Frequent Amplification of the Telomerase Reverse Transcriptase Gene in Human Tumors

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Abstract

Activation of telomerase is a crucial step during cellular immortalization and malignant transformation of human cells and requires the induction of the catalytic component, human telomerase reverse transcriptase (hTERT), encoded by the hTERT gene. It is poorly understood how the hTERT gene is activated in human cancer cells. In the present study, we examined the hTERT gene copy number in human cancer cell lines and in primary tumor tissues. Amplification of the hTERT gene was observed in 8 of 26 (31%) tumor cell lines and in 17 of 58 (30%) primary tumors examined (8 of 21 lung tumors, 3 of 10 cervical tumors, 5 of 19 breast carcinomas, and 1 of 8 neuroblastomas). In addition, 13 of 26 (50%) cell lines and 13 of 58 (22%) primary tumors displayed gain of hTERT gene copies with 3–4 copies/cell. The present findings imply that the genetic event probably contributes to the dysregulation of telomerase activity occurring in human tumors.

Introduction

Telomerase is a ribonucleoprotein DNA polymerase that synthesizes TTAGGG telomeric repeat sequences essential for genomic stability (for reviews, see Refs. 1 and 2). In humans, telomerase activity is widespread in many tissues throughout fetal development but becomes repressed in most somatic cells before or shortly after birth. Without telomerase activity, somatic cells exhibit progressive shortening loss of telomeric sequences with each cell division because of “the end replication problem” and eventually enter senescence when a critical length of telomeres is reached. It is believed that such telomere shortening acts as a mitotic clock that records the replicative history and sets a finite life span for normal somatic cells (1, 2). In contrast, malignant cells are capable of escaping the senescent checkpoint and undergoing unlimited proliferation. Compelling evidence has accumulated that the maintenance of telomere length is required to acquire replicative immortality and that the activation of telomerase is the most common mechanism through which cancer cells stabilize their telomere size and subsequently sustain their infinite growth (1, 2). Indeed, activation of telomerase is widely observed in human malignancies (3), and the malignant transformation of normal human cells has been shown to be achieved by reconstitution of telomerase activity (8–11). Recent attention has therefore been focused on the regulatory mechanisms of hTERT expression, presumably at the transcriptional level. Because gain or loss of genetic material occurs frequently in human malignancies, and gene amplification is one important mechanism for oncogene activation, we investigated the possibility of amplification of the hTERT gene and the potential influence of the hTERT gene alteration on its expression in human tumors.

Materials and Methods

Cell Lines and Cell Culture. The human tumor cell lines used in this study and their origins are listed in Table 1. The cell lines were maintained in either RPMI 1640 or Iscove’s Dulbecco’s medium (Life Technologies, Inc., Paisley, United Kingdom) containing 10% FCS, 2 mM L-glutamine, and 100 units/ml penicillin.

Tumor Specimens. The primary tumors that we examined included 24 lung, 10 cervical, and 19 breast carcinomas and 8 neuroblastomas. Of patients with lung cancers, 13 had NSCLC, and 11 had SCLC. Fifteen of those patients had local lymph node metastases. Nine of 10 cervical carcinomas were invasive and squamous (1 well, 6 moderately, and 2 poorly differentiated, respectively), and one was an in situ adenocarcinoma. Of breast carcinomas, 11 were poorly differentiated, and the remainder were moderately differentiated. Axillary lymph node metastases were found in nine of the patients. All of the breast tumors had been analyzed previously for c-myc, MDM2, and C-erbB oncogene amplification and p53 protein expression. Two of eight neuroblastomas exhibited N-myc amplification.

FISH. The specific hTERT gene probe, isolated from the PAC clone RPCI-6 135 M06 (Pieter de Jo; Roswell Park Cancer Institute, Buffalo, NY), was directly labeled with Spectrum Orange fluorophore-conjugated dUTP using standard nick translation. The marker probe 685A18 (PAC library RPCI-4; Pieter de Jo; Roswell Park Cancer Institute), located on chromosome band 5q31, was labeled with Spectrum Green-dUTP (Vysis Inc., Downers Grove, IL). The labeled probes were then coprecipitated using ethanol/sodium acetate together with Cot-1 (Life Technologies Inc., Grand Island, NY) and carrier DNA. FISH was performed on slides prepared by cytospin (cell lines), imprint touch preparation (breast carcinomas), or 4 μm formalin-fixed, paraffin-embedded tumor sections (lung and cervix). After a paraformaldehyde fixation (cell line and breast carcinsomas) or deparaffinization and a mild digestion with pepsin (lung and cervical cancers), the slides were rinsed in 2× standard SSC, dehydrated in graded ethanol, and air dried. Ten μl of hybridization

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The abbreviations used are: hTERT, human telomerase reverse transcriptase; β2-M, β2-microglobulin; DAPl, 4′,6-diamidino-2-phenylindole; dms, double minutes; FISH, fluorescence in situ hybridization; NSCLC, non-small cell lung carcinoma; RT, reverse transcription; SCLC, small cell lung carcinoma; SKY, spectral karyotyping.
petitive PCR for hTERT mRNA was performed as described previously (12).

RNA Extraction, RT, and Competitive PCR. Total cellular RNA was extracted using the ULTRASPEC-II RNA kit (Biotecx Laboratory, Uppsala, Sweden) and Moloney murine leukemia virus reverse transcriptase. The cDNA was synthesized using random primers (N6; Pharmacia, Uppsala, Sweden). cDNA was synthesized using random primers (N6; Pharmacia, Uppsala, Sweden). cDNA was synthesized using random primers (N6; Pharmacia, Uppsala, Sweden). cDNA was synthesized using random primers (N6; Pharmacia, Uppsala, Sweden). cDNA was synthesized using random primers (N6; Pharmacia, Uppsala, Sweden).

Table 1 The hTERT copy number in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>hTERT copies/nucleus</th>
<th>hTERT:5q31 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>Lung (NSCLC)</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>H23</td>
<td>Lung (NSCLC)</td>
<td>4</td>
<td>1.4</td>
</tr>
<tr>
<td>H125</td>
<td>Lung (NSCLC)</td>
<td>7</td>
<td>3.5</td>
</tr>
<tr>
<td>H157</td>
<td>Lung (NSCLC)</td>
<td>4</td>
<td>1.0</td>
</tr>
<tr>
<td>U1752</td>
<td>Lung (NSCLC)</td>
<td>8</td>
<td>2.0</td>
</tr>
<tr>
<td>H69</td>
<td>Lung (NSCLC)</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>U12285</td>
<td>Lung (NSCLC)</td>
<td>6</td>
<td>3.0</td>
</tr>
<tr>
<td>U2020</td>
<td>Lung (NSCLC)</td>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>578T</td>
<td>Breast</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td>MDA</td>
<td>Breast</td>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>Breast</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>T-47D</td>
<td>Breast</td>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td>HL60</td>
<td>Leukemia</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>K562</td>
<td>Leukemia</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>H9</td>
<td>Lymphoma</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>U266</td>
<td>Myeloma</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>RPMI</td>
<td>Myeloma</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix</td>
<td>5</td>
<td>1.2</td>
</tr>
<tr>
<td>Saos-2</td>
<td>Osteosarcoma</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>Lan1</td>
<td>Neuroblastoma</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>Lan2</td>
<td>Neuroblastoma</td>
<td>&gt;40</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Lan5</td>
<td>Neuroblastoma</td>
<td>8</td>
<td>1.3</td>
</tr>
<tr>
<td>SK-N-AS</td>
<td>Neuroblastoma</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>SK-N-BE</td>
<td>Neuroblastoma</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>SHEP1</td>
<td>Neuroblastoma</td>
<td>5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

mixture containing 20 ng of each probe, 55% formamide, 10% dextran, and 2× SSC were applied to each slide. The slides were sealed with coverslips and denatured with the probe mixture simultaneously. The hybridization was performed at 37°C overnight (14–16 h) in a humidified chamber. The coverslips were then removed, and the slides were washed twice in 2× SSC for 5 min at 72°C, dehydrated in graded ethanol, air dried, and mounted in anti-fade solution containing DAPI (Vector Laboratories Inc., Burlingame, CA). The samples were evaluated under a Zeiss epifluorescence microscope equipped with the corresponding wavelength filter, charge-coupled device camera, and an image capturing and analyzing system. Signals of both hTERT and the 5q marker were counted from at least 100 nonoverlapping nuclei. Amplification was defined as ≥5 hTERT gene copies/nucleus in at least 40% (cell lines) or 20% (primary tumors) of the cells. hTERT was performed in the same manner as a control on normal human lymphocytes in both interphase and metaphase.

SKY. SKY combined with FISH for the hTERT gene was carried out on the neuroblastoma cell line Lan2 and the lung cancer cell line U1752. The SKY probe mixture and hybridization reagents were prepared by Applied Spectral Imaging (Migdal Ha’Emek, Israel). Before denaturation of the SKY probe, 50 ng of Spectrum Orange-labeled hTERT probe were added into the mixture. The hybridization and detection were performed according to the manufacturer’s protocol with the probe mixture hybridized to the slide for 2 days at 37°C. Metaphase cells were captured using the SD200 spectral imaging system Spectral Cube (Applied Spectral Imaging) mounted on a Zeiss Axioskop II fluorescence microscope with a triple bandpass optical filter (Chroma Technology) for red, green, and blue. DAPI images were acquired using a DAPI filter. Eighteen metaphases were analyzed using the SKY view software (Applied Spectral Imaging). The DAPI display was inverted by the SkyView software to produce a transformation to G-band-like patterns. The location of the hTERT probe could be detected in all metaphases. Because Spectrum Orange has an emission wavelength of 590 nm, the hTERT locus is detected as green and white signals by the spectral colors and the classified colors, respectively.

Results

The hTERT Gene in Normal Human Lymphocytes. The hTERT gene has previously been mapped to 5p15.33 (8). In the present study, we isolated a PAC clone that covered the genomic region encoding hTERT. Using this sequence as a hTERT probe, we performed FISH analysis on metaphase cells derived from normal human lymphocytes. As shown in Fig. 1, the normal metaphases displayed two hTERT gene copies (red signals) located on band 5p15.33. An identical result was obtained from analysis of normal human fibroblasts (data not shown).

Because the FISH probe for the centromere of chromosome 5 cross-hybridizes with the centromere of chromosome 19 (data not shown), we chose a specific sequence at 5q31 as a marker probe (Fig. 1, green signals).
Fig. 2. SKY combined with FISH for the hTERT gene on the neuroblastoma cell line Lan2 and lung cancer cell line U1752. A, a–c show the same metaphase. a, hTERT gene amplification on dms in Lan2 cells is detected by the spectral colors (green granules) and (b) visible by the inverted DAPI display (gray granules). c, this metaphase is tetraploid and has two normal chromosomes 5, two derivative chromosomes 5 containing chromosome 2 material, and more than 50 dms carrying the hTERT gene. B, a–d show the same metaphase. The U1752 cell line contains several chromosomal aberrations visualized by the inverted DAPI display (a) and characterized in detail by the spectral colors (b) and by the classified colors (c), d, this metaphase has two normal chromosomes 5, one chromosome 5 with a deletion of 5p distal to the hTERT gene, and three marker chromosomes carrying chromosome 5p material containing the hTERT gene.

signals). A 1:1 ratio of the hTERT marker was obtained in normal cells when the 5q31 probe was used as a marker for chromosome 5.

Amplification of the hTERT Gene in Human Tumor Cell Lines.

We then determined the hTERT gene copy number in human tumor cell lines. A total of 26 cell lines representing various origins were examined (Table 1). Five cell lines (19%) carried two hTERT and two 5q31 marker gene copies, respectively, whereas hTERT gene amplification was identified in 8 (31%) cell lines derived from carcinomas of the lung (H125, U1285, and U1752), breast (578T), and cervix (HeLa) and neuroblastomas (Lan2, Lan5, and SHEP1). The hTERT copy number in these cells varied between 5 and >50 per cell. We found a significant heterogeneity among cells from the same cell lines.

To characterize hTERT gene amplification in further detail, combined FISH/SKY analysis was performed on metaphases from the neuroblastoma cell line Lan2 and lung cancer cell line U1752. Interestingly, the hTERT signals came predominantly from dms, and more than 50 hTERT signal-containing dms were seen in the Lan2 cell (Fig. 2A). The FISH analysis on metaphases of Lan2 cells showed an identical result (data not shown). Unlike Lan2 cells, U1752 cells did not exhibit dms amplification of the hTERT locus. As shown in Fig. 2B, U1752 cells acquired a number of derivative chromosomes containing 5p material from which the hTERT signals were derived.

In addition to the hTERT gene amplification observed in 8 tumor cell lines, we found that 13 of 26 cell lines (50%) had 3–4 hTERT signals/cell with a hTERT:5q31 marker ratio of ~2, which was considered to be a low grade of amplification. Six of them exhibited an equal increase in both the hTERT and 5q31 marker signals and thus kept a 1:1 ratio of the hTERT:5q31 marker, indicating chromosome 5 aneusomy. In the remaining cell lines, the increased number of hTERT signals was slightly or moderately higher than that of the 5q31 marker signals, resulting in a hTERT:5q31 signal ratio of >1 but <2, likely due to the acquisition of extra 5p material.

To determine whether the amplification of the hTERT gene affects the expression of hTERT and telomerase activity, we examined the levels of hTERT mRNA and telomerase activity in the tumor cell lines using competitive RT-PCR and a telomerase PCR ELISA kit, respectively. hTERT expression was well compatible with telomerase activity in those cell lines (data not shown). No correlation was found between the hTERT expression or telomerase activity and gene copy number when all cell lines were compared as one group. However, in
subgroups of the cell lines such as neuroblastomas, the highly hTERT gene-amplified cell line Lan2 expressed 5-fold greater hTERT mRNA and 3-fold higher telomerase activity than SK-N-AS and SK-N-BE cells carrying only three hTERT copies (Fig. 3, A–C). These cell lines were further analyzed for the levels of hTERT protein using immunohistochemistry. Compared with SK-N-AS and SK-N-BE cells, Lan2 and Lan5 cells displayed a much stronger nuclear hTERT staining (Fig. 3D). A positive association of hTERT expression/telomerase activity with the hTERT copy number was similarly observed in lung cancer cell lines (data not shown). Interestingly, hTERT expression and telomerase activity were undetectable in the osteosarcoma cell line Saos-2 that exhibits a normal number of hTERT signals (Fig. 3).

**Table 2** hTERT gene amplification in human primary tumors

<table>
<thead>
<tr>
<th>Tumors</th>
<th>Normal</th>
<th>Amplified</th>
<th>Low-grade amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTERT gene copies</td>
<td>2–4</td>
<td>5–20</td>
<td>3–4</td>
</tr>
<tr>
<td>Lung n = 21</td>
<td>10</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Breast n = 19</td>
<td>5</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Cervix n = 10</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Neuroblastoma n = 8</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total n (%)</td>
<td>28 (48)</td>
<td>17 (30)</td>
<td>13 (22)</td>
</tr>
</tbody>
</table>

Normal in hTERT gene is defined as two hTERT and 5q31 signals per nucleus with a 1:1 ratio of hTERT:5q31 in more than 80% tumor cells. Amplification of the hTERT gene is defined as ≥5 hTERT signals per nucleus in more than 20% of tumor cells. Low-grade amplification is defined as 3–4 hTERT signals per nucleus.
hTERT amplification in human tumors

...gene copies (between 3 and 4), accompanied by an increased number of 5q31 marker signals (a ratio of the hTERT:marker signals between 1 and <2). Moreover, the heterogeneity of the hTERT signals was readily seen in most of the primary tumors with hTERT gene amplification.

The effect of hTERT gene amplification on its protein expression in primary tumors was further evaluated. hTERT immunohistochemical staining was performed on imprints of frozen breast tumor tissues and paraffin-embedded tumor sections from lung and cervical carcinomas, respectively. In breast carcinomas, 4 of 5 tumors carrying a normal number of hTERT signals exhibited a weak hTERT protein staining (≤1+), whereas a moderate or strong expression of the hTERT protein (≥2+) was found in 8 of 11 specimens with an aberrant number of hTERT signals. In contrast, the same pattern could not be identified in lung and cervical carcinomas.

We wanted to further determine the relationship between hTERT amplification and clinicopathological variables in primary tumors. In lung carcinomas, the mean size (diameter) of tumors with hTERT gene amplification was greater than those carrying normal hTERT signals (5.6 ± 3.0 versus 3.8 ± 1.0 cm, mean ± SD), but the difference was not statistically significant (P = 0.064, Mann-Whitney U test). Gene amplification was found in both SCLC and NSCLC. In the breast and cervical carcinomas, alteration of the hTERT gene was not related to tumor size, differentiation status, axillary lymph node metastases, and other oncogene amplification (c-myc, MDM2, and C-erb2). However, 7 of 14 breast cancer samples with the hTERT alteration displayed overexpression of the tumor suppressor p53 protein, whereas 5 tumors with normal hTERT signals were all negative for p53.

Discussion

The stabilization of telomeres through activation of telomerase is the most common mechanism for cancer cells to escape the senescence checkpoint and achieve replicative immortality (1, 2). Because telomerase activity is controlled predominantly by the expression of its rate-limiting component, hTERT, elucidation of the regulatory mechanisms of hTERT expression is critical to the understanding of telomerase activation in human tumors. In the present study, hTERT gene copy number in human malignancies was examined. We identified that hTERT gene amplification occurred in more than 30% of human malignant cells including both in vitro cultured tumor cell lines and four kinds of primary solid tumors. Typically, the hTERT gene is amplified in dms, paired, and spherical chromatin bodies without a centromere. In addition, a substantial fraction of tumors acquired extra hTERT gene copies or a low grade of amplification due to aneusomy 5 and/or gains of 5p. The data suggest that the hTERT gene may be a frequent target for amplification in the formation and development of human cancers.

It has long been debated whether the expression of telomerase activity in human tumors is due to reactivation after the genetic alteration, or whether targets for human carcinogenesis are rare telomerase-positive stem cells that selectively expand during tumor progression (14, 15). The present finding provides evidence that the genetic events such as hTERT amplification may be an important driving force to activate telomerase in at least certain types of cancers. The in vitro model of cellular transformation will allow us to vigorously test this hypothesis. The formation of dms is readily observed in in vitro transformed cells at precrisis and crisis just before telomerase is activated (16). It will be interesting to examine whether the dms produced in these transformed cells contain the amplicons of the hTERT locus, or whether any other forms of hTERT amplification are present in these cells.

In many tumors, the inhibitory mechanisms of hTERT expression are defective due to either acquisition of positively acting transcription factors or loss of active repressors of hTERT transcription. Increased hTERT gene copies in those tumors are very likely to contribute to the up-regulation of hTERT expression. In tumor cell lines derived from neuroblastomas, the levels of hTERT expression and telomerase activity were several-fold higher in the hTERT-amplified Lan2 cells than in the cell lines without the gene amplification. Similarly, high levels of hTERT expression were closely associated with hTERT gene amplification in breast cancers. These results suggest that hTERT amplification plays a role in the up-regulation of hTERT expression. However, this pattern could not be seen in lung and cervical carcinomas. One possible explanation might be the intrinsic problems with immunohistochemical analysis performed on the paraffin-embedded tissues. It has been demonstrated that staining of paraffin-embedded sections is significantly affected by antigen loss, fixation and processing, etc, which is exemplified by the HER/2neu oncogene frequently amplified in breast and other carcinomas. A number of observations have shown a lack of strict correlation between HER/2neu amplification as determined by FISH and its expression when paraffin-embedded tumor tissues were used for the measurement of HER/2neu protein levels (17). Therefore, caution should be taken when evaluating the protein expression as determined on paraffin-embedded tumor sections. On the other hand, the regulatory mechanisms of hTERT expression may operate at multiple levels. For example, c-myc oncogene and estrogens are capable of transcription-
ally activating hTERT expression (18–21). Because each tumor cell line or primary tumor has different genetic characteristics (such as myc amplification), it is not surprising that hTERT amplification is not strictly related to the levels of hTERT expression.

Based on the results obtained from a limited number of tumor specimens, we found no clear association of the hTERT amplification with clinicopathological characteristics. We are aware that these preliminary data are unlikely to be conclusive. For instance, the present data show that the hTERT gene amplification seems to occur independently of myc amplification and differentiation status in breast carcinomas. However, both myc expression and cellular differentiation have been implicated in the regulation of hTERT expression/telomerase activity (12, 13, 18–20). Thus, additional studies recruiting larger cohorts of cancer patients are required to ascertain a relationship between the hTERT amplification and clinical/biological variables. Interestingly, all of the five breast tumors that carried a normal number of hTERT signals were negative for p53 protein staining, whereas 7 of 14 specimens with extra copies of the hTERT locus overexpressed p53, suggesting a strong association of hTERT alteration with overexpression of the p53 tumor suppressor protein in breast cancers. Roos et al. (22) previously showed that high levels of telomerase activity were more frequently observed in the breast cancers with p53 overexpression. Taken together, the increase in hTERT copy number might be an important factor that up-regulates the telomerase activity observed in breast cancers overexpressing p53.

In summary, the present study reveals a high frequency of hTERT gene amplification in human tumors, which indicates that the hTERT gene may be a target for amplification during the transformation of human malignancies and that this genetic event probably contributes to a dysregulation of hTERT/telomerase occurring in a subset of human tumors.

References

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