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計畫名稱: 滋養層細胞疾病之尾部酵素活性

Telomerase Activity in Gestational Trophoblastic Diseases

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Abstract

Objective: To evaluate telomerase activity in gestational trophoblastic neoplasms and pregnancies. **Methods:** We used a semi-quantitative telomeric repeat amplification protocol assay to measure telomerase activity. **Results**: Telomerase activity was detected in placental tissue in 33 out of 45 (73.3%) early pregnancies, 9 of 27 (33.3%) spontaneous abortions, 11 of 37 (29.7%) late pregnancies, 15 of 18 (83.3%) cases of hydatidiform mole, and 3 of 3 cases of choriocarcinoma. Although telomerase activity was present less frequently in normal pregnancies as the gestation period increased (80.7% *vs.* 30.8%, $p<0.0002$), this phenomenon was not present for hydatidiform mole (76.9% *vs.* 100%). Among the 15 cases of hydatidiform mole with positive telomerase activity, the two patients who developed persistent GTT were pregnant for ≧10 weeks. **Conclusion:** Trophoblast senescence is present in normal pregnancy but not in hydatidiform mole. Early management of hydatidiform mole may decrease the chances of developing persistent GTT.

1. Introduction

Telomeres, which are TTAGGG repeats at the distal ends of the human chromosome, are essential for complete DNA replication. As human cells divide, telomeres become progressively shorter through replication-dependent sequence loss at DNA termini. This shortening protects the ends of chromosomes from degradation and illegitimate recombination [1, 2]. However, the progressive shortening of telomeres in somatic cells also results in chromosomal instability, eventually leading to senescence. One possible cause of the shortening of human telomeres is the repression of telomerase [3].

Telomerase is a cellular reverse transcriptase that adds 5'-d(TTAGGG)-3' hexameric repeats onto the 3' ends of chromosomes, thus helping to provide genomic

stability in highly proliferative normal, immortal, and tumor cells by maintaining the integrity of the chromosome ends, the telomeres. Telomerase activity is associated with the majority of malignant human cancers. In cancer cells, augmentation of telomerase activity is apparently necessary to balance telomere loss with the *de novo* synthesis of telomeric DNA so as to maintain sufficient telomere length to ensure proliferation [4,5]. In a review study, the author suggested that telomerase activity could be a potentially valuable diagnostic and prognostic tool for human solid tumors [6].

Gestational trophoblastic neoplasm is a category that includes several diseases of the female genital system, including complete hydatidiform mole, partial hydatidiform mole, and choriocarcinoma. All of these diseases possess a neoplastic trophoblast. Recent studies have found that telomerase activity is present in some cases of hydatidiform mole, and that the presence of telomerase activity may be associated with the development of persistent GTT [7,8]. However, the possibility of a significant connection between telomerase activity and GTT has not been adequately addressed in the existing literature on these diseases.

Normal human somatic cells have a limited life span both *in vitro* and *in vivo*. It has been hypothesized that this limited capacity for proliferation is regulated by telomere length [9, 10]. Although telomerase activity in these cells is repressed, normal trophoblasts are known to proliferate in placental tissue from pregnant patients. In fact, telomerase activity has been found in first- and second- trimester placental tissue specimens [11]. However, whether trophoblasts from molar tissue samples and from normal pregnancy placental tissue samples possess the same level of telomerase activity as the pregnancy progresses has rarely been investigated.

In this study, we will look for the presence of telomerase activity in trophoblasts from cases of normal pregnancy and from cases of gestational trophoblastic neoplasm in order to determine whether this measure is of clinical significance.

2. Methods

Tissue Samples The study material consists of 73 tissue samples of trophoblasts that came from 14 women with gestational trophoblastic neoplasms (11 hydatidiform mole, 3 choriocarcinoma), 33 women who underwent a legal abortion for social reasons during early normal pregnancy (5 to 12 weeks), 13 women who gave birth during a late normal pregnancy (35-42 weeks), and 13 women who had a surgical evacuation because of a spontaneous abortion (6 to 13 weeks).

We began by washing these tissue samples in an ice-cold wash buffer (10 mM Hepes-KOH (pH 7.5), 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol), and then used liquid nitrogen to shock freeze the samples into smaller

pieces. Afterwards, we used disposable surgical knife blades to slice flakes from these frozen tissue specimens, which had previously been prepared on sterile petri dishes; these flakes were immediately transferred to homogenization tubes containing a 200 μl ice-cold lysis buffer (10 mM tris-HCl (pH7.5), 1 mM $MgCl_2$, 1mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM ß-mercaptoethenol, 0.5% CHAPS (Sigma), and 10% glycerol). The flakes were then homogenized on ice with a motorized pestle until they reached a uniform consistency. After incubating the lysate on ice for 30 minutes, it was centrifuged at 16,000 g for 20 minutes at 4°C using Eppendorf tubes. We then carefully removed the supernatant and measured the protein concentration by Bradford assay [12]. Finally, we used liquid nitrogen to shock freeze the tissue extracts into aliquot parts, and then stored them at –80°C.

Telomerase repeat amplification protocol (TRAP) reaction

We performed a TRAP assay using the Telomerase PCR ELISA assay in a 50 µl reaction mixture according to the manufacturer's protocol (Boehringer Mannheim Biochemicals, Mannheim, Germany). In this primer-extension based assay for detecting telomerase activity, the telomerase-reaction product is amplified by PCR [13], and a photometric enzyme immunoassay is used. We began by mixing a 25µl reaction solution, which contained a Tris-buffer, a biotin-labeled P1-TS primer, a P2 primer, nucleotides, and a Tag polymerase, with 3 µl of cell extract. We then added sterile water to the result, until a final volume of 50 µl was reached. After a 30-minute incubation period at 25°C for telomerase-mediated extension of the P1-TS primer, the reaction mixture was heated to 94 °C for 5 minutes and immediately subjected to 33 PCR cycles at 94 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 90 seconds. After adding 5 µl of the amplification product and 20 µl of a denaturation reagent containing sodium hydroxide (<0.5%) to 225 µl of hybridization buffer (digoxigenin-labeled detection probe), we mixed the resulting combination thoroughly.

Hybridization and ELISA procedure

We then transferred 100 µl of the mixture to a well made of streptavidin-coated microtiter plate. We incubated the microtiter plate at 37°C on a shaker (300 rmp) for 2 hours, and then washed it 3 times with 250 µl of washing buffer. After adding 100 µl of anti-digoxigenin-peroxidase and incubating at room temperature for 30 minutes, while shaking at 300 rpm, we removed the solution. We then added 100 µl of a substrate solution containing 3,3', 5,5'-tetramethyl benzidine and incubated at room temperature for 10-20 minutes for color development while still shaking at 300 rpm. Finally, we added 100 µl of 5% sulfuric acid to stop the reaction. Using a microtiter reader, we measured the absorbance of the samples at 450 nm. Absorbance values were reported as the $A_{450\ nm}$ reading against blank (reference wavelength $A_{690\ nm}$). We

regarded samples as telomerase-positive if the difference in absorbance (ΔA) was higher than 0.2 units. For negative controls, we incubated 5 μl of cell extract with DNase-free RNase at a concentration of 1 μg/μl for 20 min at 37°C. The maximum value of absorbance for the negative control should be 0.25 units; if the value was higher, the whole test, including TRAP, was repeated. A cell extract prepared from immortalized telomerase-expressing human kidney cells was used as a positive control. Since the absorbance readings of the positive controls should be higher than 1.5 units, the test was repeated if the values were lower.

3. Results

Telomerase activity in cases of normal pregnancy, spontaneous abortion, and in gestational trophoblastic disease is shown in Table 1. Fifteen of the eighteen (83.3%) cases of hydatidiform mole and all three cases of choriocarcinoma tested positive for telomerase activity. Of the 45 trophoblasts from early pregnancies (5 to 12 weeks gestation) that we examined for telomerase activity using the TRAP assay, 33 (73.3%) were positive for telomerase. In contrast, only 11 of the 37 (29.7%) late-pregnancy trophoblasts (36 to 41 weeks gestation) expressed telomerase activity. Trophoblasts from early spontaneous abortions also exhibited telomerase activity, but at a low level; only nine of the 27 (33.3%) were found to exhibit telomerase activity. Telomerase activity was not detected in the placental tissue from the 2 cases of late-pregnancy intrauterine fetal death.

We observed significant telomerase activity in trophoblasts that came from early-stage pregnancies. A comparison of different pregnancies for positive telomerase activity in the early gestation period between <10 weeks and ≧10 weeks is shown in Table II. In early normal pregnancies without symptoms of abortion, telomerase activity was present less frequently (25/31, 80.7% *vs* 8/14, 30.8%) as the gestation period advanced from <10 weeks to ≧10 weeks. However, this phenomenon was not present in cases of hydatidiform mole (10/13, 76.9% *vs* 5/5, 100%) and spontaneous abortion (7/18, 38.9% *vs.* 2/9, 22.2%, Figure 1).

Only two patients out of 15 with hydatidiform mole with positive telomerase activity developed persistent GTT, and both of them were pregnant for ≧10 weeks. However, none of the 3 patients with negative telomerase activity developed persistent GTT.

4. Discussion

In a normal pregnancy, the early chorion is composed of a proliferating trophoblastic mass. Chorionic development begins soon after the implantation of the blastocyst. During the invasion of the trophoblast into the myometrium, the cytotrophoblast continues to proliferate, resulting in the growth of the trophoblast and the subsequent development of the chorion and placenta. It was this proliferation of

the trophoblast that prompted us to examine telomerase activity in the chorion of normal pregnancies.

In our study, we found that the presence of telomerase activity was at its highest during the early period of pregnancy, with activity decreasing significantly after the first trimester. However, telomerase was barely present in cases of abortion, while in cases of fetal demise there was no telomerase activity. Our findings suggest that in normal pregnancies, telomerase activity in the trophoblast is critically regulated over the course of gestation. Unfortunately, it is not clear in cases of fetal demise and placental senescence whether telomerase activity is the cause or the result. In a recent report that evaluated telomerase activity in the placenta in cases with and without fetal growth retardation, such activity was detected more often in cases without fetal growth retardation [11]. From our results and from the literature, we conclude that telomerase activity can not only be expressed in normal trophoblasts, but can also play a role in placental senescence.

The category of gestational trophoblastic disease contains hydatidiform mole, invasive mole and choriocarcinoma. Since some cases of hydatidiform mole in our study progressed from a benign state to malignancy, and since telomerase activity has recently been noted to be present in some cases of hydatidiform mole [7, 8], we believe that the presence of telomerase activity may be associated with the development of persistent gestational trophoblastic diseases. In addition to these findings, we discovered that, in contrast to what we found in normal pregnancies, in cases of hydatidiform mole telomerase activity was detected more often in the later stages of pregnancy. Furthermore, the two cases of hydatidiform mole that developed persistent GTT were diagnosed and treated at a later stage ($\geqq$10 weeks), and both were telomerase positive. Our results suggest that the presence of telomerase activity is a critical step in the oncogenesis of these malignancies, and that the early termination of a hydatidiform mole may decrease the incidence of the subsequent development of persistent GTT.

In this study, we found that telomerase activity is present more often as the gestation period increases in cases of hydatidiform mole. However, in normal pregnancies the reverse was true. We conclude that placental senescence is present in the trophoblasts of normal pregnancies but not in the trophoblasts of hydatidiform mole. Our findings also suggest that the early diagnosis and evacuation of a hydatidiform mole may decrease the incidence of persistent GTT.

References

[1] Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989; 59: 521-529.

[2] Counter CM. The roles of telomere and telomerase in cell life span. Mutat Res 1996; 366: 45-63.

[3] Cerni C. Telomeres, telomerase, and myc. An update. Mutat Res 2000; 462:31-47.

[4] Collins K, Kobayashi R, Grieder CW. Purification of Tetrahymena telomerase and cloning of genes encoding the two protein components of the enzyme. Cell 1995; 81: 677-686.

[5] Counter CM, Hirte HW, Bacchetti S, Harley CB. Telomerase activity in human ovarian carcinoma. Proc Natl Acad Sci USA 1994; 91: 2900-2904.

[6] Vasef MA, Ross JS, Cohen MB. Telomerase activity in human solid tumors. Diagnostic utility and clinical applications. Am J Clin Pathol 1999; 112: 68-75.

[7] Bae SN, Kim SJ. Telomerase activity in complete hydatidiform mole. Am J Obstet Gynecol 1999; 180: 328-333.

[8] Sukcharoen N, Mutirangura A, Limpongsanurak L. Telomerse activity in complete hydatidiform mole. J Reprod Med 1999; 44: 465-470.

[9] Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB. Telomere length predicts replicative capacity of human fibroblasts. Proc Natl Acac Sci USA 1992; 89: 10114-10118.

[10] Harley CB. Telomere loss: mitotic clock or genetic time bomb? Mutat Res 1991; 256: 271-282.

[11] Isuzu T, Kudo T, Sato T, Nishiya I, Ohyashiki K, Nakagawara K. Telomerase and proliferative activity in placenta from women with and without fetal growth restriction. Obstet Gynecol 1999; 93: 124-129.

[12] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities. Anal Biochem 1976; 72: 248-254.

[13] Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PLC et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 226: 2011-2015.