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Effect of ellagic acid on the expression of human telomerase reverse transcriptase (*hTERT*) α+β+ transcript in estrogen receptor-positive MCF-7 breast cancer cells

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Abstract

Objectives: To evaluate the potential of ellagic acid to inhibit the expression of human telomerase reverse transcriptase (*hTERT*) α+β+ splice variant in MCF-7 breast cancer cells.

Design and methods: MCF-7 cells were incubated with ellagic acid (10^{-9} M–10^{-5} M) in the absence and in the presence of 17β-estradiol (10^{-8} M), a known inducer of *hTERT* transcription, and *hTERT* α+β+ mRNA expression was quantified by real-time RT-PCR. 17β-estradiol and ICI182780, a known estrogen antagonist, served as positive and negative controls respectively.

Results: Ellagic acid, when alone, increased *hTERT* α+β+ mRNA while its coexistence with 17β-estradiol reduced significantly the 17β-estradiol-induced increase in *hTERT* α+β+ mRNA, implicating thus both its estrogenic and anti-estrogenic effects in breast cancer cells.

Conclusions: The potential of ellagic acid to down-regulate the 17β-estradiol-induced *hTERT* α+β+ mRNA expression may be a mechanism via which ellagic acid exerts, at least in part, its chemopreventive effects in breast cancer.

Keywords: Ellagic acid; Human telomerase reverse transcriptase [*hTERT*] α+β+ transcript; MCF-7 cells; Polyphenol; Breast cancer cells

Introduction

In recent years, research on the role of telomerase in human carcinogenesis has grown exponentially [1,2] and a very strong association between telomerase activation and malignancy has been established. Telomerase is a promising tumor marker and target for cancer therapy and many studies have provided strong evidence on its direct role in early oncogenic transformation [3–6]. Activation of telomerase is the most common mechanism through which cancer cells stabilize their telomere size and subsequently sustain their unlimited growth [6–8]. The *in vitro* malignant transformation of normal human cells was achieved by reconstitution of telomerase activity by induction of human telomerase reverse transcriptase (*hTERT*) gene expression in combination with other oncogenes [4,9]. The growing interest in the potential of telomerase as a diagnostic and prognostic tumor marker is based on the observation that more than 85% of most human tumors express telomerase activity [10]. The observation that functional hTERT protein is critical for acquisition of telomerase activity [11] has led to the wide application of *hTERT* mRNA determination through standard RT-PCR procedures for the identification of telomerase-positive samples. Essentially all major types of cancer have been screened for the presence of *hTERT* mRNA in a variety of clinical specimens [12].

hTERT has been shown to contain at least 6 alternative splicing sites [13–17]; however, only the full-length *hTERT* transcript is associated with telomerase activity [13–15]. The α splice site causes a 36-bp deletion within the conserved reverse transcriptase motif A [13] and the splice variant was found to be a dominant negative inhibitor of telomerase activity [18]. The β splice site causes a 182-bp deletion leading to a nonsense mutation truncating the protein before the conserved reverse transcriptase motifs, resulting in a non-active TERT protein and catalytically inactive telomerase [13–15]. Alternative splicing of *hTERT* may be a novel mechanism of telomerase regulation

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[19], and *hTERT* mRNA splicing patterns have been studied in immortal human cells [20] and benign and malignant breast tumors [21] in an attempt to elucidate their biological role. The expression of the α-negative splice variant can inhibit telomerase activity [22] in telomerase-positive cells and causes telomere shortening and eventually cell death. The function and biological role of *hTERT* splice variants however is still unknown [2,15–23].

hTERT is among the important target genes via which estrogens induce mammary carcinogenesis. The *hTERT* promoter contains an imperfect palindromic estrogen-responsive element (ERE), and it has been shown that 17β-estradiol (E2) induces *hTERT* expression in MCF-7 cells via an estrogen receptor-ERE dependent mechanism [24]. More importantly, compounds with selective estrogen receptor modulator (SERM) properties such as tamoxifen and raloxifen have been shown to down-regulate the E_2-induced up-regulation of *hTERT* mRNA by direct interaction of estrogen receptor (ER) with the ERE in the *hTERT* regulatory region in MCF-7 cells [25–27].

Ellagic acid, a polyphenol found in berries, grapes and nuts, exhibits important health promoting effects via its antioxidant, antiproliferative, chemopreventive and antiatherogenic activities [28–32]. Ellagic acid has been shown to inhibit cancer cell growth, including breast cancer cell growth, by inducing apoptosis, by regulating matrix metalloproteinases, vascular endothelial growth factor expression and angiogenesis [33]. In addition, it prevents and repairs DNA damage [34,35]. Ellagic acid exhibits SERM-like properties, i.e. it favors bone cell function and exerts anti-estrogenic effects in MCF-7 cells, increasing like ICI182780 the levels of insulin growth factor binding protein-3 (IGFBP-3), a known antigrowth promoter in breast cancer [36,37]. However, the effect of ellagic acid on *hTERT* gene expression in breast cancer is yet unknown. Due to the key role of *hTERT* gene expression in mammary carcinogenesis, and because of the prevalence of ellagic acid in health promoting foods, in the present study we investigated whether ellagic acid has the ability to inhibit *hTERT* gene expression in ER-positive breast cancer MCF-7 cells. For this, we have used our recently developed real-time RT-PCR method for the quantification of *hTERT* α+β+ mRNA, since this splicing variant has been shown to be strongly associated with tumor development and progression [38].

Methods

Cell culture and incubation of compounds

MCF-7 cells (ATCC Cell Bank) were maintained in Dulbecco's Minimal Essential Medium (DMEM-Invitrogen) supplemented with 10% fetal bovine serum (FBS-Invitrogen). Cells were plated in 6-well plate at initial density 100,000 cells/well in DMEM Phenol Red (PR) free, supplemented with 10% FBS dextran-coated charcoal treated (FBS-DCC) in order to eliminate endogenous steroids. After 48 h media were changed and fresh DMEM PR free 10% FBS-DCC containing 10^{-7} M–10^{-8} M, 10^{-9} M, 10^{-10} M 17β-estradiol (SIGMA) or 10^{-5} M–10^{-9} M ellagic acid (Fluka) was added. Co-incubation of 17β-estradiol (10^{-8} M) with ellagic acid (10^{-5} M–10^{-9} M) also occurred. Additionally, 10^{-8} M ICI182780 (Tocris) and 10^{-6} M or 10^{-8} M tamoxifen (Sigma) were used as control of anti-estrogenic activity. After 24 h further incubation, cells were collected by scraping, centrifuged (1200 rpm, 5 min) and the cell pellet was used for RNA isolation.

Total RNA isolation and cDNA synthesis

Total RNA isolation was performed by using Trizol LS reagent (Invitrogen, USA) according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a laminar flow hood, under RNase-free conditions. The isolated RNA was dissolved in RNA storage buffer (Ambion, USA) and stored at −70 °C until use. RNA concentration was determined, by using the Nanodrop-1000 Spectrophotometer (NanoDrop Technologies, USA). Reverse transcription of RNA was carried out with the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen, USA) according to the manufacturer's instructions, using 1 μg of total RNA as template. RNA integrity was tested in the cDNA preparation by real-time PCR amplification of the PBGD (porphobilinogen deaminase) as we have previously described [39].

Real-time PCR

For the amplification of *hTERT* α+β+ we have previously designed a set of primers (TE1/2, KAT4b) spanning exon–intron junction so that nonspecific target transcript detection in any trace of contaminating genomic DNA is completely avoided [38]. TE1/2 was designed to amplify α+ splice variant (gene position: 2181–2199) and KAT4b was designed to amplify β+ splice variant (gene position: 2510–2527). A Taqman probe was also designed (gene position: 2241–2269). Quantification of *hTERT* splice variant mRNA α+β+ was performed in the LightCycler (Roche). Real-time PCR was performed in a total volume of 10 μL per reaction. We placed 1 μL of cDNA into a 9-μL reaction mixture that contained 0.1 μL of Taq DNA polymerase (5 U/μL, Platinum DNA Polymerase; Invitrogen), 1 μL of the supplied 10× PCR buffer, 0.5 μL of $MgCl_2$ (50 mmol/L), 0.2 μL of dNTPs (10 mmol/L; Fermentas), 0.15 μL of bovine serum albumin (10 g/L; Serva), 0.5 μL of the TE1/2 sense primer, 0.5 μL of the KAT4b antisense primer (3 μmol/L), and 1 μL of the TaqMan probe (3 μmol/L); finally, DEPC-H2O was added to a final volume of 10 μL. The cycling protocol was an initial 5-min denaturation step at 95 °C, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 65 °C for 20 s, and extension at 72 °C for 20 s.

Normalization of data

We evaluated all our data with respect to *hTERT* α+β+ over expression by normalizing to the expression of PBGD and using the $2^{-\Delta\Delta Ct}$ method, as described in detail by Livak and Schmittgen [40]. We used a cut off value of 2.00, with samples having a $2^{-\Delta\Delta Ct}$ value>2.00 considered positive for over expression. This number for the cut off is in accordance to the

Relative Quantification Software (Roche Molecular Diagnostics) recommendations for the LightCycler. More specifically, the expression of *hTERT* α+β+ is expressed as a relative ratio to PBGD used as a reference gene, which is then normalized to the expression of the same gene, as measured in the vehicle control MCF-7 used as a calibrator. Thus, using the $2^{-\Delta\Delta Ct}$ approach, we have related the real-time PCR analytical signal (Cp) of the target transcript (e.g. *hTERT*) in each MCF-7 under the effect of natural or chemical compounds to the vehicle control (under the absence of compounds).

Results

Effect of 17β-estradiol, tamoxifen and ICI182780 on the expression of hTERT α+β+

We studied the effect of 17β-estradiol (10^{-7} M–10^{-8} M, 10^{-9} M, 10^{-10} M), ICI182780 (10^{-8} M) and tamoxifen (10^{-8} M, 10^{-6} M) on the expression of *hTERT* α+β+ in the MCF-7 breast cancer cell line. We found that 17β-estradiol increases significantly the expression of *hTERT* α+β+ at all concentrations tested and that ICI182780 at the concentration of 10^{-8} M (ΔΔCp=0.22, P=0.0034) reduces the expression of *hTERT* α+β+ in a significant way (Fig. 1). On the other hand tamoxifen at the concentration of 10^{-6} M significantly reduces the expression of *hTERT* α+β+ (ΔΔCp=0.41, P=0.0096), while at the concentration of 10^{-8} M it does not decrease significantly the expression of *hTERT* α+β+ (ΔΔCp=0.48, P=0.0752) (Fig. 1). Co-incubation of E2 (10^{-8} M) with ICI182780 (10^{-8} M) and tamoxifen (10^{-6} M, 10^{-8} M) reduces the expression of E2-induced *hTERT* α+β+ significantly (Fig. 1).

Effect of ellagic acid on the expression of hTERT α+β+

We studied the expression of *hTERT* α+β+ in MCF-7 cancer cell line using ellagic acid at concentrations ranging from

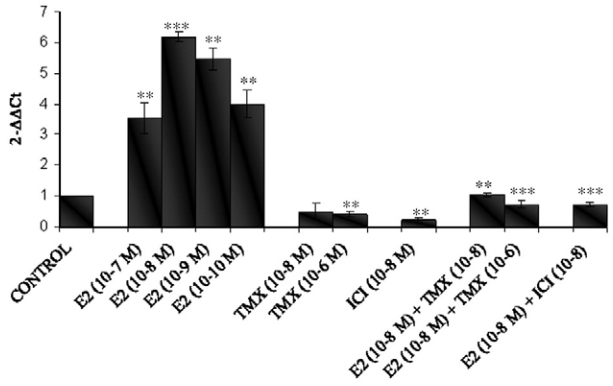

Fig. 1. Effect of 17β-estradiol (E2), ICI182780 and tamoxifen on the expression of *hTERT* α+β+ in MCF-7 cancer cells. Cells were treated with 17β-estradiol (10^{-7} M, 10^{-8} M, 10^{-9} M, 10^{-10} M), ICI182780 (10^{-8} M) and tamoxifen (10^{-6} M, 10^{-8} M). Co-incubation of E2 (10^{-8} M) with ICI182780 (10^{-8} M) and tamoxifen (10^{-6} M, 10^{-8} M) was also carried out. Results are expressed using the $2^{-\Delta\Delta Ct}$ method in relation to vehicle control. Columns and bars represent mean±SD of the results of three experiments. *Significantly different from vehicle control (*p<0.05, **p<0.01, ***p<0.001).

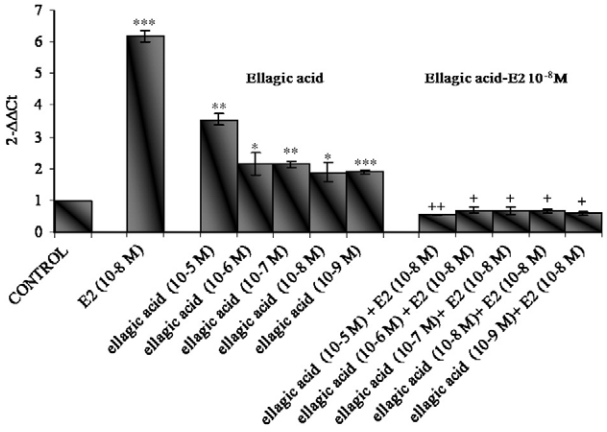

Fig. 2. Effect of 17β-estradiol (E2) and ellagic acid on the expression of *hTERT* α+β+ in MCF-7 cancer cells. Cells were exposed to vehicle control, 17β-estradiol (E2) (10^{-8} M) and ellagic acid (10^{-5} M–10^{-9} M). Co-incubation of 17β-estradiol (10^{-8} M) with ellagic acid (10^{-5} M–10^{-9} M) also occurred. Results are expressed using the $2^{-\Delta\Delta Ct}$ method in relation to vehicle control. Columns and bars represent mean±SD of the results of three experiments. *Significantly different from vehicle control (*p<0.05, **p<0.01, ***p<0.001). +Significantly different from 17β-estradiol (10^{-8} M) (+p<0.05, ++p<0.01).

10^{-5} M–10^{-9} M, and co-incubation of 17β-estradiol at the concentration of 10^{-8} M with all the different concentrations of ellagic acid (Fig. 2). As can be seen in Fig. 1, 17β-estradiol significantly increased the expression of *hTERT* α+β+ at the concentrations of 10^{-7} M (ΔΔCp=3.52, P=0.0139), 10^{-8} M (ΔΔCp=6.19, P=0.0004), 10^{-9} M and 10^{-10} M (ΔΔCp=4.00, P=0.0078) compared to vehicle control. Ellagic acid significantly increased the expression of *hTERT* α+β+ (ΔΔCp=3.56, P=0.0016) at the concentration of 10^{-5} M, followed by the concentration of 10^{-6} M and 10^{-7} M (ΔΔCp=2.13, P=0.0310, ΔΔCp=2.14, P=0.0024 respectively) in relation to vehicle control. However, according to the $2^{-\Delta\Delta Ct}$ method, ellagic acid at the concentrations of 10^{-8} M–10^{-9} M did not result in overexpression of *hTERT* α+β+ (ΔΔCp=1.88, P=0.0359 and ΔΔCp=1.89, P=0.0009 respectively). Co-incubation of 17β-estradiol (10^{-8} M) with ellagic acid (10^{-5} M–10^{-9} M) reduced the expression of *hTERT* α+β+ in relation to the effect of 17β-estradiol at the concentration of 10^{-8} M in a significant manner (Fig. 2). Particularly, ellagic acid at the concentration of 10^{-5} M when co-incubated with 17β-estradiol reduces the expression *hTERT* α+β+ significantly (ΔΔCp=0.54, P=0.0160) in relation to the expression of 17β-estradiol alone. Moreover, ellagic acid at the concentration of 10^{-6} M–10^{-9} M reduces significantly the expression *hTERT* α+β+ (ΔΔCp=0.68, 0.69, 0.66, and 0.58, respectively) in relation to the 17β-estradiol alone (P=0.0327, P=0.0410, P=0.0134, and P=0.0055).

Discussion

Breast cancer is a high incidence cancer and one of the leading causes of cancer death among women [41,42]. Chemoprevention i.e. the cancer prevention by administering synthetic chemical compounds or bioactive food components is

nowadays considered the ideal strategy for reducing cancer on society. Polyphenols are naturally occurring substances, and among the promising bioactive components to reduce breast cancer risk [43]. Ellagic acid, a naturally occurring polyphenol, is found in abundance in nature as food component, it is effectively absorbed and after ingestion the plasma concentrations are at around 0.05–50 μM [44–48].

In this study, we investigated the possibility that ellagic acid possesses anticancer properties via modulation of *hTERT* α+β+ mRNA expression which is an important prognostic indicator of telomerase activity and a prognostic marker for cancer progression, and is highly expressed in cancer cell lines and clinical cancer samples [20,38]. Of note, we have previously demonstrated that MCF-7 cells treated with different antineoplastic agents were found positive for variable *hTERT* splicing variants and that doxorubicin and 5-fluorouracil but not taxol, cisplatin or etoposide, repressed completely *hTERT* β+ gene expression in MCF-7 cells [49].

We assessed the biological activity of ellagic acid in MCF-7 cells at a concentration range 10^{-9} M–10^{-5} M, which are physiologically achievable concentrations. We used as a positive control 17β-estradiol (10^{-8} M), which is known to induce *hTERT* expression and cell growth in MCF-7 cells [24]. Tamoxifen (10^{-6} M, 10^{-8} M), a SERM compound and ICI182780 (10^{-8} M), a known anti-estrogen, served as negative controls, since they are known to inhibit the *hTERT* expression and cellular growth in MCF-7 cells [24–25].

Our data support that 17β-estradiol up-regulates *hTERT* α+β+ gene expression while tamoxifen and ICI182780 down-regulate *hTERT* α+β+ mRNA. To our knowledge, this is the first report demonstrating that 17β-estradiol (10^{-8} M) up-regulates and that ICI182780 (10^{-8} M) or tamoxifen (10^{-6} M, 10^{-8} M) down-regulate *hTERT* α+β+ mRNA expression. Earlier studies have shown that 17β-estradiol up-regulates *hTERT* mRNA while tamoxifen and ICI182789 down-regulate the expression of *hTERT* [24,25]. However, in all these reports *hTERT* was amplified by using primers that amplify all *hTERT* splicing variants since they anneal onto a region common for all transcripts. Therefore, we may hypothesize that the regulation of *hTERT* by estrogens and SERMs [24–27], may be due, at least in part, to sample positivity in *hTERT* α+β+ mRNA. However, this needs to be further elucidated.

Ellagic acid, in the absence of 17β-estradiol, induces *hTERT* α+β+ mRNA expression, while its coexistence with 17β-estradiol inhibits the 17β-estradiol-induced *hTERT* α+β+ mRNA expression. Such data implicate that ellagic acid acts bifunctionally exerting both estrogen agonistic effects and antagonistic effects on *hTERT* α+β+ mRNA expression in breast cancer cells, via an ER dependent mechanism. In agreement, several studies support that phytoestrogens and plant polyphenols may act either as estrogen agonists or estrogen antagonists depending on the absence or the presence of 17β-estradiol [50,51]. In a previous study, we evaluated the estrogenic/anti-estrogenic activity of ellagic acid and demonstrated that it exhibits SERM-like properties. We also showed that ellagic acid, similarly to tamoxifen, displays a small but significant estrogenic activity via ERα, whereas it is a complete estrogen antagonist via ERβ in HeLa cells cotransfected with an ERE-driven luciferase reporter gene and an ERα or ERβ expression vector [36]. In view of the above, and because *hTERT* promoter contains EREs, we may hypothesize that ellagic acid regulates *hTERT* α+β+ mRNA expression, at least in part, in an ER–ERE dependent manner. It is important to mention that tamoxifen and raloxifene, which are known SERMs widely used in the therapy of breast cancer, mediate their 17β-estradiol-antagonistic effects on the *hTERT* promoter via an ERE-dependent mechanism [25,26].

Ellagic acid may activate *hTERT* via extragenomic effects i.e. via several membrane-initiated signaling mechanisms. To this effect, an indirect activation of *hTERT* by estrogens has been demonstrated via Akt/PI3K/NF-kB signaling pathway in MCF-7 cells [26]. Similarly, raloxifene has been reported to inhibit the 17β-estradiol-induced up-regulation of *hTERT* mRNA not only via an ERE-dependent mechanism but also by the PI3K/Akt/NF-kB cascade [26]. In addition, the MAP kinase cascade is involved in the tamoxifen-induced activation of *hTERT* in endometrial cells [25]. In light of above, we may hypothesize that ellagic acid may mediate its inhibitory effects on 17β-estradiol-induced *hTERT* α+β+ mRNA via an ERE-dependent mechanism, as well as via the PI3K/Akt/NF-kB or the MAP kinase signal transduction cascade. However, future studies are needed to further evaluate the exact mechanism of action.

In conclusion, our study reveals that ellagic acid is an inhibitor of *hTERT* α+β+ in MCF-7 cells and suggests that the ellagic acid-induced down-regulation of *hTERT* α+β+ may be a mechanism through which ellagic acid exerts, at least in part, its chemopreventive effects in breast cancer. Furthermore, our results support earlier findings about the possible important role of the alternate transcripts of *hTERT* in the regulation of telomerase and raise questions about the impact of *hTERT* α+β+ splice variant expression in 17β-estradiol-induced malignant transformation of breast tissue.

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