Telomerase activity and the subunit of telomerase in hydatidiform mole and their relationship with the development of postmolar tumor

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Summary

Objectives: To investigate the pattern of telomerase activity and the subunit of telomerase in normal placentae and GTD, and to determine the prognostic significance of telomerase activity and the subunit of telomerase in GTD.

Methods: Telomerase activity human telomerase (hTERT) and human telomerase (hTR) expression were analyzed in the initial uterine evacuation specimen of 63 hydatidiform moles (HMs), 42 normal human placental tissues, 17 malignant gestational trophoblastic tumors, primary cultures of normal villi and JAR cell lines by use of the polymerase chain reaction-based telomeric repeat amplification protocol (TRAP) assay and reverse transcription-polymerase chain reaction (RT-PCR) methods.

Results: Telomerase activity was 100% in primary cultures of normal villi and JAR cell lines and in less than 60-day early placental villi, while only 9.1% in greater than 60-day placental villi, 27% in HMs and 58% in malignant trophoblastic tumors. High levels of hTR could be found in all groups. hTR expression was detected in all cases of < 60-day placental villi, in 72.7% > 60-day placental villi, in 87.3% in HMs and 100% in malignant trophoblastic tumors. Telomerase activity and hTERT expression had significant differences among the groups. Telomerase activity was associated with serum hCG levels but not related to other clinical risk factors.

Conclusions: Telomerase activity may be correlated with the development of trophoblastic tumors, and hTERT may be a useful diagnostic marker for detecting the existence of malignant trophoblastic cells.

Key words: Telomerase activity; hTERT; hTR; Gestational trophoblastic disease; Hydatidiform mole.

Introduction

Repetitive telomere sequences are present at the ends of eukaryotic chromosomes which protect the ends from damage and rearrangement. Progressive shortening of telomeric sequences is associated with cell division owing to the end replication problem involved in DNA replication. Most normal somatic cells lack telomerase activity. The telomeres of tumor cells have to be maintained in order to sustain their continuous proliferative capacities, and telomerase is commonly activated in human tumors for this purpose [1, 2]. Telomerase is a reverse transcriptase composed of two primary components: human telomerase protein catalytic subunit (hTERT) and human telomerase RNA (hTR). It is believed that the telomere length in cancer cells is regulated by both the telomerase complex and the proteins that bind to the telomeric DNA repeats [3, 4].

Hydatidiform mole (HM) is a very commonly encountered disease in the west district of Guangdong province in China. HM is diagnosed histologically by the presence of hydropic swelling of the chorionic villi and proliferation of trophoblasts. Outcome of patient follow-up after months or even years of using successive serum β-human chorionic gonadotrophin (hCG) measurements allows early detection of persistent gestational trophoblastic cell neoplasia. However, not every patient can come to the hospital for a timely follow-up, which makes treatment too late for some patients. In fact, HM is associated with substantial risk (10-15%) of developing persistent gestational trophoblastic disease (GTD) and may even develop into choriocarcinoma eventually. The mechanisms involved in the pathogenesis and progression of HM are poorly understood. Except for the serial assays of serum and urine hCG, there is no reliable predictor for the early detection of persistent trophoblastic disease.

In this study, we assessed and compared telomerase activity, hTERT and hTR in normal placental villi in different gestational stages, HM, invasive mole, choriocarcinoma, primary culture normal villi and JAR cell lines using the TRAP assay and RT-PCR [5-7] .

Material and Methods

Clinical samples

Tumor samples and HM were obtained from patients who underwent surgery from October 1997 to March 2002. The samples were washed with 0.9% sodium chloride, frozen with liquid nitrogen and stored at –80°C. Sixty-three samples of HM, 42 normal placental villus specimens (20 early pregnant villi samples less than 60 days' gestation and 22 greater than 60 days' gestation) and 17 invasive mole and choriocarcinoma samples were the subjects of this study. Both invasive mole and choriocarcinoma were regarded as malignant gestational tro-

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phoblastic tumor (MGTT). All tissues were confirmed by histopathological examination. Diagnoses were carried out using well established diagnostic criteria [8]. Primary cultured normal villi and JAR choriocarcinoma cell lines (Shanghai Institute of Biologic Sciences, Chinese Academy Sciences) were also included in the study.

Extraction of telomerase

Telomerase was extracted from sections of frozen tissues or cell pellets according to a previously published protocol [9]. Briefly, each frozen section or cell pellet was extracted with 200 μl of ice cold 1 x CHAPS lysis buffer. Before adding lysis buffer to the tissues, they were homogenized with liquid nitrogen. All precautions against RNase contamination were observed. After incubated on ice for 30 minutes, the homogenized lysate was centrifuged at 12,000 g for 20 minutes at 4°C and protein concentration was measured by Bradford assay [10]. Finally, the supernatant was stored at –80°C until use. The telomerase activity in the extract was then assessed by the TRAP [5-7].

Telomerase assay-TRAP reaction

We performed a TRAP assay using the Telomerase assay kit (Roche Co.) according to the manufacturer's protocol. In this primer-extension based assay for detecting telomerase activity, the telomerase reaction product is amplified by PCR [1]. Each extract was assayed in 50 μl of reaction mixture containing 10 x CHAPS lysis buffer, 1 μl of TS primer, 1 μl TRAP primer mix, Taq polymerase 2U, with 3 μl of cell (tissue) extract. Then sterile water was added until a final volume of 50 μl was reached. A 30-minute incubation period at 30°C was allowed for the telomerase mediated extension of the TS primer. The reaction mixture was then heated at 94°C for three minutes and subjected to 35 PCR cycles (94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec). The PCR products were then electrophoresed on a 12.5% polyacrylamide gel. The HeLa cell extract was also included in each telomerase assay as a positive telomerase control to adjust for interassay variation.

Determination of hTERT and hTR by RT-PCR

Total cellular RNA was extracted from cells and tissues with Trizol (Invitrogen Co.). hTERT primers were 5'-cggaagagtgtctggagc aa-3'(antisense strand), 5'-ggatgaagcggagtctgga-3' (sense strand); and the final amplification product was 145 base pairs. The hTR primers were 5'-tctaaccctaactgagaagggggtag-3' (antisense strand), 5'-gtttgctagaatgaacggtggaag-3' (sense strand) and the final amplification product was126 base pairs. The sequences of the internal primers of β-actin were 5'- gaaactaccttcaactccatc-3' (antisense strand) and 5'- gaagcatttgcggtggacgat-3' (sense strand). The final amplification product was 300 base pairs. Reverse transcription and PCR reactions were carried out with the two-step Reverse Transcription System kit (Promega Co.).

Statistical analysis

Statistical analysis was performed with the chi-square test and the Fisher exact test in SAS (SAS Institute Inc., China) and State softwares. Significance was accepted at $p < 0.05$.

Results

Telomerase activity was 100% in the primary culture of normal villi and JAR cell lines and in < 60-day early placental villi, while only 9.1% in > 60-day placental villi, 27% in HM and 58% in MGTT. It appears in HM and is enhanced in MGTT. Telomerase activity in < 60-day villi was significantly higher than in > 60-day term villi , HM and MGTT ($p < 0.01$; two-tailed Fisher's exact test). The difference between MGTT and HM was also statistically significant ($p < 0.05$). Telomerase activity in < 60-day term villi was significantly lower than in > 60-day normal villi, HM and MGTT ($p < 0.01$). RNase pretreated extracts did not show telomerase activity (Figure 1).

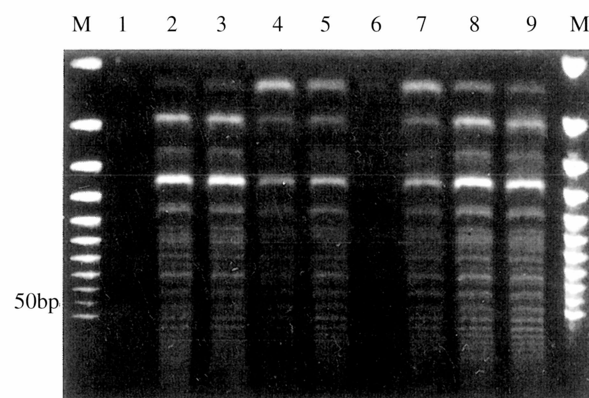

Figure 1. — Telomerase activity in human placental and trophoblastic tumors M 25bp DNA marker; 1. Negative control; 2. Positive control; 3. Primary cultured normal villi; 4. JAR cell line; 5. Less than 60 days normal villus; 6. > 60-day normal villi; 7. HM; 8. Invasive HM; 9. Choriocarcinoma.

hTERT expression was detected in all cases of < 60-day placental villi, 31.8% in > 60-day placental villi, 31.7% in HM and 70.6% in malignant trophoblastic tumors. hTERT in < 60-day villi was significantly higher than > 60-day term villi, HM and MGTT ($p < 0.01$). The difference between malignant GTT and HM was statistically significant ($p < 0.05$) and hTERT in > 60-day term villi was significantly lower than < 60-day normal villi HM and MGTT ($p < 0.01$). However high levels of hTR could be found in all groups. hTR expression was detected in all cases of < 60-day placental villi, 72.7% in > 60-day placental villi, 87.3% in HM and 100% in malignant GTT; hTR showed no significant difference among groups (Figure 2).

The samples were classified into those with positive or negative telomerase activity, TERT, hTR and are summarized in Table 1, together with their clinical risk factors which are summarized in Table 2. Telomerase activity was associated with serum hCG levels but was not related to other clinical risk factors such as age, size of uterus, ovarian luteinzing cysts. hTERT and hTR had no association with clinical risk factors (Table 2).

Table 1. — *Telomerase activity, hTERT, hTR in trophoblastic and other tissues.*

Types	numbers	telomerase activity		hTERT		hTR	
		+	–	+	–	+	–
< 60-day normal villi	20	20	0	20	0	20	0
≥ 60-day normal villi	22	2	20	7	15	16	6
HM	63	17	46	20	43	55	8
MGTT	17	10	7	12	5	17	0
JAR Cell Line	1	1	0	1	0	1	0

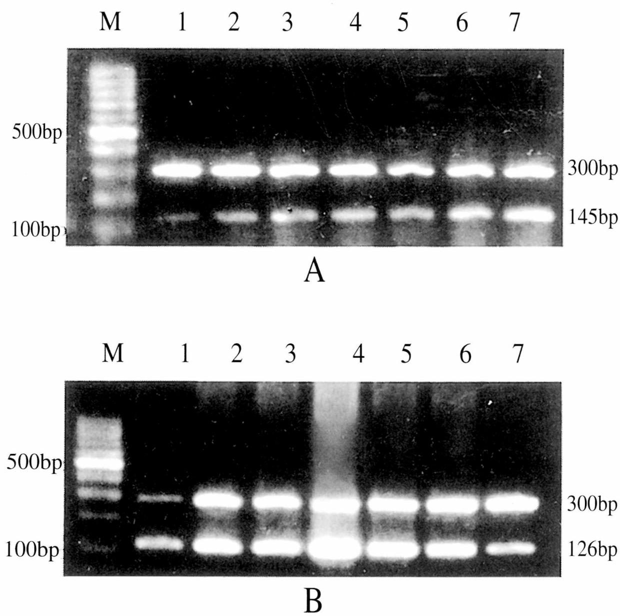

Figure 2. — Expression of hTERT and hTR in human placental and trophoblastic tumors.
Panel A. Expression of hTERT in human placental and trophoblastic tumors. M 100 bp DNA marker; 1. Primary culture villi; 2. JAR cell lines; 3. < 60-day normal villi; 4. > 60-day normal villi; 5. HM; 6. Invasive HM; 7. Choriocarcinoma.
Panel B. Expression of hTR in human placenta and trophoblastic tumors. M 100 bp DNA marker; 1. Primary culture villis; 2. JAR cell lines; 3. < 60-day normal villi; 4. > 60-day normal villi; 5. HM; 6. Invasive HM; 7. Choriocarcinoma.

Table 2. — *The relationship between telomerase activity, hTERT, hTR and clinical risk factors.*

Types	numbers	telomerase activity +	telomerase activity −	hTERT +	hTERT −	hTR +	hTR −
Age > 40	17	7	10	7	10	17	0
< 40	46	10	36	13	33	38	8
Size of uterus:							
> normal pregnancy	48	13	35	15	33	40	8
≤ normal pregnancy	15	4	11	5	10	15	0
Ovarian cyst:							
+	18	4	14	8	10	14	4
−	45	13	32	12	33	41	4
Serum β-hCG:							
≥ 10^5 mIU/ml	27	14	13	10	17	24	3
< 10^5 mIU/ml	36	3	33	10	26	31	5

Discussion

Telomeres, the ends of human chromosomes, seem to function in chromosome stabilization during replication by protecting the chromosomal ends against exonucleases and ligases [11]. They consist of several thousand copies of a repeat nucleotide sequence 5"-TTAGGG-3".With each cycle of DNA replication, one end of the linear chromosome develops a short 8-12 bp. It has been proposed that telomere shortening may function as a "mitotic clock" [12]. Telomerase is a rionucleoprotein that synthesizes telomeric DNA. Somatic cells exhibit progressive loss of telomeric sequences; in contrast, the majority of cancerous tissues have demonstrable telomerase activity, thus stabilizing the telomere undergoing unlimited proliferation, even if an alternative pathway [13], or individual differences in telomere shortening [14] has been proposed. Telomerase activity in GTD has been studied by other researchers. In Chen *et al's.* study, telomerase activity was detected in 11 of 30 early placentas (36.7%), one of five term placentas (20%), five of 27 HMs which regressed spontaneously (18.5%), and six of eight HMs which developed persistent trophoblastic disease (75%). The positive rate was different, but the variational trend was similar. The difference was related to the positive criteria [15].

In the present study, the relative level of hTR and hTERT in human normal placental tissues as well as JAR cell lines and GTD tissue were determined by RT-PCR. hTR was cloned as a template RNA of telomerase and was expressed in pre-crisis cell lines and non-neoplastic tissue, as well as in immortalized cell lines or tumor specimens, and the expression level was not correlated with the level of telomerase activity [2]. With the cloning of both genes coding for RNA and the protein component of telomerase, only the expression of hTERT was closely correlated with telomerase activity [16]. Screening of hTERT expression using Northern blotting, *in situ* hybridization and mainly RT-PCR in various tumors and healthy tissues, has confirmed these initial findings [17].

It has been suggested that germline tissues and tumor cell lines express higher levels of hTR than normal somatic cells and tissues [2], More recent studies have indicated that the regulation of hTR synthesis is not strictly correlated with that of the telomerase enzyme itself, and expression of hTR in normal somatic cells that are telomerase negative have been reported [2, 18]. Our results that hTR could be found in normal early pregnancy villi and term placental tissue are in accordance with previous observations of others. hTR expression was detected in all cases of < 60-day placental villi, in 72.7% > 60-day placental villi, in 87.3% in HM and 100% in malignant trophoblastic tumors.

It is known that trophoblastic cells from fetal villi can aggregate and invade maternal tissue during early pregnancy, and many features of this invasion of trophoblastic cells were no different from the invasion of tumor cells [19, 20]. Over-expression of certain oncogenes has been reported in malignant trophoblastic cells as well as in normal trophoblastic cells found in normal human placental tissue [21, 22]. Over-expression of certain oncogenes has been reported to be highest in first-trimester placental tissue with a gradual decrease as gestational age increased [23, 24]. Our data showed that telomerase activity was much higher in early human pregnant placental tissue, and became either very weak or undetectable when the placental tissue approached term. Telomerase activity and hTERT expression showed significant difference among the groups, but there was no significant difference in hTR expression among groups. Telomerase activity was associated with serum hCG levels but not with other clinical risk factors such as age,

size of uterus or ovarian luteinzing cysts; hTERT and hTR had no association with clinical risk factors.This differential expression of telomerase is in agreement with the notion that telomerase activity is correlated with the proliferative activity of trophoblastic cells and placental tissue [25]. Telomerase activity was not detected in RNase pretreated extracts of the cell lines or tumor tissue.

Conclusions

Telomerase activity was very strong in choriocarcinoma tissue, and cell lines may be useful as corroborative support for cytological and pathological examinations to identify malignant trophoblastic cells. Further studies will be necessary to clarify the oncogenic role of telomerase and to address the putative modulation of hTERT expression by other factors.

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