadava D, Whitlock E, Kane S (2007) The green tea polyphenol, epigallocatechin-3-gallate inhibits telomerase and induces apoptosis in drug-resistant lung cancer cells. Biochem Biophys Res Commun 360:233–237
11. Shervington A, Patel R, Lu C, Cruickshanks N, Lea R, Roberts G, Dawson T, Shervington L (2007) Telomerase subunits expression variation between biopsy samples and cell lines derived from malignant glioma. Brain Res 1134:45–52
12. Bigelow R, Cardelli J (2006) The green tea catechins (-)-epigallocatechin-3-gallate (EGCG) and (-)-epicatechin-3-gallate (ECG) inhibit HGF/Met signalling in immortalized and tumorigenic breast epithelial cells. Oncogene 25:1922–1930
13. Yokoyama S, Hirano H, Wakimaru N, Sarker K, Kuratsu J (2001) Inhibitory effect of epigallocatechin-gallate on brain tumor cell lines in vitro. Neurooncol 3:22–28
14. Boldrini L, Pistolesi S, Gisfredi S, Ursino S, Ali G, Pieracci N (2006) Telomerase activity and hTERT mRNA expression in glial tumors. Int J Oncol 28:1555–1560
15. Lin S, Li W, Shih J, Hong K, Pan Y, Lin J (2006) The tea polyphenols EGCG and EGC repress mRNA expression of human telomerase reverse transcriptase (hTERT) in carcinoma cells. Cancer Lett 236:80–88
16. Yokoyama M, Noguchi M, Nakao Y, Pater A, Iwasakaa T (2004) The tea polyphenol, (-)-epigallocatechin gallate effects on growth, apoptosis, and telomerase activity in cervical cell lines. Gynecol Oncol 92:197–204
17. Kim H, Kang Y, Kwak S, Ding G, Bae S, Park E et al (2005) Effects of cell growth inhibition on the combination of cisplatin with green tea extracts. Korean J Gynecol Oncol 16:347–353
18. Chisholm K, Bray B, Rosengren R (2004) Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. Anticancer Drugs 15:889–897
19. Jodoin J, Demeule M, Beliveau R (2002) Inhibition of the multidrug resistance P-glycoprotein activity by green tea polyphenols. Biochim Biophys Acta 1542:149–159
20. Stammler G, Volm M (1997) Green tea catechins (EGCG and EGC) have modulating effects on the activity of doxorubicin in drug-resistant cell lines. Anticancer Drugs 8:265–268
21. Qian F, Wei D, Zhang Q, Yang S (2005) Modulation of P-glycoprotein function and reversal of multidrug resistance by (-)-epigallocatechin gallate in human cancer cells. Biomed Pharmacother 59:64–69
22. Zhang X, Mar V, Zhou W, Harrington L, Robinson M (1999) Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev 13:2388–2399
23. Jagadeesh S, Banerjee P (2006) Telomerase reverse transcriptase regulates the expression of a key cell cycle regulator, cyclin D1. Biochem Biophys Res Commun 347:774–780
24. Kondo Y, Kondo S, Tanaka Y, Haqqi T, Barna B, Cowell J (1998) Inhibition of telomerase increases the susceptibility of human malignant glioblastoma cells to cisplatin-induced apoptosis. Oncogene 16:2243–2248
25. Adachi S, Nagao T, Ingolfsson H, Maxfield F, Andersen O, Kopelovich L et al (2007) The inhibitory effect of (-)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells. Cancer Res 67:6493–6501
26. Jung Y, Kim M, Shin B, Chay K, Ahn B, Liu W et al (2001) EGCG, a major component of green tea, inhibits tumour growth

by inhibiting VEGF induction in human colon carcinoma cells. Br J Cancer 84:844–850
27. Shimizu M, Deguchi A, Joe A, Mckoy J, Moriwaki H, Weinstein I (2005) EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. J Exp Ther Oncol 5:69–78
28. Bertolini F, Fusetti L, Rabascio C, Cinieri S, Martinelli G, Pruneri G (2000) Inhibition of angiogenesis and induction of endothelial and tumor cell apoptosis by green tea in animal models of human high-grade non-Hodgkin's lymphoma. Leukemia 14:1477–1482