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Increased Placental Telomerase mRNA in Hypertensive Disorders of Pregnancy

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Abstract

Objective—We assessed hTERT mRNA levels in normal versus preeclamptic placental samples, examining hTERT expression levels in different clinical manifestations of hypertensive disorder of pregnancy.

Methods—We performed a single-site, prospective case-control study of hTERT mRNA levels in placentas from term and preterm pregnancies with hypertensive disorders compared with unaffected pregnancies. Placental biopsies were collected from 61 patients (preeclamptic: 32; non-preeclamptic (control): 29). Total RNA from placenta was isolated and reversely transcribed to c-DNA. A probe-specific real-time quantitative PCR assay was employed to determine the relative expressional levels of hTERT mRNA levels in these placentas from both unaffected and affected pregnancies with different categories of hypertensive disorders including preeclampsia, severe preeclampsia, eclampsia and HELLP syndrome (Hemolysis, Elevated Liver function tests, Low Platelet).

Results—The average ratio of hTERT mRNA levels was 1.73 in the preeclamptic group and 1.02 for control group ($p < 0.0001$). The hTERT expression levels were elevated for each of the different categories of hypertensive disorders of pregnancy compared with control: HELLP syndrome 1.86, severe preeclampsia 1.81, eclampsia 1.71 and mild preeclampsia 1.63. In addition, hTERT levels were higher in severe than mild preeclampsia ($p < 0.01$).

Conclusions—Elevated hTERT mRNA expression is observed in placentas from pregnancies with different clinical manifestations of hypertensive disorders of pregnancy. The patho-physiological significance of this finding awaits further studies.

Keywords

Telomeres; Telomerase; Hypertensive Disorder of Pregnancy; hTERT; Preeclampsia

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Introduction

Preeclampsia affects 8% of all pregnancies and is the cause of considerable maternal and fetal morbidity and mortality (1). Possible etiologies have been suggested and include abnormal trophoblast invasion, coagulation abnormalities, vascular endothelial damage, cardiovascular maladaptation, immunologic and genetic predisposition (2). Despite intensive research, the pathophysiology and etiology of preeclampsia is unknown. Preeclampsia develops in the presence of placenta (3) and delivery of the placenta is the most effective way to treat preeclampsia. Therefore, studies of placental/trophoblast biology are essential for understanding the pathological mechanisms involved in preeclampsia.

Telomeres consist of repetitive DNA, TTAGGG repeats, at the end of linear chromosomes (4). They have an important role in genomic stability, preventing both end-to-end fusions and the loss of useful genetic information. Telomeres shorten with each cell division of cultured human somatic cells. When they become critically short telomeres trigger cell cycle arrest, termed “replication senescence” (5). As such, telomeres are “molecular clocks” in cultured cells. *In vivo*, telomere length in proliferating cells might be an index of biological age (5,6). Shortened telomeres are associated with cardiovascular disease (7,8), essential hypertension (9), diabetes (10), atherosclerosis (11). However, telomeres can also be shortened through environmental effects (12).

Telomerase, a reverse transcriptase, compensates for the loss of telomeric DNA by adding telomeric repeats DNA onto the chromosome end (13). Human telomerase is composed of two sub units, a catalytic component, namely the telomerase reverse transcriptase (TERT), and a RNA component (hTR), the template for telomere elongation (14). The expression of the human telomerase catalytic subunit (hTERT) usually correlates with telomerase activity (15, 16), which is robust during embryogenesis, in the germ line (17), but not in somatic tissues (14). The placenta, displays a higher telomerase activity in the first than the third trimester. This finding is consistent with the higher proliferation of cytotrophoblasts in the first trimester than the third trimester (18,19). Placentas from patients with normal pregnancy and preeclampsia express hTERT (20). Moreover, hypoxia induces placental telomerase activity through increased hTERT expression (21). The purpose of this work was to further explore the relation between hTERT expression and preeclampsia.

Methods

Sample Collection and Clinical Background

Fresh placental tissue was collected over one year from 61 delivered patients in the following categories: healthy term deliveries (n = 18), spontaneous preterm deliveries without other complications (n = 11), preeclamptic patients (n = 32, preterm n = 24, term n = 8). Patients that had any other medical problems were excluded and the control group included only low risk pregnancies. Tissue was obtained from the maternal site of the placenta, about 2–3 inches from the area of the cord insertion. Specimens were cut into small pieces and washed twice with ice-cold D-PBS (phosphate buffered Saline without calcium and magnesium, Invitrogen, Carlsbad, CA, USA). Samples were immersed in RNAlater (Ambion, Austin, TX, USA) overnight at 4°C and transferred to –80°C for long-term storage.

Patients' clinical and demographic data were obtained to include: age, body mass index (BMI), blood pressure, obstetrics history, and history of smoking, previous history of hypertension or preeclampsia, clinical diagnosis at time of delivery, birth weight of the newborn, and mode of delivery. Patients were recruited and classified according to the criteria and definitions of the different hypertensive categories based on the National High Blood Pressure Education

Program Working Group on High Blood Pressure in Pregnancy and ACOG classification (22,23).

Preeclampsia (mild) was defined as a pregnancy-specific syndrome that occurs after 20 weeks of gestation and is characterized by systolic pressure of 140 mmHg or higher or diastolic pressure of 90 mmHg or higher, occurring with proteinuria (>300 mg of protein over 24 h or a random dip-stick urine determination of >1+ protein or >30 mg/dL). Severe preeclampsia was defined as blood pressure of 160/110 mmHg and proteinuria of more than 5 grams over 24 h or >3+ on 2 random urine samples collected 4 h apart. Eclampsia was defined as new-onset of grand mal seizures in presence of preeclampsia, and HELLP syndrome is the development of hemolysis, elevated liver enzymes and low platelet count. The study was approved by Temple University Institutional Review Board and informed consents were obtained.

RNA Isolation

Total RNA of placenta samples was isolated with TRIZOL reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) except that 0.1 volume of 0.1% DEPC water was added together with 0.3 volume of chloroform for each one volume of TRIZOL reagent in the isolation process due to the previous preservation of tissue RNA with RNAlater reagent. At the end of the procedure, RNA pellets were briefly air-dried for 5–10 min, dissolved in RNase-free Resuspension Solution (Ambion, Austin, TX, USA), and heated at 60°C. RNA concentration was quantified by UV absorbance at OD260.

Quantitative Real-time RT-PCR

Total RNA was transcribed to cDNA according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Each 5 µg Total RNA isolated from the placenta samples was dissolved in 0.1% DEPC water to make a final volume of 8 µl and denatured at 65°C for 5 min with 1 µl 50 ng/µl random hexamers and 1 µl 10 mM dNTP mix. Annealing was done at 25°C for 10 min with 2 µl 10 × RT Buffer, 4 µl 25 mM MgCl₂, 2 µl 0.1 M DTT, 1 µl 40 U/µl RNaseOUT and 1 µl 200 U/µl Superscript III RT. cDNA was synthesized at 50°C for 50 min and the reaction was terminated at 85°C for 5 min. RNA was removed with 1 µl RNaseH per 20 µl reaction volume at 37°C for 20 min. Primers and probes' sequences of Homo sapien telomerase catalytic subunit (hTERT) and β-actin (24,25) were designed for real-time PCR as follows: hTERT: Forward Primer: gccttaagagccacgtc, Reverse Primer: ccacgaactgtcgcatgt, Probe: ctccagcc β-actin: Forward Primer: ccaaccgcgagaagatga, Reverse Primer: ccagagcgctacagggatag, Probe: ccagcgtg.

Real-time PCR was performed with Roche Light Cycle 4.0 software as follows: one term placental sample from the control group was used as the calibrator. The relative expression level of hTERT mRNA was assessed by comparing it to β-actin level from the same sample (24,25). Calibrator from a normal placenta was used, as it allows for comparisons to be made among the samples, examining them in different times and therefore diminishing the experimental variation. The levels from different samples were calculated in comparison to the levels of the calibrator and the results obtained were referred to as the "ratio".

cDNA samples were pre-incubated at 95°C for 10 min and amplified for 45 cycles, which included denaturation at 95°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 1 s. Samples were cooled down at 40°C for 30 s. Crossing point (Cp) value of each sample was obtained. mRNA level of each sample was calculated using the relative quantification method: relative expression level = $2^{(Cp \text{ hTERT} - Cp \text{ } \beta\text{-actin})}$.

A prearranged placental sample from the control group was included in each run as the calibrator. It diminishes the experimental variation and therefore allows for comparisons among samples which were examined at different times. The calibrator normalized ratio = relative expression level (sample)/ relative expression level (calibrator) (26). A higher ratio is indicative of increased level of hTERT RNA expression.

Statistical Analysis

Differences in hTERT levels (ratios) were analyzed using analysis of covariance with BMI used to adjust hTERT levels. The null hypothesis was that there would be no difference in hTERT levels between the patient groups. Prior to analysis, hTERT data were tested for normality using the Shapiro–Wilk test. The data were found to be significantly non-normal. In order to apply ANOVA methods, a ‘normalized-rank’ transformation was applied to the data. The rank-transformed data were analyzed using a mixed-model ANOVA adjusting for BMI as a covariate followed by multiple comparisons to detect significant differences between patient groups. Multiple pair-wise comparisons used the Bonferroni adjustment to avoid compounding of Type 1 error. Differences were considered statistically significant if the adjusted *p*-value was ≤ 0.05 .

Results

Clinical Background

Sixty-one patients were recruited to this study; 32 comprised the preeclampsia/eclampsia (PE/E) group and 29 were the control group. In the PE/E group 13 (40.63%) patients were diagnosed with mild preeclampsia and 15 (46.88%) patients were diagnosed with severe preeclampsia, including 3 (9.4%) patients that had HELLP syndrome and 2 of them also presented with intrauterine fetal death (IUFD). Four (4/32, 12.5%) patients had eclampsia. There were 24 (75%) patients in the PE/E group who delivered prematurely (<37 weeks gestation) at mean gestational age of 32 weeks and 8 (25%) patients delivering at term (≥ 37 weeks gestation) at mean gestational age of 38 weeks. Eleven patients (37.9%) in the control group had preterm labor and delivery and 18 (62.1%) patients delivered at term (Table 1).

The demographic variables and clinical data of the study subjects are summarized in Table 1. The average age of the patients was 22.5 years in the control group and 23.8 years in the study group. The mean gestational age was 34 in the study PE/E and 37 in the control group ($p = 0.1932$). The mean BMI of the patients in the PE/E group (32.7) was higher than in the control group (29.2). More patients in the PE/E group were African-American (23/32, 71.86%) compared to the control group (12/29, 41.38%). There were 10 (31.25%) patients who were primigravidas in the study group and 10 patients were primigravidas (34.48%) in the control group. We also examined the birth weights of the newborns. In the PE/E group, 20 (62.5%) newborns' birth weights were lower than the 10th percentile while in the control group, all the babies were above the 10th percentile.

hTERT mRNA Expression

hTERT mRNA levels were elevated in the PE/E group versus the control group. The ratio of hTERT mRNA in the PE/E group (1.73) was significantly higher than the control group (1.02) ($p < 0.0001$) (Figure 1). The mRNA levels of hTERT were also significantly higher when we compare the hTERT mRNA between the PE/E and control group according to different gestational age (preterm: 1.75 versus 0.84, $p < 0.0001$, term: 1.67 versus 1.12) (Figure 2). The age of the patients, gravida, parity, gestational age, birth weight, race, mode of delivery, and smoking status were not demonstrated to have a significant effect on the hTERT mRNA expression in the study or the control group.

hTERT mRNA Levels and Different Categories of Preeclampsia

The average ratios of hTERT mRNA among mild preeclampsia, severe preeclampsia, HELLP syndrome and eclampsia were 1.63, 1.81, 1.86, and 1.71, respectively, compared with 1.02 for the control group. The hTERT mRNA in all PE/E subgroups were significantly higher than the control group ($p < 0.0001$). The levels of hTERT mRNA was significantly higher in the severe preeclampsia group and in the HELLP group compared with mild preeclampsia ($p < 0.01$) (Figure 3). There were 3 patients with HELLP syndrome and 2 of them had IUFD. In the subgroup analysis, the average ratio of hTERT mRNA in these 3 patients (1.86) was higher than the preeclamptic patients without HELLP syndrome (1.63) ($p < 0.01$) (Figure 4).

hTERT mRNA Levels and Body Mass Index

We found a linear relationship between BMI and hTERT independent of the pre-eclampsia groups. The BMI of the 5 groups was significantly different. To adjust for this difference in BMI, we used an analysis of covariance to adjust the hTERT mRNA levels for the between group comparisons. When adjusting for BMI, hTERT mRNA was significantly higher in the control term patients compared with control preterm patients ($p < 0.0001$) and the hTERT mRNA expression level in the preterm preeclampsia group was higher than the control preterm group ($p < 0.0001$). In addition, after adjusting for BMI the hTERT mRNA level in the severe preeclampsia group was higher than the mild preeclampsia group ($p < 0.01$).

Discussion

In this study we observed an increase in the relative expression of hTERT mRNA placentas in patients belonging to the PE/E group. Moreover, we observed a positive association between the severity of PE/E and hTERT expression. Our study also showed a lower hTERT mRNA levels in placentas of mothers delivering preterm newborns than in those with term pregnancy. High levels of hTERT mRNA and telomerase activity are expressed during early stages of pregnancy, perhaps due to rapid proliferation of trophoblast, that invade the endometrium (27). As pregnancy progresses from the first trimester to term, telomerase is down regulated in tandem with diminished proliferation of villous cytotrophoblasts (19,28). We have thus showed that beyond 20 weeks gestation in the normal pregnancy and relative to pregnancies complicated with hypertensive disease, there is lower expression of hTERT mRNA (20,21).

In contrast to our results and using different methods and material, Lehner et al. reported that overall intensity of hTERT staining of the trophoblast was relatively stable throughout all stages of placental development including in preeclampsia (20). They had 5 patients with preeclampsia at mean gestational age of 28.4 weeks. Two of the specimens from these women were negative for the hTERT protein and the others showed a diffuse granular cytoplasmic distribution. In light of our findings, it is only reasonable to re-study the levels of the telomerase protein levels in correlation with hTERT mRNA in the placentas of normal pregnancies and those complicated by PE/P.

Our results are consistent with the findings that hTERT is increased in preeclamptic placenta (21). However, the stratification of hTERT mRNA based on preeclampsia, eclampsia and HELLP syndrome has not been observed before. The increase in hTERT mRNA, means increase in hTERT gene expression that may or may not correlate with increase in telomerase activity associated with cell proliferation. Telomerase activity and hTERT expression, have a role, not only in healthy conditions such as cell growth and proliferation at the beginning of the pregnancy but also in pathological conditions of cell immortalization, cancer progression and as demonstrated in our work in hypertensive diseases of pregnancy such as preeclampsia, HELLP and eclampsia (29).

It was previously shown that low oxygen tension-triggered trophoblast proliferation can result in early onset preeclampsia (30). The placental exposure to hypoxia is associated with an increased risk of IUFD in patients with preeclampsia (31). Hypoxia is a key player in pathological conditions but also has a role in healthy conditions, such as the early weeks of pregnancy development which was already shown to be associated with increased expression level of hTERT mRNA. This phenomenon may stem from telomeric damage that heightens telomerase activity by up-regulating hTERT via hypoxia-inducible factor 1 α (HIF-1 α) and direct enhancement of endogenous hTERT expression (32). This could be one of the mechanism through which telomerase attenuate hypoxia-induced DNA damage (33).

There are few potential explanations to the increased hTERT mRNA expression in placentas of patients with preeclampsia. First, the upregulation of hTERT is a biologic response to counteract apoptosis in the placenta (34). Higher levels of hTERT mRNA in normal term pregnancy compared with preterm as well as in severe preeclampsia compared with the milder form of the disease could be a response of telomerase, induced by apoptosis of the placenta (35,36). Similar to hypoxia, oxidative stress also up-regulates telomerase activity in an attempt to protect the cells and overcome increased apoptosis and may play a role in these pre-eclamptic placentas (12).

Smoking was shown to have a protective effect from preeclampsia. We had a limited number of patients that were smokers in both groups and that might explain why we did not find significant effect of smoking on hTERT mRNA expression levels. However, increased BMI was associated with increased expression of hTERT mRNA in the PE/E and control groups. Fitzpatrick et al. did not find associations between cigarette smoking and BMI and telomeres length in cells from adults, postmenopausal, not pregnant women (7). On the other hand Valdes et al. studied telomere length in white blood cells in healthy, white, younger, non-pregnant women and found that both obesity and smoking are associated with shortened WBC telomere length (37). Obesity is important risk factors in many age-related diseases and is associated with increased oxidative stress, which increased the rate of telomere erosion per replication and inflammation, which enhances white blood cell turnover (37). These above mentioned studies did not report on pregnant patients and therefore their findings may not be relevant to the pregnant population. Our study has shown that patients with increased BMI had higher hTERT mRNA. A link may be suggested that obesity is associated with telomeres attrition which causes increased in telomerase activity and increased hTERT mRNA expression level as was found in our study.

The limitation of our study include the relative small number of patients in each of the PE/E subgroups and in the control group, the lack of data on hTERT gene polymorphism in our population and in ethnically diverse group. The lack of information on the effect of treatment with magnesium sulfate, used as seizure prophylaxis or as tocolytic agent, on hTERT mRNA levels could influence our results. However, these medications were not reported in the literature to have any effect on hTERT mRNA.

Telomere attrition rate in somatic cells was introduced as a biological indicator of growth and aging and as a model that provides a better understanding of the etiology of diseases such as essential hypertension (9). The same model may be applied for hypertensive disorder of pregnancy, a unique disease of pregnancy that its development and biology are associated with aging of the placenta and is limited to the 40 weeks of gestation. Preeclampsia is linked to inflammation and oxidative stress and therefore, increased expression of hTERT mRNA may be part of the process resulting from telomere attrition and premature aging of the placenta that are occurring in this disease (9). Our study findings of increased hTERT mRNA in hypertensive disease of pregnancy can be explained by the known biological role of telomerase in human

cells. There is a strong evidence that forced expression of hTERT gene in human somatic cells is sufficient to produce telomerase activity that helps to circumvent the senescent stage (38).

Conclusions

We demonstrated increased hTERT mRNA in different categories of hypertensive disorders of pregnancy. Large-scale studies that concurrently examine hTERT mRNA, telomere length, and telomerase activity in placentas, cord blood, and maternal blood might shed light on the role of telomere biology in preeclamptic pregnancies.

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Figure 1. hTERT mRNA levels in non-preeclamptic control & preeclamptic groups

The average of hTERT mRNA ratios in the preeclamptic group is 1.73. The average of hTERT mRNA ratios in the non-preeclamptic control group is 1.02. The average ratios of hTERT mRNA is increased in preeclamptic group ($p < 0.0001$) compared to control.

Figure 2. hTERT mRNA levels in control & study group

The average of hTERT mRNA ratios in the study group ($n = 32$) by gestation of delivery, 1.83 for 24 patients at <37 weeks preterm, and 1.70 for 6 patients at ≥ 37 weeks term gestation. The control group ($n = 29$) gestation of delivery, 0.84 for 11 patients at <37 weeks preterm, and 1.12 for 18 patients at ≥ 37 weeks, term gestation. The average ratios of hTERT mRNA is increased in preeclampsia in both preterm ($p < 0.0001$) and term ($p < 0.0001$) groups compared to controls.

Figure 3. hTERT mRNA levels and different categories of preeclampsia

The average of hTERT RNA ratios in each subgroup of preeclampsia was elevated compared to control ($p < 0.0001$).

Figure 4. hTERT mRNA levels& HELLP Syndrome

The average expression ratio of hTERT mRNA in HELLP syndrome was higher than in mild preeclampsia without HELLP syndrome ($p < 0.0001$) and eclampsia ($p < 0.001$), but showed no difference from the severe preeclampsia group without HELLP syndrome.

Table 1

Demographic characteristics and clinical data of study group compared with control group.

	Control group	Study group	p-Value
Number	29	32	
AGE (Mean±SD)	22.52 ± 5.36	23.81 ± 6.35	0.4193
Range	16–36	14–38	
Gestational Age (Mean±SD)	37.16 ± 2.45	34.07 ± 4.14	0.1932
BMI (Mean±SD)	29.23 ± 5.48	32.72 ± 6.81	0.0378
Range	19.80–42.00	15.60–45.70	
RACE			
African-American	12 (41.38%)	23 (71.88%)	0.0287
Non African-American	17 (58.62%)	9 (28.12%)	
PARA			
0	11	18	0.3399
I	10	5	
II	7	5	
III	1	1	
IV	0	2	
V	0	1	
GRAVIDA			
I	11	12	0.2748
II	7	6	
III	6	3	
IV	3	5	
V	2	4	
VI	0	2	
MODE OF DELIVERY			
Vaginal Delivery	19	12	0.5303
C. Section	10	20	
SMOKING HISTORY	1	3	0.6141
BIRTH WEIGHT (Mean±SD)	2916.83 ± 724.84	2052.64 ± 886.94	<0.0001
Range	1855–4130	550–3995	
SUBGROUP			
Preeclampsia	N/A	13	
Severe Preeclampsia		12	
Eclampsia		4	
HELLP syndrome		3	