Catalytic subunit of telomerase expression is related to RNA component expression

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Abstract Telomere length is maintained by the ribonucleoprotein enzyme telomerase. The RNA component of telomerase (hTR) is widespread, and only the expression of the mRNA encoding the catalytic protein subunit (hTRT) is correlated with telomerase activity. We have studied the level of expression of hTR and hTRT in four different models of neoplastic and preneoplastic lesions using the RT-PCR method on RNA extracted from paraffin-embedded human tissues after microdissection. The expression at the mRNA level was compared with the enzymatic activity. Our results suggest that there may be a reciprocal control at the transcriptional level of the expression of hTRT and hTR which in turn is associated with tumor progression.

Key words: Telomerase; Cancer; Preneoplastic lesion; Reverse transcription polymerase chain reaction; Paraffin-embedded tissue

1. Introduction

Telomerase is a ribonucleoprotein detected in immortalized cells and in most tumors studied [1]. Its activity seems to be associated with tumor progression, but it is also possible to find telomerase-positive cells in normally regenerating human tissues [2]. These facts suggest that telomerase is connected with proliferation and plays a permissive rather than an initiating role in cancer.

The control of telomerase expression and activity is still unclear. There are many suggestions that could explain this control, such as alternative splicing of the enzyme gene transcripts [3] or relationship with the expression of retinoblastoma or myc genes [4–6]. Another possible control could be connected directly with the ribonucleoprotein components.

The relationship between the cellular levels of the components of the ribonucleoprotein is not clear. The RNA component presents a widespread expression [7,8], whilst the catalytic component shows a restricted pattern of expression, related to the enzyme activity [9]. A multistage tumorigenesis study in transgenic mice has shown that the RNA component of telomerase is up-regulated in the first stages of tumorigenesis, even in precancerous lesions, but the telomerase enzymatic activity is detectable only in the later stages of tumor progression [10]. This fact suggests that a down-regulation of the RNA component of the ribonucleoprotein is present only at the appearance of the enzymatic activity.

The RNA component (hTR) and the catalytic component (hTRT) of human telomerase have been sequenced [7,9] and we have analyzed their expression at the RNA level in human tumors and precancerous lesions using RNA extracted from paraffin-embedded tissues. The aim of this study was to show in human models the association between the expression of the telomerase RNA component and the protein catalytic component.

2. Materials and methods

2.1. Tissues

Fresh surgical homogeneous tissues fragments were divided into two parts. One half was paraffin-embedded for RT-PCR analysis and the other was frozen and stored at −80°C for the telomeric repeat amplification protocol (TRAP) assay. We collected 10 samples: three hyperplastic lymph nodes, two lymph nodes with metastases, a metastatic lymph node with extensive necrosis, two breast carcinomas, a breast cancer local recurrence and a urinary bladder wall with severe neutrophil infiltration and edema. Frozen tissues were powdered in liquid nitrogen. Total RNA from paraffin-embedded human tissues with cancer of liver, brain and breast was extracted as already reported [11] or using Total Quik RNA (Talent, Italy). The use of paraffin-embedded tissues allowed a correct identification and localization of the lesions; the tissues were microdissected and only the selected cells were subjected to RNA extraction. We analyzed 14 cases of primary carcinoma of the liver and 16 cases of glioma of the brain. RNA was also extracted from 28 breast carcinomas and 18 cases of precancerous (CIN2) and nine cases of in situ carcinomas of the cervix uteri.

2.2. Northern analyses

Northern blot analyses were carried out on total RNA extracted from HeLa cells as a positive control, a renal tumor, a breast cancer and a mammary tissue. Northern blot analysis and probe preparation were carried out according to standard methods [12]. Total RNA for each sample (10 μg) was fractionated on a 1% formaldehyde agarose gel and blotted on a positively charged nylon membrane. The membranes were tested with ^{32}P-labeled hTR and hTRT amplicons (using the oligonucleotides reported in Section 2.4) purified from low-melting agarose gel. For the same specimens also RT-PCR for hTR and hTRT and TRAP assay on protein lysates was performed.

2.3. Trap assay

Extracts of tissue proteins were obtained incubating the powder in ice-cold lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol) for 30 min. Telomerase activity was assayed by a modified TRAP method to avoid direct primers interactions [13], using 10 μg of protein lysate for each sample. The PCR products were analyzed on a 15% non-denaturing polyacryl-

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Abbreviations: hTR, RNA component of human telomerase; hTRT, mRNA of the catalytic subunit of human telomerase; RT-PCR, reverse transcription PCR; TRAP, telomeric repeat amplification protocol; CIN, cervix intraepithelial neoplasia

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amide gel. The level of radioactivity of each lane of the gel was measured using a computerized phospho imaging system (Istant Imager, Packard Instrument Company).

2.4. RNA analysis

For the hTR and hTRT analysis a DNase digestion of RNA was performed to avoid genomic DNA contamination. For RT-PCR analysis were used specific oligonucleotides [7] (hTR1: 5′-TAGGCGCCGTGCTTTTGCT-3′; hTR3: 5′-GCTCTAGAATGAACGGTG-3′) with the amplification of a 99 base fragment for the RNA component and for the catalytic component of the telomerase [9] (hTRT1: 5′-AGCCAGTCTCACCTTCAA-3′; hTRT3: 5′- ACAGGCTGTGACACTTCA-3′). For hTRT the amplicon was 92 bases long. The dot blot of the amplified material was hybridized with an internal probe (hTR2: 5′-CGCTGTTTTTCTCGCTGACTTTCAGCG-3′) for RNA component and (hTRT2: 5′-GGAACATGCGTCGCAAACTCTTTG-3′) for mRNA of the catalytic component. The results were analyzed by a computerized phosphor imaging system. Relative quantification of the specific RNA was performed, after standardization of the RNA degradation level on the β-actin expression levels, with initial RNA quantity and amplification conditions consistent with a linear relationship between the log of the target RNA and the log of the amplified product [14,15]. These conditions were satisfied with 45 ng of total RNA per test and with five cycles of amplification of 95°C/1 min, 55°C/1 min, 72°C/1 min and 40 cycles of 95°C/30 s, 55°C/30 s and 72°C/30 s for the RNA component. For the catalytic component the conditions were 250 ng of total RNA and five cycles of amplification of 95°C/1 min, 55°C/1 min, 72°C/1 min and 30 cycles of 95°C/30 s, 55°C/30 s and 72°C/30 s.

3. Results and discussion

The limited amount of RNA of reasonable molecular weight that can be obtained from paraffin-embedded tissue sample makes RT-PCR the only methodology capable of measuring hTR and hTRT mRNA. To validate this technique and establish its correlation with enzymatic activity we compared in three fresh human tissue specimens (a renal cancer, a breast cancer and a normal mammary tissue) the RT-PCR-based method with the TRAP assay and Northern blot. For every one of the three specimens we found a positive qualitative correlation for both the telomerase components in Northern blot and RT-PCR with enzyme activity (Fig. 1).

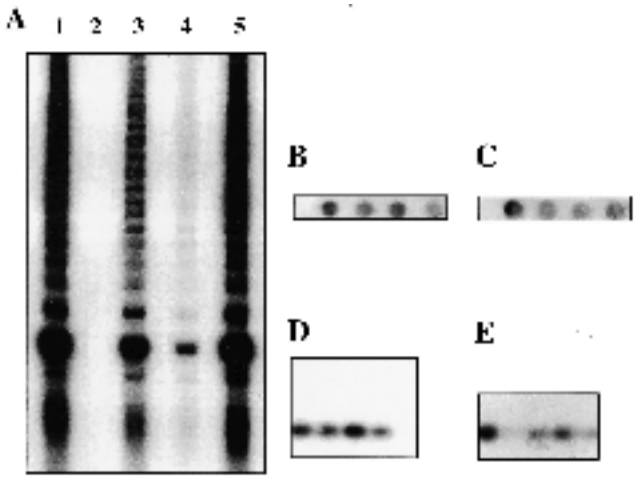

Fig. 1. A: TRAP assay for telomerase activity (lanes 1, 2: positive and negative control; lane 3: renal cancer; lane 4: mammary gland; lane 5: breast carcinoma). B: Dot-blot relative of hTR expression (negative control, positive control, renal tumor, breast carcinoma and mammary gland). C: Dot-blot relative of hTRT expression (negative and positive controls, renal tumor, mammary tissue and breast carcinoma). D: Northern blot relative of hTR (positive control, renal tumor, breast cancer and mammary gland, negative control). E: Northern blot relative of hTRT (positive and negative controls, renal tumor, breast carcinoma and mammary tissue).

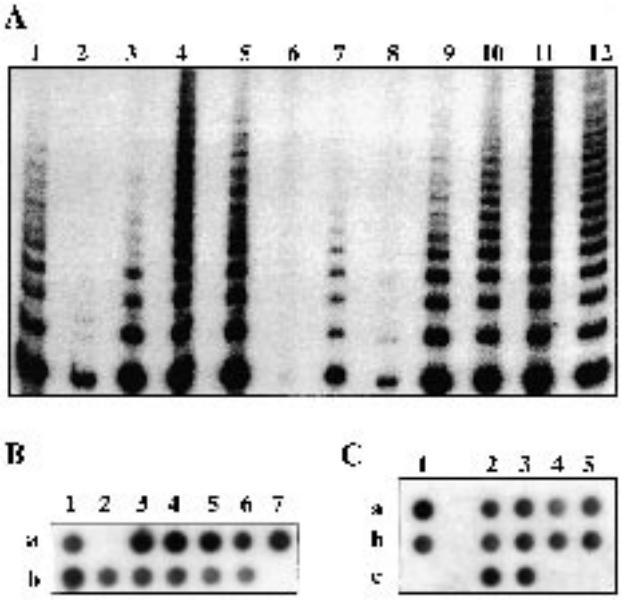

Fig. 2. A: TRAP assay for telomerase activity (lanes 1, 2: positive and negative control; lanes 3, 5: lymph nodes with metastasis; lanes 4, 11, 12: hyperplastic lymph nodes; lane 6: lymph node with necrotic metastasis; lane 7: breast carcinoma; lane 8: urinary bladder inflammation; lane 9: breast carcinoma recurrence; lane 10: breast carcinoma). B: Dot-blot relative of hTR expression (a1, a3: positive controls; a2: negative control; a4, a5: lymph nodes with metastasis; a6: lymph node with necrotic metastasis; a7: breast carcinoma; b1: breast carcinoma recurrence; b2: urinary bladder inflammation; b3: breast carcinoma; b4, b5, b6: hyperplastic lymph nodes). C: Dot-blot relative of hTRT expression (a1, b1: positive controls; a2, a3: lymph nodes with metastasis; a4: lymph node with necrosis; a5: breast carcinoma; b2: urinary bladder inflammation; b3: breast carcinoma recurrence; b4: breast carcinoma; b5, c2, c3: hyperplastic lymph nodes; c4: negative control).

To establish the quantitative relationship between methods, telomerase activity was detected with TRAP analysis in fresh tissues of 10 human tissues and the results were compared with RT-PCR-based method in the same paraffin-embedded cases. We found a good quantitative correlation between activity measured with TRAP and hTRT mRNA expression in eight cases ($r = 0.68$). The other two cases, a lymph node with

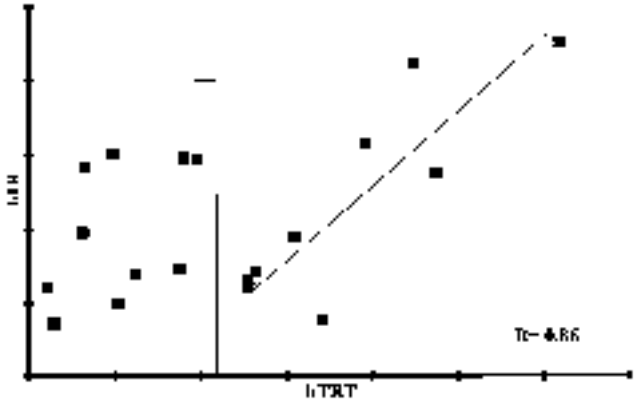

Fig. 3. Relative standardized quantitation of hTR and hTRT mRNA in brain tumors.

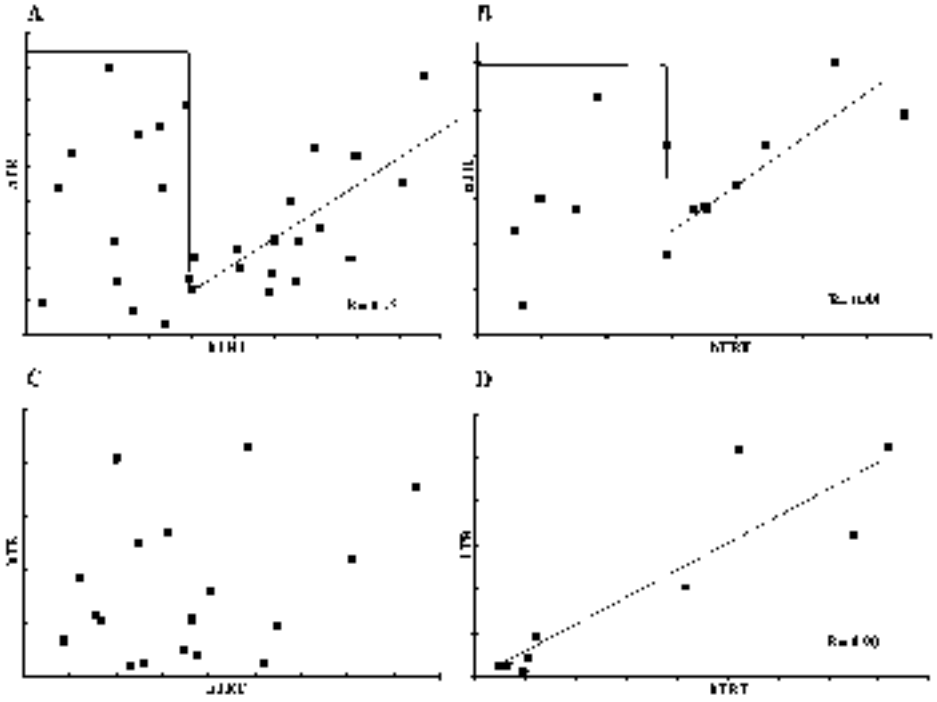

Fig. 4. Relative standardized quantitation of hTR and hTRT-mRNA in breast carcinomas (A), liver tumors (B), cervix uteri precancerous dysplasias (C) and cervix uteri in situ carcinomas (D).

a necrotic cancer metastasis and a urinary bladder wall with severe neutrophil infiltration, were positive for hTRT mRNA expression but negative or with a very low activity with TRAP analysis. In both cases necrosis and severe neutrophil infiltration led to proteolysis and protein degradation that affected the TRAP assay (Fig. 2). No quantitative correlation was found between the enzyme activity and the expression of the RNA component of telomerase.

We studied the telomerase RNA component (hTR) and the mRNA of the catalytic component (hTRT mRNA) in four types of human cancer. The expression of hTR and the level of the hTRT mRNA were compared in brain, liver, and breast cancers, and in cervix uteri precancerous lesions.

In gliomas of the brain the level of expression of hTR appears unrelated to the hTRT mRNA for lower levels of expression of the latter (Fig. 3). When the catalytic subunit mRNA presented higher expression we found a good linear correlation between the two components ($r = 0.86$). The second model, the liver hepatocarcinomas, showed a similar pattern (Fig. 4A) with no correlation at lower levels of protein mRNA and a linear relationship with the RNA component at higher levels of protein expression ($r = 0.88$). The same behavior as the previous two models was found in breast carcinomas ($r = 0.75$) (Fig. 4B). There was a linear relationship between hTR and hTRT expression also for cervix uteri in situ carcinomas ($r = 0.90$) (Fig. 4D), but in cervical precancerous dysplasias there was no correlation between the two levels of expression (Fig. 4C).

To study human tissues the availability of extensive archives of paraffin-embedded tissues from biopsies and autopsies is an invaluable resource that unfortunately was not tapped until now for telomerase-tumor progression associations. Obviously it is impossible to measure enzyme activity in fixed tissues but we have developed a reliable RT-PCR method to quantify the amounts of hTR and hTRT [14,15].

It was already stressed that the correlation between hTRT mRNA and the level of telomerase activity remains to be clarified [16]. Our data suggest that there is a good quantitative correlation between the activity and the expression of the enzyme, with some limitations due mostly to proteolysis processes that can affect the TRAP analysis. In the two cases in which activity could not be shown, there were cellular processes that could be ascribed to protein degeneration and lysis, i.e. necrosis and neutrophil infiltration. A significant correlation between hTRT expression and telomerase activity has already been reported in the literature [17].

In this study we analyzed the presence of the telomerase RNA component (hTR) and its relationship with the catalytic component (hTRT) expression in four different groups of human tumors. Once the correlation between hTRT mRNA levels and telomerase activity was established, it was possible to focus on the relationship that exists between the RNA and the catalytic components of the ribonucleoprotein in paraffin-embedded tissues, which does not allow the detection of telomerase activity, but has the advantage of the sure recognition and collection of the lesions, which can then be easily microdissected with the analysis confined to the cells involved. It was recently reported that in many tumors previously scored as telomerase negative, the activity was detected after careful histological confirmation with an adjacent cryosection of the lesions and microdissection [18].

Our data indicated that in four different neoplastic models a relationship exists between the structural RNA and the mRNA encoding the catalytic component of telomerase. When the mRNA of the catalytic subunit reaches a certain level of expression the RNA component is linearly correlated.

This correlation is absent for low or absent expression of the protein component. The meaning of this high expression of the RNA component in cases without an evident protein component expression must be further investigated. The simplest explanation for this different relationship between the two components of telomerase could be ascribed to a different proportion of cycling cells in tissues. It was recently shown in preneoplastic and neoplastic early lesions that the amount of hTRT mRNA present within individual cells increased gradually during progression and also the number of expressing cells increased within the same tumor [15]. It was also suggested that the level of activity of telomerase could correspond to the percentage of primarily immortal or mortal cancer cells within the tissues [19]. That telomerase activity is connected with the continuous progression of the tumor is shown by the fact that telomerase reactivation occurs during progression in multistep human colorectal carcinogenesis [20]. On the other hand, the level of expression of the RNA component of the ribonucleoprotein seems to be unrelated to the activity of the enzyme. In lung precancerous lesion the expression of hTR was not directly connected to the telomerase activity and highly expressed from in situ non-infiltrative tumors with low enzyme activity [21]. The same discrepancy was noted in multistage tumorigenesis in transgenic mice in which the up-regulated RNA component did not parallel the amount of telomerase activity detected in precancerous cells [10]. Also in the mouse the telomerase RNA component is up-regulated in early hyperproliferative stages of tumor progression before telomerase activity is apparent [22]. The cervix uteri model confirms these data and gives a clearer picture that there is no relationship between RNA and protein component of telomerase in precancerous lesions. A good linear relationship was instead found for in situ carcinomas (Fig. 4). A stoichiometric ratio of the two component of the ribonucleoprotein telomerase is probably reached only in the first step of a true cancer lesion but not in the precancerous one.

It is known that the telomerase catalytic subunit binds the structural RNA and this complex interacts with the telomeres. This RNA-protein interaction may not be only a catalytic subunit-template interaction but could be also a regulatory loop. There are systems where regulation is achieved by binding of RNA to a transcription factor. Transcription factor IIIA binds to 5S RNA to regulate its synthesis [23], another example is reported for HIV in which TAT protein transactivation is achieved by binding an RNA sequence [24]. The changes observed in hTR RNA and in hTRT mRNA levels would be consistent with a regulation of telomerase RNA synthesis by the level of the telomerase catalytic subunit present.

Hoffman and collaborators found in kidney fetal tissues alternative splicing of hTRT transcripts as a mechanism controlling the telomerase activity [3], others found a possible transcriptional control connected with the expression of retinoblastoma protein and the myc gene [4–6]. Some authors have shown inhibition of telomerase activity by treating cells with 9-hydroxyellipticine (an antitumor alkaloid) irrespective of p53 status [25]. We suggest there is another possible transcriptional control, a cross-regulation between the RNA and catalytic components of telomerase. High expression of hTR could down-regulate the expression of the protein component, when enough hTRT is expressed to form a stoichiometric complex with the RNA component, then the relationship hTR/hTRT levels will tend to be linear.

The suggestion of these regulatory loops between the two components of telomerase could contribute to explain the role of this enzyme in tumor progression.

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