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Expression Profile of the Putative Catalytic Subunit of the Telomerase Gene

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Abstract

Telomerase, a ribonucleoprotein complex, adds hexameric repeats called telomeres to the growing ends of chromosomal DNA. The enzyme telomerase activity is present in a vast majority of tumors but is repressed in most normal tissues. Recently, two groups have reported the molecular cloning of the putative catalytic subunit (hEST2/hTRT) of the telomerase gene. We investigated the expression of this gene in diverse tumor-derived cell lines and tumors as well as in various normal tissues. The expression of hEST2/hTRT was detectable in tumor-derived cell lines, primary breast tumors, pancreatic tumors, and kidney tumors. Furthermore, the expression of hEST2/hTRT was down-regulated in response to a differentiation inducer. However, several normal tissues also expressed varying levels of hEST2/hTRT. Early passage cultures of endothelial fibroblasts and some epithelial cells also expressed the telomerase gene, albeit at low levels. In contrast, the expression of TLP1/TP1, the human homologue of *Tetrahymena* p80 telomerase subunit, was similar in all of these samples. Our results indicate that the differences in expression of hEST2/hTRT in tumor *versus* normal cells are relative and are not absolute.

Introduction

Eukaryotic chromosomes are made up of an array of tandem repeats of the hexanucleotide 5′~~-TTAGGG-3′~~, called telomeres, which protect the ends of the chromosomes against numerous challenges (for a review, see Refs. 1–3). Putative functions of telomeres include protecting the ends of the chromosomes against exonucleases and ligases, preventing the activation of DNA damage check points, and countering the loss of terminal DNA segments that occurs when linear DNA is replicated (for a review, see Refs. 1–3). A ribonucleoprotein enzyme called telomerase is required to add the hexanucleotide to the ends of the chromosomes (1–3). Numerous published reports have documented the involvement of telomerase function in cellular senescence and acquisition of immortality in cancer cells (for a review, see Ref. 3). In human cells, telomerase activity is detected in fetal and adult testis and in ovarian follicles and in certain somatic tissues (4–10). Telomerase activity is inactivated or repressed in the majority of human somatic tissues (4); in contrast, in most tumors, telomerase activity is readily detectable (4–10). These findings suggest that telomerase is active in germ cells and in rare somatic cells with high proliferative potential.

The RNA component of the telomerase complex (TR) has been cloned from several species, including humans (11, 12). The expression of hTR, however, is not a predictor of telomerase activity, because hTR is expressed in all cells (13). Similarly a newly described gene, TLP1/TP1, which is presumably the human homologue of the *Tetrahymena* telomerase p80 gene, is also ubiquitously expressed (14, 15); thus none of these genes can be used as a prognostic indicator of transformation. In this context, a recently described gene, hEST2/hTRT, offers considerable promise. hEST2/hTRT is a human homologue of the yeast *Saccharomyces cerevisiae* gene EST2 that is the catalytic subunit of the yeast telomerase gene (16). The hEST2/hTRT gene has been postulated to be the catalytic subunit of the human telomerase gene. This gene was shown to be expressed in six of six tumor-derived cell lines but was absent in six of six primary fibroblasts. In addition, it was shown to be down-regulated in differentiated cells *in vitro* and up-regulated after immortalization (17, 18). These results are consistent with the regulation of telomerase activity in response to diverse stimuli (13, 19–21). However, the expression of hEST2/hTRT was detected in some telomerase-positive as well as telomerase-negative normal tissues (17), so at this point, it is not clear whether the expression of hEST2/hTRT is strictly correlatable with telomerase positivity.

To clarify whether hEST2/hTRT expression would be indicative of the transformed status of the cells and tissues, we investigated a panel of normal and tumor-derived cell lines and tissues by RT-PCR.[2] Our results show that although differences between normal and tumor cells exist in hEST2/hTRT expression, the differences are essentially relative, and most normal cells do express the hEST2/hTRT gene.

Materials and Methods

Cell Lines and Tissues. All of the tumor cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained as recommended. HL-60 cells were induced to differentiate with phorbol ester treatment as described previously (20). Telomerase activity was measured by the telomeric repeat amplification protocol as described previously (20, 22). Breast tumor RNAs were isolated at the Stiftung Tumorbank, Basel. These tumors were histopathologically characterized, and several molecular markers including estrogen receptor, progesterone receptor, cathepsin D, urokinase-type plasminogen activator, p53, DNA content, and S-phase content were analyzed. In addition, the complete clinical history of each tumor, including its differentiative status, size, lymph node status, and degree of invasiveness, was compiled.

The normal tissue RNAs were either from Clontech Laboratories, (Palo Alto, CA or from Strategene (La Jolla, CA). The early passage normal human cells were from Clonetics, Inc. (San Diego, CA) and were maintained as recommended. Pancreatic tumor cDNAs as well as normal colon and ovary cDNAs were from Strategene. Multiple tissue cDNA panels were from Clontech Laboratories.

RNA Isolation and RT-PCR. RNA was isolated from tissues and cell lines by RNAzol (Biotecx, Inc.), as described previously (22). PCR primers were as follows: (*a*) hEST2/hTRT, 5′-CGGAAGAGTGTCTGGAGCAA-3′ (sense) and 5′-GGATGAAGCGGAGTCTGGA-3′ (antisense); amplicon, 145 bp (18); (*b*) TLP1/TP1, 5′-TCAAGCCAAACCTGAATCTGAG-3′ (sense) and 5′-CCCGAGTGAATCTTTCTACGC-3′ (antisense); amplicon, 264 bp (15); and (*c*) GAPDH, 5′-CCATGGAGAAGGCTGGGG-3′ (sense) and 5′-CAAAGTTGTCATGGATGACC-3′ (antisense); amplicon, 208 bp (22). The cDNAs were synthesized from total RNAs using random hexamers as described previously (22) with 10 μCi of [^{32}P]dCTP and amplified using the PCR profile described by Nakamura *et al.* (18) The amplified products were separated by electrophoresis on either 3% agarose gel or 8% PAGE and detected by autoradiography. In some instances, the products were visualized

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[2] The abbreviations used are: RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

by staining the gel with either ethidium bromide or Sybergreen (Molecular Probes, Inc.). Due care was taken to prevent PCR carryover contamination including physical separation of the reaction area from the analysis area. Each RT-PCR was repeated three to four independent times using different preparations of RNA, and representative results are shown. Internal control GAPDH RT-PCR was done on all of the samples simultaneously. The RT-PCR products were confirmed at least once by DNA sequencing.

Results

hEST2/hTRT Expression in Tumor-derived Cell Lines and Tumor Tissues. Diverse human solid tumor-derived cell lines (n = 19) including carcinomas of the colon, breast, pancreas, prostate, and ovary (n = 3–5/organ type) were analyzed for the expression of hEST2/hTRT (Fig. 1*A*). All of these cell lines expressed similar levels of hEST2/hTRT. The TLP1/TP1 levels were also comparable in all of these samples except in MCF7 breast carcinoma and PC3 prostate carcinoma cell lines, in which TLP1/TP1 levels were barely detectable.

The expression of hEST2/hTRT was shown to be down-regulated by retinoic acid-induced differentiation of HL-60 cells (17). We and several others have recently demonstrated that *in vitro* differentiation of tumor and normal cells results in pronounced down-regulation of telomerase activity (20, 21). Taken together, these results suggest that down-regulation of the enzyme activity may result from repression of gene expression. Hence, we investigated the expression of hEST2/hTRT in response to TPA treatment of HL-60 cells (Fig. 1*B*). A pronounced down-regulation of hEST2/hTRT expression but not of TLP1/TP1 (p80 homologue) or of the internal control GAPDH gene was seen in the treated cells. In these cells, telomerase activity was significantly down-regulated (data not shown). These results corroborated the findings of Meyerson *et al.* (17).

We next investigated a panel of breast carcinomas (n = 22) for expression of hEST2/hTRT (Fig. 2*A*). The majority of these tumors were positive for hEST2/hTRT expression, but there were clear differences in the levels of hEST2/hTRT, despite the fact that the levels of TLP1/TP1 and GAPDH were similar in most of these samples. In addition to the breast tumors, three of three pancreatic tumors and three of three kidney tumors (Fig. 2*B*) were positive for hEST2/hTRT, and no differences in the levels of TLP1/TP1 were seen, as described above (Fig. 2*A*).

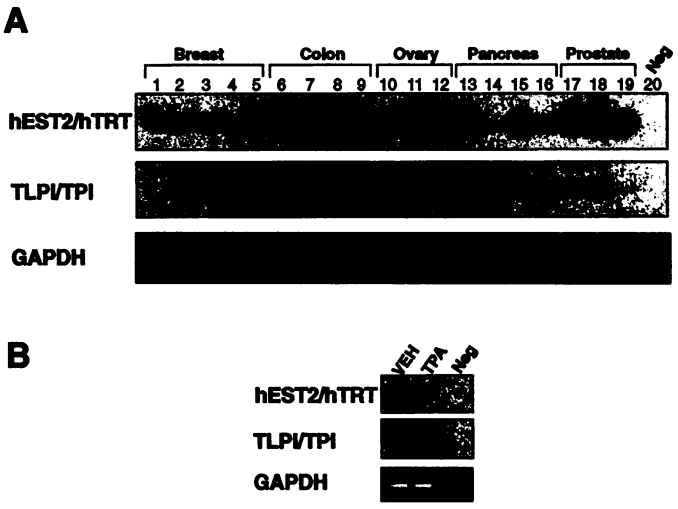

Fig. 1. hEST2/hTRT expression and regulation in tumor-derived cell lines. *A*, RNA from tumor-derived cell lines including breast carcinomas (*Lane 1*, MCF7; *Lane 2*, T47D; *Lane 3*, MDA435; *Lane 4*, ZR75; and *Lane 5*, MDA 231), colon carcinomas (*Lane 6*, SW 480; *Lane 7*, HCT 116; *Lane 8*, RKO; and *Lane 9*, SW 837), ovarian carcinomas (*Lane 10*, OVCAR; *Lane 11*, OV 1063; and *Lane 12*, MDA 2724), pancreatic carcinomas (*Lane 13*, HPAC; *Lane 14*, CAPAN 2; *Lane 15*, PANC 1; and *Lane 16*, BXPC3), and prostate carcinomas (*Lane 17*, LnCap; *Lane 18*, DU 145; and *Lane 19*, PC3) was analyzed by RT-PCR for hEST2/hTRT, TLP1/TP1, and GAPDH, and the products were visualized by autoradiography. *Lane 20*, *Neg*, template − negative control. *B*, RNA from HL-60 cells treated with vehicle (0.01% ethanol) or TPA (10 ng/ml) for 24 h. was analyzed by RT-PCR as in *A*. *Neg*, template − negative control.

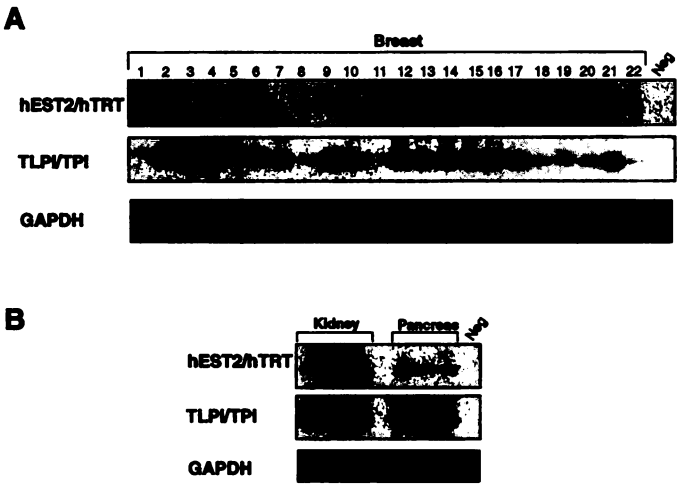

Fig. 2. hEST2/hTRT expression in primary tumors. RNA from primary breast tumors (n = 22), shown in *A*, or kidney tumors (n = 3) and pancreas tumors (n = 3), shown in *B*, was analyzed by RT-PCR for expression of hEST2/hTRT, TLP1/TP1, and GAPDH. *Neg*, template − negative control.

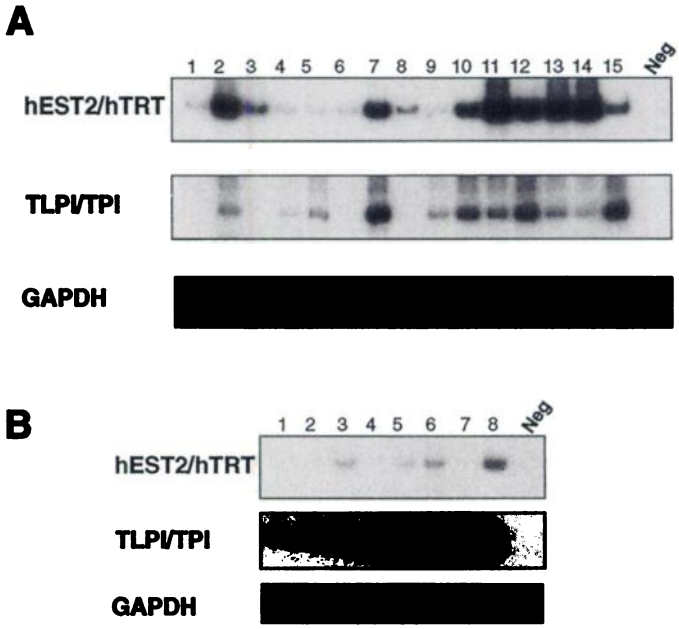

Fig. 3. Expression analysis of hEST2/hTRT in normal cells. *A*, RNA from normal human tissues. *Lanes 1–15* (brain, breast, colon, heart, kidney, liver, lung, ovary, pancreas, prostate, small intestine, spleen, stomach, testis, and uterus, respectively) were analyzed by RT-PCR for hEST2/hTRT, TLP1/TP1, and GAPDH expression. *Neg*, template − control. *B*, RNA from early passage human cell lines. *Lanes 1–8* (smooth muscle cells, dermal fibroblasts, WI38 fibroblasts, SV WI 38 virally transformed fibroblasts, prostate epithelial cells, renal epithelial cells, mammary epithelial cells, and vascular endothelial cells) were analyzed as described above. *Neg*, template − control.

Expression of hEST2/hTRT in Normal Human Tissues and Early Passage Primary Cells. Our observation of widespread hEST2/hTRT expression in a large number of tumor-derived cell lines and in representative primary tumor samples and its down-regulation in response to an inducer of differentiation corroborated the findings of Meyerson *et al.* (17). Encouraged by these findings, we next investigated the expression of hEST2/hTRT in diverse normal tissues (Fig. 3*A*). High levels of hEST2/hTRT expression were detected in the

spleen, testis, stomach, and small intestine. Surprisingly, all other organs (see Fig. 3*A*) also expressed readily detectable levels of hEST2/hTRT, albeit at varying levels. Some of the organs that showed positivity in our analysis (testis, intestine, and colon) had also been reported as positive for hEST2/hTRT expression by Meyerson *et al.* (17). In contrast, organs such as prostate, pancreas, heart, brain, and liver that showed weak positivity in our analysis were reported as negative in the study of Meyerson *et al.* (17). The expression of TLP1/TP1 in all of these tissues was similar, except that very little expression was detected in the brain, colon, lung, and ovary, consistent with earlier studies (14, 15). Independent cDNA preparations from normal human tissues (Clontech Multiple-Tissue cDNA; MTC panels) showed similar results (data not shown).

Telomerase activity and hence the expression of the putative telomerase genes in the tissues could result from lymphocytic contamination. To eliminate this possibility, we next investigated several early passage cultured human cells for the expression of hEST2/hTRT (Fig. 3*B*). All of these cells were negative for telomerase activity as monitored by the telomeric repeat amplification protocol assay,[3] with the exception of the human umbilical vascular endothelial cells (7). However, low levels of hEST2/hTRT expression were detected in the telomerase-positive human umbilical vascular endothelial cells as well as in telomerase-negative renal epithelial cells, prostate epithelial cells, and WI38 fibroblasts. In contrast, the smooth muscle cells, dermal fibroblasts, and mammary epithelial cells were completely negative for the expression of hEST2/hTRT. Similar to other cells in the study, no differences were seen in the levels of TLP1/TP1 in any of these samples. These results suggested that the detection of hEST2/hTRT expression in the tissues could not be attributable to the lymphocytic contamination and raise the possibility that certain normal cells may express the hEST2/hTRT gene, despite the absence of telomerase activity.

Discussion

Telomerase has attracted considerable interest as a possible target for therapeutic intervention of cancer. The apparent absence of measurable telomerase activity in many (but not all) normal tissues suggests that telomerase inhibitors might have a place in cancer therapy (3). However, despite a strong causal link between telomerase activity and cancer, a definitive proof of principle that inhibition of telomerase activity would effectively inhibit tumor growth has yet to be established.

The enzyme telomerase function is implicated in cellular senescence and immortalization of normal cells (1). One of the known roles of telomerase is maintenance of telomeric integrity. Telomerase activity is present in germ cells but is repressed in most somatic cells, and the mechanism of repression is not yet known. Major questions remain, such as whether the repression is at the level of the enzyme activity or at the gene expression level, *i.e.*, whether the activity of the enzyme might be physically blocked by a natural repressor, or the inactivation occurs at the expression level of one of the genes of the RNP complex, or, alternatively, whether the inactivation might be a result of some posttranslational modification of a component of the RNP complex.

Although some of the genes involved in the human telomerase RNP complex have been cloned, analysis to date indicates that their expression does not correlate with the telomerase activity. This includes the RNA component of the RNP complex, hTR, which is expressed in all of the cells irrespective of their immortalization status (13), and TLP1/TP1, the p80 homologue of the *Tetrahymena* telomerase gene, which is also ubiquitously expressed (14, 15). In this context, the recent identification of a putative telomerase gene termed hEST2/hTRT offers considerable promise for being a marker for immortalization (17, 18). This gene, hEST2/hTRT, shows homology to two related telomerase genes, EST2p (16), from a yeast known as *S. cerevisiae*, and p123 (23), from a ciliate, *Euplotes aediculatus*.

All three of these genes (hEST2/hTRT, EST2p, and p123) share considerable sequence homology, including the reverse transcriptase motifs (17, 18). hEST2/hTRT was shown to be expressed in tumor cell lines and in a limited number of tumors. In contrast, no expression was detected in normal fibroblast-derived mortal cell lines (18) or in some normal tissues (including the heart, brain, placenta, liver, skeletal muscle, breast, ovary, and prostate; Ref. 17). However, expression was detected in several other normal tissues, including the thymus, testis, colon, and intestine (17). Furthermore, virtually 100% of the tumor cell lines analyzed and a limited number of primary tumors were positive for hEST2/hTRT expression. In addition, Meyerson *et al.* (17) showed that the expression of hEST2/hTRT was down-regulated in a differentiated HL-60 promyelocytic cell line and up-regulated after *in vitro* immortalization of cultured cells. Based on these results to date regarding the expression profile, the sequence homology to yeast and ciliate catalytic subunit of telomerase, and regulation of gene expression, it has been inferred that the hEST2/hTRT gene is likely to be the catalytic subunit of the telomerase RNP complex.

Reasoning that a detailed expression profiling of the hEST2/hTRT gene in diverse tumor-derived cell lines and tumors would be informative, we analyzed a panel of tumor-derived cell lines, tumors, several normal human tissues, and cultured normal cells for the expression of hEST2/hTRT. As a control, we also included TLP1/TP1 (putative human homologue of *Tetrahymena* telomerase p80 subunit) in the analysis for gene expression profiling.

We used the RT-PCR method of analysis for the expression profiling following the conditions described by Nakamura *et al.* (18). Although generally corroborating the studies of both Nakamura *et al.* (18) and Meyerson *et al.* (17) with reference to the tumor cell lines, our findings differed with regard to the expression profiling of hEST2/hTRT in normal tissues. Several normal tissues (heart, brain, liver, prostate, pancreas, and ovary) that had been found to be negative by the RNase protection assay of Meyerson *et al.* (17) were positive in our method of detection by RT-PCR. Our conditions were identical to those of Nakamura *et al.* (18). Regardless of the final method of detection of the PCR-amplified products (^{32}P incorporation or Sybergreen), we detected the hEST2/hTRT gene product in these normal tissues. Using the conditions of Nakamura *et al.* (18), we also detected the expression in certain mortal cell types (endothelial, fibroblast, and epithelial), but some cell types were clearly negative. Use of nonoverlapping sets of PCR primers for hEST2/hTRT gave identical results (data not shown).

Our results of down-regulation of hEST2/hTRT expression in response to TPA treatment of HL-60 cells corroborate those of Meyerson *et al.* (17). Thus, two different inducers of differentiation [retinoic acid in Meyerson *et al.* (17) and TPA in this study] caused the down-regulation of hEST2/hTRT expression. Whether the down-regulation of hEST2/hTRT expression is a general consequence of differentiation induction and parallels the down-regulation of telomerase activity seen in diverse cell types (20, 21) remains to be clarified.

In contrast to hEST2/hTRT expression, the expression of TLP1/TP1, which is presumably the other subunit of telomerase, was not affected by the TPA treatment. Thus, all of the evidence to date indicates that this subunit is unlikely to be rate limiting for enzyme activity. It is unclear whether these two are the only subunits needed

[3] R. Hsiao and R. Narayanan, unpublished observations.

for the enzyme (telomerase) activity, which awaits the reconstitution experiments.

Our study raises several issues that need to be clarified before a clear link between the hEST2/hTRT expression and transformed status of the cell can be established. For example, what is the functional consequence of the varying levels of hEST2/hTRT expression seen in both normal and tumor tissues? Can the low levels of expression seen in some tissues be due to contamination of lymphocytes, or, alternatively, can these low levels be due to the fact that certain cell types within these tissues express the hEST2/hTRT gene? It is unlikely that the low levels are due to carryover contamination of the RT-PCR reactions, because we also detected the expression in some (but not all) of the early passage-cultured human cells. By the same token, can the same definition of normality that is used for analysis of gene expression profiling with other genes be used for analysis of telomerase-related genes? Does the RNA expression correspond to the protein expression/activity? A recent study, for example, demonstrated that despite the ubiquitous RNA expression of a gene called CYP1B1 (a cytochrome P-450 gene), the protein expression was seen in a tumor-specific manner (24). While this study was in progress, Killian *et al.* (25) cloned the hEST2/hTRT gene (hTCS1) from a human colon carcinoma cell line. Using RT-PCR-based expression profiling, these authors showed a complex, alternative splice-based expression in diverse tissue types; expression was detected in certain normal tissues including colon, testis, and peripheral blood lymphocytes. Consistent with our findings, some of the tumors analyzed by Killian *et al.* (25) were clearly negative for the expression of this gene.

Clearly, future experiments based on *in situ* expression of the RNA and protein as well as knockout of hEST2/hTRT gene expression will help to clarify the prognostic potential of the hEST2/hTRT gene in cancers. Our results indicate, however, that the differences in expression of the hEST2/hTRT gene in tumors *versus* normal cells are likely to be relative and not ± differences. A simultaneous measurement of telomerase activity and hEST2/hTRT expression at the RNA and protein levels in a large number of samples is needed before correlation with the transformed status of the cell.

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