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Effect of antineoplastic agents on the expression of human telomerase reverse transcriptase beta plus transcript in MCF-7 cells

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Abstract

Objectives: To evaluate the effect of antineoplastic agents on the expression of human telomerase reverse transcriptase (hTERT) splice variants in MCF-7 cells.

Design and methods: We have developed a luminometric hybridization assay for hTERT beta plus transcript. MCF-7 cells were isolated before and after treatment with antineoplastic agents. A combination of nested RT-PCR and the developed luminometric hybridization assay was used for the specific detection of hTERT beta plus transcript in treated and untreated MCF-7 cells. Amplification of all hTERT splicing variants by nested PCR in the same samples was also performed.

Results: MCF-7 cells treated with taxol and etoposide were found positive for all hTERT splicing variants, while the expression of hTERT beta plus transcript did not differ significantly before and after exposure. MCF-7 cells treated with doxorubicin and 5-fluorouracil did not express any of hTERT splicing variants. In the presence of cisplatin, three splicing variants of hTERT were detected.

Conclusions: The developed hybridization assay is highly sensitive and specific for the detection of hTERT beta plus transcript in clinical samples.

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Keywords: hTERT; Splice variants; MCF-7; Antineoplastic agents; Luminometric hybridization assay

Introduction

In recent years, a very strong association among telomerase activation, cell immortalization [1,2] and malignancy [3] has been established making this enzyme one of the most promising general tumor markers [4,5] and targets for cancer therapy [6,7]. Telomerase is a cellular ribonucleoprotein reverse transcriptase that synthesizes telomeric DNA onto chromosomal ends, using a segment of its own RNA as a template [8]. Two main components are required for core enzymatic activity of telomerase *in vitro*: human telomerase RNA (hTR), which contains the template for reverse transcription, and human telomerase reverse transcriptase

(hTERT), which consists the enzyme's catalytic subunit [9]. Expression of the functional hTERT protein is a rate-limiting step for acquisition of telomerase activity [9–12] and the *in vitro* malignant transformation of normal human cells, achieved by induction of hTERT gene expression, in combination with other oncogenes, was important for the elucidation of its role in human carcinogenesis [13].

Recently, the observed differences in telomerase activity in normal vs. tumor derived cells led to the hypothesis that telomerase may represent a suitable target for highly specific anticancer therapies, so several classes of telomerase inhibitors were prepared and evaluated [14,15]. Most of these inhibitors act through transcriptional repression of hTERT [16]. Other studies have evaluated the presence of telomerase activity in telomerase-positive cell lines exposed *in vitro* to various antineoplastic agents like tamoxifen, 5-fluorouracil, adriamycin, taxotere, doxorubicin and cisplatin [17–24]. Most of these studies show that resistance of neoplastic cells to chemotherapeutic agents can be monitored by following

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telomerase activity. In recent years, work is also being carried out to explore the therapeutic opportunities presented by hTERT [6,7].

The development of the sensitive “telomeric repeat amplification protocol” (TRAP) assay by Kim et al. [3] enabled the wide screening of many clinical samples for the presence of telomerase activity. However, telomerase activity assays have serious limitations in the handling of many clinical samples on a routine basis since they are laborious and highly affected by the presence of protein activity inhibitors, proteases or RNAses in clinical samples [25,26]. hTERT RT-PCR has been used for the identification of telomerase-positive samples in a variety of clinical specimens since it is insensitive to the presence of proteases and protein inhibitors [27]. However, post-transcriptional regulatory mechanisms controlling the activity of telomerase and especially alternative splicing of hTERT must be seriously taken into account before designing a new assay for hTERT [28,29]. The presence of multiple alternatively spliced hTERT transcripts, corresponding to the full-length message as well as to spliced messages with critical reverse transcriptase motifs deleted has been shown by using primers within the reverse transcription domain of hTERT [28,29]. The α splice site causes a 36-base (12 amino acids) deletion within the conserved reverse transcriptase motif A, whereas the β splice site causes a 183-base deletion resulting in a nonsense mutation truncating the protein before the conserved reverse transcriptase motifs B, C, D and E, thus resulting in a nonactive hTERT protein and catalytically inactive telomerase complex. Four hTERT splicing variants, α^+/β^+ , α^-/β^+ , α^+/β^- , α^-/β^- , of 457, 421, 275 and 239 bp, respectively, have been detected [28,29]. The beta plus hTERT splicing variant is expressed at very low concentrations even in cancer cell lines, since it was found that about 5% of total hTERT mRNA is in hTERT α^+/β^+ form, 80–90% in α^+/β^- form, 5–15% in α^-/β^- form and less than 1% in α^-/β^+ form [30].

In the present study, we evaluate the effect of five commonly used antineoplastic agents, cisplatin, 5FU, doxorubicin, taxol and etoposide, on the expression of hTERT splice variants and especially beta plus transcript in MCF-7 cells isolated before and after exposure to these drugs. For the specific detection of hTERT beta plus transcript in treated and untreated MCF-7 cells, we have developed a methodology that is based on combination of nested RT-PCR and a sensitive luminometric hybridization assay. Nested RT-PCR for the amplification of all hTERT splicing variants in the same samples was also performed.

Materials and methods

Cell culture

Telomerase-positive MCF-7 cells were grown in DMEM/NUT.MIX F12 medium (Invitrogen, Life Technologies)

supplemented with 10% fetal bovine serum (FBS), 10 mg/l bovine insulin and 40 mg/l gentamicin sulphate. MCF-7 cells (2×10^6) were plated in T75 flasks and after 24 h their medium was replaced by culture medium containing the following antineoplastic agents: taxol (Bristol Myers Squib, USA), doxorubicin (Ebwe Arzneimittel, Austria), 5FU (Roche), etoposide (VP-16, Sigma, USA), H7 (Sigma) and cisplatin (CDDP, Sigma). MCF-7 cells were exposed in the antineoplastic agent containing culture medium for 24 h. The culture medium was then replaced by fresh one without drugs, cells were left for 24 h in the fresh medium, harvested and washed with PBS. During the entire period of cell culture, untreated cells were in the exponential phase of growth. Cell counts were performed by the trypan blue exclusion method using a hemocytometer. The IC_{50} of the antineoplastic agents used was determined using the Cell Titer96 Aqueous one solution cell proliferation kit (Promega, USA). All drugs were used at their IC_{50} concentrations.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from MCF-7 cells using Trizol LS reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. All preparation and handling steps of RNA took place in a chemical fume hood, under RNase-free conditions. The isolated RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and stored at -80°C until use. RNA concentration was determined by absorbance readings at 260 nm with the HITACHI UV-VIS (U-2000) spectrophotometer. RNA integrity was tested by PCR amplification of the actin housekeeping gene, as previously described [31]. Reverse transcription was carried out with the SUPERScript RT-PCR System (Invitrogen, Life Technologies), according to the manufacturer's instructions. Total RNA (3 μg) was used as starting material for cDNA synthesis.

Oligonucleotide sequences and labeling

All primers and hybridization probe sequences used in this study were firstly evaluated in the computer (in silico) by using the PRIMER PREMIER 5 software program (Premier Biosoft International, Palo Alto, CA, USA) and are listed in Table 1. All oligonucleotides were synthesized at the Lab of Microchemistry of FORTH, (FORTH, Crete, Greece). The sequence of primers LT5, b2164, 2620 has been previously reported [28,29] while KAT3 primer was in silico designed to anneal into the beta plus splicing region (2342–2524) of hTERT mRNA. The hybridization probe E1 was designed to specifically anneal into the beta plus splicing region of hTERT mRNA and used for the detection of biotinylated PCR products. This probe was tailed enzymatically with Dig-11-dUTP (Roche Molecular Biochemicals, USA), by using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals), as previously described

Table 1
Sequences and positions of primers and hybridization probes used

Use	Name	Oligonucleotide sequence ^a (5'–3')	Nucleotide position
Forward	LT5	CGGAAGAGTGTCTGGAGCAA	1784–1803
Reverse	2620	CGCAAACAGCTTGTCTCCATGTC	2597–2620
Forward ^b	b-2164 ^b	GCCTGAGCTGTACTTTGTCAA	2164–2184
Reverse	KAT3	TAGGACTTGCCCTGATG	2512–2529
Hybridization Probe ^c	E1	GGTGGCACATGAAGCGTAGGAAGA	2478–2501

^a hTERT mRNA (GeneBank accession number AF015950).

^b Labeled with biotin.

^c Labeled with digoxigenin.

[31] and used in the hybridization assay without further purification.

PCR amplification

The amplification was based on a nested PCR protocol. For the first PCR, primers LT5 and 2620 (20 pmol of each) and 1.5 μ l cDNA was added in a total volume of 25 μ l of PCR mixture. This first PCR yields a 837-bp product. An aliquot of 1.5 μ l of the first PCR product was used as a template for nested PCR. In nested PCR, by using as inner primers the pairs 2164/2620 (20 pmol of each), we could amplify all hTERT transcripts, giving four splicing products α^+/β^+ , α^-/β^+ , α^+/β^- , α^-/β^- , of 457, 421, 275 and 239 bp, respectively [28,29], while by using as inner primers the pairs b2164/KAT3 (20 pmol of each), we could amplify hTERT beta plus transcript, giving two products of 366 (α^+/β^+) and 330 (α^-/β^+) bp.

The PCR reaction mixture (25 μ l) consisted of 20 mmol/l Tris–HCl (pH 8.4), 50 mmol/l KCl, 0.2 mmol/l dNTPs, 1.5 mmol/l MgCl₂ and 0.5 U Taq DNA polymerase (Platinum, Invitrogen, Life Technologies). In both cases, the first PCR reaction was initiated by a 5-min denaturation at 94°C and terminated by a 5-min extension at 72°C. The cycling protocol (repeated 25 times) consisted of denaturation at 94°C for 30 s, annealing at 60°C for 50 s and extension at 72°C for 50 s. Both nested PCR reactions were performed under the same conditions as above, the only difference being in cycling protocol (repeated 35 times). PCR amplification was performed in a Peltier Thermal Cycler (DNA Engine, PTC-200, MJ Research, USA). Amplified products were electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet (UV) light.

Hybridization assay

Opaque polystyrene microtiter wells (Microlyte 2, Dynatech, USA) were coated overnight at room temperature with 50 μ l of streptavidin (1.4 mg/l) diluted in phosphate-buffered saline (PBS, pH 7.4). During the whole assay, the wells were washed three times after each step with wash solution [50 mmol/l Tris (pH 7.4), 0.15 mol/l NaCl and 1 ml/l Tween-20], using a microtiter plate washer (Wellwash \times 4, Denley).

Five microliters of the biotinylated PCR products (nested PCR, hTERT beta plus transcript) diluted 10-fold in blocking solution [10 g/l blocking reagent (Roche Molecular Biochemicals) in 0.1 mol/l maleic acid and 0.15 mol/l NaCl, pH 7.5] were added per well and incubated at room temperature with shaking for 30 min. Subsequently, 50 μ l of 0.2 mol/l NaOH was added. After a 20-min incubation at room temperature, the non-biotinylated DNA strands were removed by washing the wells. Fifty microliters of E1 probe labeled with digoxigenin (10 μ M), 1000-fold diluted in blocking solution, were added to each well and incubated at 55°C for 30 min. Fifty microliters of an anti-digoxigenin antibody conjugated to alkaline phosphatase (ALP, 750 U/l, Roche Molecular Biochemicals, 2000-fold diluted in blocking solution) were added per well and incubated at room temperature for 30 min. After this step, the wells were washed six times and 50 μ l of the chemiluminogenic ALP-substrate LumiPhos (Aureon Biosystems, GmbH, Vienna, Austria) were added to each well. The enzymatic reaction was completed after 30 min incubation at 37°C, in the dark. The chemiluminescence signal integral was measured with a Luminometer (Mediator PhL, MEDIATORS Diagnostika GmbH, Austria) and reported as relative luminescence units (RLUs).

Results

Primer selection and PCR optimization

PCR conditions were firstly optimized (MgCl₂ concentration, number of cycles, annealing temperature and amount of DNA sample). Hot-start PCR was used in all cases so that the formation of primer dimers was completely avoided. By using primers LT5 and 2620 in the first PCR and 2164–2620 in nested PCR (Table 1), all hTERT splicing variants were amplified since these primers anneal into a region that is common for all transcripts. The selective amplification of hTERT beta plus transcript (α^+/β^+ , α^-/β^+), in the presence of all other splicing variants, was achieved by using the primer pair b2164, KAT3 in nested PCR since primer KAT3 was designed in the computer (in silico) by the PRIMER PREMIER 5 software program (Premier Biosoft

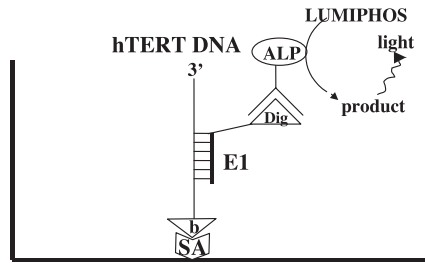


Fig. 1. Principle of the proposed luminometric hybridization assay for hTERT beta plus transcript.

International) to anneal into the beta plus splicing region of hTERT mRNA (Table 1).

Hybridization assay

The principle of the developed luminometric hybridization assay for hTERT beta plus transcript is shown in Fig. 1. Nested PCR products were biotinylated through the use of a biotin-labeled primer b2164. The biotinylated amplification products were immobilized on streptavidin-coated microtiter wells and detected through hybridization to an, in silico designed, digoxigenin labeled probe, E1 (Table 1). This probe anneals into the beta plus splicing region of hTERT mRNA, so in this way, only beta plus splicing variants of hTERT are detected. Hybridized amplicons were detected using anti-digoxigenin antibodies conjugated to alkaline phosphatase and Lumiphos, which is a highly sensitive chemiluminescent substrate. Hybridization assay conditions involving all steps such as reagent concentrations, sample and probe dilution, annealing temperature and time of incubation periods were optimized in respect to the best signal to background ratios (S/B), sensitivity and rapidity. The background is defined as the relative luminescence value (RLU) obtained when the negative control of nested PCR was assayed.

Within-run reproducibility of the hybridization assay was tested by analyzing six times each of three different PCR products, corresponding to 4, 40 and 400 MCF-7 cell equivalents, and the corresponding CVs were found to be

8.4%, 6.5% and 6.9%, respectively. To evaluate the overall reproducibility (between-run) of the hTERT beta plus splice variant luminometric hybridization assay, five different MCF-7 cell suspensions isolated from the same number of MCF-7 cells (1×10^6 , as measured in a hemocytometer) were subjected to the whole analytical procedure and analyzed in duplicate by the proposed hybridization assay. The mean RLUs were: 17,969 + 2423, and CV = 13.5% ($n = 5 \times 2$) which was quite satisfactory as it included all steps such as total RNA extraction, cDNA synthesis, nested PCR and detection of biotinylated PCR products by the proposed luminometric hybridization assay.

The detection limit was found to be 4 MCF-7 cell equivalents since this concentration (RLUs: 34,612 \pm 2485, $n = 4$) could be clearly distinguished from the background (RLUs: 1,098 \pm 168, $n = 4$). The luminometric hybridization assay was more sensitive than agarose gel detection of nested PCR products, where 40 MCF-7 cell equivalents could be detected and this is in accordance with our previous results for CK-19 mRNA detection [31].

Effects of antineoplastic agents on hTERT expression

The proposed methodology was applied for the evaluation of hTERT splicing pattern in MCF-7 cells before and after exposure to five commonly used antineoplastic agents, taxol, etoposide, doxorubicin, 5-fluorouracil and cisplatin. The drug concentrations used in our study correspond to those capable of inhibiting MCF-7 cell growth by 50% (IC_{50}), as evaluated by the Cell Titer 96 Aqueous one solution cell proliferation kit and are shown in Table 2. Total RNA was extracted from the same number of MCF-7 cells isolated before and after exposure to these drug concentrations. The same cDNA samples were analyzed in triplicate for (a) hTERT beta plus transcript by the developed luminometric hybridization assay and (b) for the presence of all hTERT splice variants by nested RT-PCR and agarose gel detection of the PCR products.

hTERT splicing patterns for these samples as evaluated by the developed methodology are also summarized in Table 2. As can be seen in Table 2, MCF-7 cells treated

Table 2
Effect of antineoplastic agents on the expression of hTERT splicing variants in MCF-7 cells

Antineoplastic agent ^a	Dose (μ g/ml)	hTERT beta plus transcript, (RLUs \pm SD)	hTERT splicing variants			
			α^+/β^+	α^-/β^+	α^+/β^-	α^-/β^-
Doxorubicin	4	2389 \pm 79	–	–	–	–
Positive control	–	26,549 \pm 1933	+	+	+	+
5-Fluorouracil	10	2093 \pm 367	–	–	–	–
Positive control	–	16,467 \pm 1774	+	+	+	+
Taxol	20	23,633 \pm 2563	+	+	+	+
Positive control	–	20,219 \pm 471	+	+	+	+
Etoposide	20	21,668 \pm 602	+	+	+	+
Positive control	–	19,308 \pm 1821	+	+	+	+
Cisplatin	6	13,676 \pm 1515	+	+	+	–
Positive control	–	22,932 \pm 2074	+	+	+	+

^a All experiments were performed in triplicate.

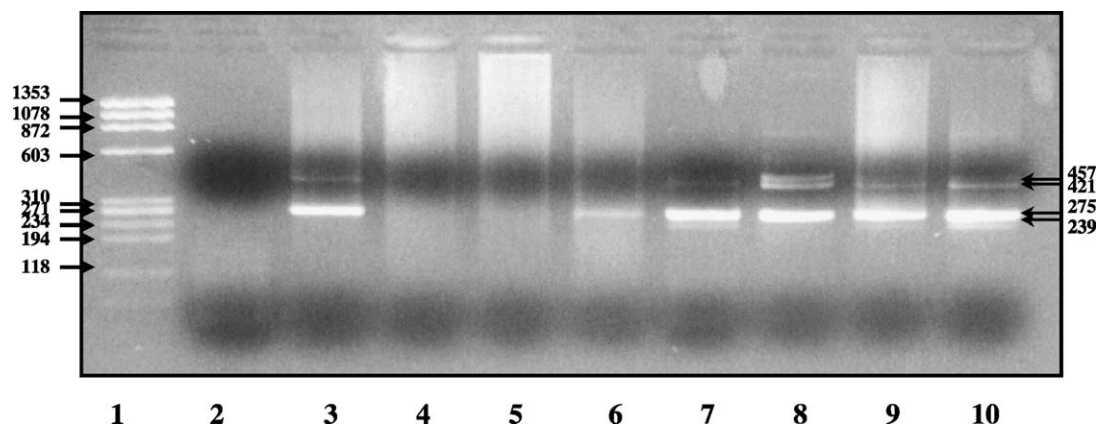


Fig. 2. hTERT splicing variants in MCF-7 cell line before and after treatment with antineoplastic agents. Lane 1: Φ X 174 DNA marker, lane 2: nested PCR negative control, lane 3: MCF-7 cell line (positive control), lane 4: doxorubicin (4 μ g/ml), lane 5: 5-fluorouracil (10 μ g/ml), lane 6: H7 (9 μ g/ml), lane 7: cisplatin (6 μ g/ml), lane 8: taxol (20 μ g/ml), lane 9: etoposide (20 μ g/ml), lane 10: MCF-7 cell line (positive control).

with 20 μ g/ml of taxol and those treated with 20 μ g/ml of etoposide were found positive for all hTERT splicing variants (α^+/β^+ , α^-/β^+ , α^-/β^- , α^+/β^-), while the expression of hTERT beta plus transcript as evaluated by the luminometric hybridization assay (RLUs) did not differ significantly before and after exposure to these drugs. MCF-7 cells treated with 4 μ g/ml of doxorubicin and those treated with 10 μ g/ml of 5-fluorouracil did not express any of hTERT splicing variants while the expression of hTERT beta plus transcript, as evaluated by the luminometric hybridization assay (RLUs). In the presence of cisplatin (6 μ g/ml), three splicing variants of hTERT were detected (α^+/β^+ , α^-/β^+ , α^+/β^-), while the expression of hTERT beta plus transcript as evaluated by the luminometric hybridization assay (RLUs) was lower after exposure to this drug. The characteristic hTERT splicing variants for these samples can be seen in Fig. 2.

Discussion

The observation that the presence of catalytically active telomerase is closely related to hTERT expression [9–13] has led to many studies on the expression of hTERT mRNA in a variety of clinical specimens [27]. Alternate splicing of hTERT is critical for the expression of catalytically active telomerase since only the α^+/β^+ , α^-/β^+ splicing variants encode for catalytically active hTERT protein [28,29]. These transcripts are expressed at very low concentrations in immortal human cells since they account for less than 6% of total hTERT mRNA [30]. So far, most studies on the expression of hTERT in clinical specimens are based mainly on the amplification of hTERT by using primers that amplify all hTERT splicing variants since they anneal onto a region common for all transcripts.

The hypothesis that telomerase may represent a suitable target for highly specific anticancer therapies has led to the preparation and evaluation of several classes of potent

telomerase inhibitors [14–16]. Many studies have also evaluated the effect of common antineoplastic agents on telomerase activity [17–24]. It has been stated that tumor resistance to chemotherapeutic agents can be monitored by following telomerase activity and that telomerase can be a novel marker of chemotherapy failure [17]. However, the effect of antineoplastic agents on the expression of hTERT beta plus splicing variant has not yet been studied.

In this study, we firstly developed a new method for the determination of hTERT beta plus transcript that is based on RT-PCR and luminometric hybridization assay. This method enables specific amplification of hTERT beta plus transcripts by using *in silico* designed primers and hybridization probes specific for the hTERT beta plus splicing variant. The proposed hybridization assay is not quantitative since it is based on luminometric detection of nested PCR products. By principle, nested PCR cannot be quantitative since first PCR products have already reached the PCR plateau phase before entering the second PCR. However, in this case, nested PCR was necessary to achieve the required sensitivity for hTERT beta plus splice variant since this is expressed at very low concentrations even in cancer cell lines [30]. High sensitivity is achieved by the combination of nested PCR, enzymatic amplification and the use of a sensitive chemiluminescent reaction for monitoring the enzymatic activity of alkaline phosphatase, which is used as an enzyme label. The proposed hybridization assay is performed in a simple way in microtiter wells with instrumentation commonly used for ELISA assays in many clinical laboratories. The method is more practical to perform than analysis based on gel electrophoresis since it allows simultaneous detection of large number of samples.

We have applied this methodology for the determination of the hTERT beta plus splicing variant in the MCF-7 breast cancer cell line before and after exposure to five commonly used antineoplastic agents. We observed a complete repression of hTERT expression in MCF-7 cells treated with IC50 doses of 5FU and doxorubicin. Our results agree with other

studies that showed a dramatic cytotoxicity and decrease of telomerase activity in MCF-7 cells treated with agents that arrest cells at S phase such as 5FU [21]. We did not observe a significant decrease of hTERT mRNA expression in MCF-7 cells treated with agents that damage DNA such as cisplatin. Similar results concerning telomerase activity were also reported for cisplatin [18,20,24]. We observed no reduction in the expression of hTERT beta plus transcript in the presence of agents that interfere with tubulin assembly such as taxol and Top II inhibitors such as etoposide. This is in accordance to that already reported for telomerase activity [18,24]. It would be interesting to check for telomerase activity all these samples. Unfortunately, this was not possible, because the amount of sample available was too small to perform two totally different sample extraction procedures, such as protein extraction for telomerase activity and total RNA extraction.

In conclusion, the developed hybridization assay is highly sensitive and specific for the detection of hTERT beta plus transcript in clinical samples.

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