Quantitative telomerase activity in malignant, benign and normal gynecological tissues

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Summary

Objective: The aim of this study was to evaluate quantitative telomerase activity in malignant, benign and normal gynecological tissue samples by using the Telomerase-PCR ELISA kit, and to determine a cut-off level for malignancy by this quantitative method.

Materials and Methods: Fifty gynecological tumors, 27 benign gynecological disorders and 29 normal tissues were analyzed by the Telomerase-PCR ELISA kit. All tissues were confirmed by a pathologist. A ROC (receiver operator characteristic) curve was drawn to determine a threshold level best discriminating malignant tissues from benign pathologies and normal tissues. Telomerase activity was compared in malignant, benign and normal tissues.

Results: The mean level of telomerase activity of the malignant tumor samples (1.03 ± 0.53 units) was significantly ($p < .001$) higher than the normal tissues (0.13 ± 0.07 units) and benign pathologies (0.37 ± 0.25 units). The cut-off point to differentiate malignant samples from benign samples was set at 0.42 units, where the sensitivity was 93.8% and the specificity was 89.3%. Positive predictive value was 84% and negative predictive value was 89.3%. There was a significant difference in telomerase activity between malignant, benign and normal tissues within each histological group.

Conclusion: In this preliminary study, the telomerase-PCR ELISA method was found to have a high sensitivity and specificity to differentiate malignant gynecological tissues from benign tissues.

Key words: Telomerase activity; Gynecological cancers; Telomerase PCR-ELISA.

Introduction

Maintenance of telomere length is considered to be necessary for continued cell division and immortalization in germline cells, immortalized cells and tumor cells. This can be achieved by the activation of telomerase which adds telomeric hexanucleotide repeats to the ends of replicating chromosomes [1-3]. Telomerase activity has been proposed as a valuable diagnostic and prognostic tool in gynecological and other types of cancer [4-10]. Furthermore studies of premalignant lesions help define how telomerase is associated with multistep carcinogenesis [11-13]. Therefore, if detectable, telomerase assay can be potentially applied for the early detection of cancer [14] . Telomerase detection was found to be more sensitive than cytologic examination in detecting cancer cells in the peritoneal cavity of patients with ovarian carcinoma [15]. Recent studies in gynecological tumors have all used semi-quantitative telomerase assays which were based on the telomeric repeat amplification protocol (TRAP) that revealed higher telomerase activity in cancerous lesions compared to non-cancerous lesions [4-6, 16, 17] . However telomerase activity is not unique to malignant tissues. It has been reported to exist in the normal endometrium and fallopian tubes of reproductive age women [18-19] and in adult and fetal ovarian tissues [20, 21]. Therefore, although the detection of gynecological malignancies by telomerase determination may be feasable, it requires accurate quantification of telomerase activity.

The telomeric repeat amplification protocol (TRAP) developed by Kim and co-workers [21] enables the detection of cancer cells in small tissue samples and body fluids such as urine, ascites and pleural fluid specimens [22, 23]. However, the complex and time-consuming procedure of the electrophoretic radioisotope assay makes the telomerase methodology impractical as a diagnostic marker for gynecological malignancies. Non-radioisotopic quantitative ELISA-based technologies (Telomerase PCR ELISA, Roche) may have clinical potential as a diagnostic tool for the rapid analysis of large pools of samples with high sensitivity [24, 25].

The aim of this study was to evaluate the telomerase activity in malignant and benign gynecological disorders and normal tissues by using PCR ELISA assay and to determine a threshold value of telomerase which can effectively discriminate malignant gynecological tissues from benign tissues.

Materials and Methods

This study was conducted at Hacettepe University, Department of Obstetrics and Gynecology after approval from the Hacettepe University Human Investigations Committee. Informed consent was obtained from each patient. Malignant cases were staged appropriately according to the FIGO classification.

A total of 106 consecutive tissue samples (50 gynecological tumors, 27 benign gynecological disorders, 29 normal tissues) used in this study were resected surgically and stored at –70°C

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until used for assays. All tissue samples were examined by a pathologist before storage. Malignant tissues were obtained from 26 ovarian, 12 endometrial, eight cervical and four vulvar carcinoma cases. Benign tissues were obtained from 12 benign ovarian cysts, seven cases of chronic cervicitis and eight cases of myoma uteri. Normal tissues were obtained from nine ovaries, nine normal endometrial, nine cervical and two vulvar tissues resected for other benign pathologies. Telomerase activity was determined by the PCR ELISA Kit. (Telomerase PCR ELISA kit, Roche Diagnostics, Mannheim, Germany).

Before using the PCR ELISA procedure, the telomerase activity was first determined at different protein concentrations as described by Bradford *et al.* [26]. As shown in Figure 1, a linear increase was observed in the absorbances of the HeLa cell extracts with protein concentrations from 0 to 6 μg protein per assay using the TRAP-PCR ELISA procedure.

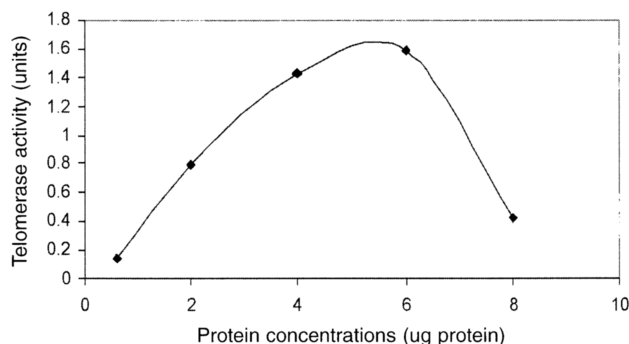

Figure 1. — The relationship between the absorbances and the protein concentrations of HeLa cell extracts. Telomerase PCR ELISA procedure was used for quantification of the telomerase activity of HeLa cell extracts. Absorbances were measured at 450 nm and expressed as unıts. The X and Y axes represent the protein concentrations (μg protein) and telomerase activity (units). Maximum absorbance value was obtained at 6 μg protein.

Telomerase activity was determined as described by Wu *et al.* [24]. The supernatants were prepared according to the instructions of the Telomerase PCR ELISA kit and 3 μl was added to the reaction mixture for PCR. After incubation for 30 min at 25°C for telomerase-mediated extension of the biotin-labeled synthetic P1-TS primer, the elongation products were amplified by using P1-TS and P2 primers during 30 PCR cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 90 sec. Following the PCR procedure, 5 μl of each sample was added to 20 μl denaturation mixture and incubated at room temperature for 10 min. After the addition of hybridization buffer (225 μl), 100 μl of this mixture was transferred to the wells of a streptavidin-coated microtiter plate and the plate was incubated at 37°C on a shaker for two hours. Following the washing procedure, peroxidase conjugated anti-digoxigenin antibody (100 μl) was added and incubated on a shaker at room temperature for 30 min. After the second washing step, TMB substrate (100 μl) was added and shaken at room temperature for 20 min. By the addition of the stop reagent (100 μl), the color of the POD substrate changed from blue to yellow. The absorbance of the samples was measured at 450 nm (with a reference wavelength of 620 nm) using an ELISA microtiter plate reader within 30 min after addition of the stop reagent.

The negative controls prepared with lysis buffer or by RNAse/heat treatment were accepted if the maximum absorbance was 0.25 $A4_{50\text{-}620\ nm}$ units. The positive control of the kit was considered valid if absorbance was 1.5 $A_{450\text{-}620\ nm}$ units or greater. The samples were regarded as telomerase negative if the difference of the absorbances ($\Delta = A_{sample} - A_{negative\ control}$) was lower than 0.2 $A_{450\text{-}620\ nm}$ units. They were regarded as weakly positive if 0.2 $A_{450\text{-}620\ nm}$ was 0.2-0.6 units and strongly positive if 0.2 $A_{450\text{-}620\ nm}$ was > 0.6 units.

Statistical analysis was performed by using a statistical program (SPSS Ver. 10.0 for Windows, Chicago, IL) for all computations. As the distribution of the results of telomerase activity was not normal, the Mann-Whitney and Kruskal-Wallis tests were used. The ROC (receiver operator characteristic) curve was created to establish the threshold value for telomerase activity with the highest sensitivity and specificity to predict malignant tissue samples. Sensitivity and specificity of various values of telomerase activity for malignancy were calculated. These calculations were displayed by graphing the sensitivity on the y-axis and false positive rate (1-specificity) on the x-axis for all possible cut-off values of telomerase activity.

Results

Mean telomerase activity was found to be 1.03 ± 0.53 units, 0.37 ± 0.25 units and 0.13 ± 0.07 units in malignant tumor samples, benign pathologies and normal tissues, respectively. There was a significant difference in telomerase activity between the three groups ($p < .001$). Mean telomerase activity in malignant, benign and normal tissues is summarized in Table 1 and Figure 2.

Table 1. — *Telomerase activity in malignant, benign and normal gynecological tissues.*

Pathologies	Telomerase activity (X ± SD)	Telomerase activity (Min.-Max.)
Normal tissues (n = 29)	0.13 ± 0.07	0.09 - 0.31
Benign group (n = 27)	0.37 ± 0.25	0.09 - 1.37
Malignant group (n = 50)	1.03 ± 0.53	0.16 -1.92

(n = Number of patients; X ± SD = Mean ± Standard deviation).

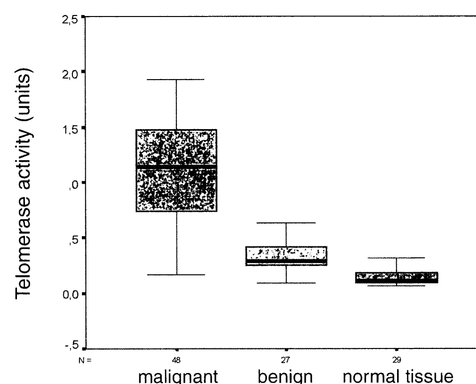

Figure 2. — Box-plot graph of telomerase activity in malignant, benign and normal groups.

Sensitivity against false positive rate (1- specificity) of telomerase activity to predict malignant tissues was plotted on a ROC curve. An ideal curve of a reasonable test should be placed on the left triangle of the ROC graph. This presumption was accomplished as seen in Figure 3. The area under the curve was 95.9 with 95% confidence interval as 91.8-99.9 ($p < .001$). The cut-off value for telomerase activity predicting malignancy was

established as 0.42 units, the value that provided the maximum sensitivity with a minimum false-positive rate. When telomerase activity was ≥ 0.42 units, the sensitivity of predicting malignancy was 93.8% and the specificity was 89.3% (Figure 3).

Positive telomerase activity (according to the defined threshold value) was detected in 93.8% (45/48) of cancer tissues, 27.6% (8/29) of benign disorders and 0% (0/29) of normal tissues by PCR-ELISA assay.

There was a significant difference in telomerase activity between malignant, benign and normal tissues within each histological group ($p < 0.001$) (Table 2).

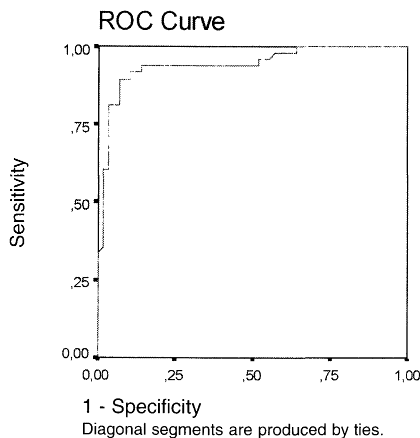

Figure 3. — A threshold telomerase activity of 0.42 units can discriminate malignant gynecological tissues with 93.8% sensitivity and 89.3% specificity. Area under ROC curve = 95.9 $p < .001$.

Table 2.— *Telomerase activity in malignant, benign and normal gynecological tissues according to histological origin.*

Tissue origin	Tissue histology	n	Mean telomerase activity by ELISA	p
Vulva and cervix	Squamous cell carcinoma	12	0.68 ± 0.36	< .001
	Chronic cervicitis	7	0.30 ± 0.12	
	Normal cervix and vulva	11	0.11 ± 0.06	
Endometrium	Endometrioid carcinoma	12	1.06 ± 0.54	< .001
	Normal endometrium	9	0.17 ± 0.07	
Ovary	Ovarian carcinoma	26	1.12 ± 0.52	< .001
	Benign ovarian cysts	12	0.43 ± 0.11	
	Normal ovaries	9	0.13 ± 0.06	

Discussion

Telomerase activity has been found in germline tissues, immortal cells, cancer cells and proliferative cells of renewal tissues such as the hematopoietic system, basal cells of the skin, hair follicules and crypt cells in the intestine [23, 27] .

In several studies, it has been reported that benign proliferative lesions as well as normal tissues in the genital tract showed no telomerase activity, thus telomerase may serve as a tumor marker for genital tract malignancies [6, 17, 28]. Others have reported telomerase activity in normal gynecological tissues [18-21]. In this study, telomerase activity was found to be high (1.03 ± 0.38 units) in the malignant group and lower (0.37 ± 0.25 units) in the benign pathologies such as ovarian cysts, myoma uteri and chronic cervicitis. Initially telomerase activity was thought to be cancer-cell specific, but now it is becoming clear that many proliferating normal cells express telomerase activity at a lower level. The low levels of activity in normal and benign lesions may be necessary for their physiological proliferation [19, 29]. On the basis of our findings, the arbitrary cut-off value was set up at 0.42 units, where the sensitivity was 93.8% and the specificity was 89.3% for malignancy. Tatsuma *et al.* assessed telomerase activity by PCR-ELISA in hepatocellular carcinomas, chronic liver disease and healthy controls and the arbitrary cut-off level was set at 0.7 units [7]. Streutker *et al.* regarded the absorbance values between 0.2-0.5 as low activity and the absorbance values higher than 0.5 units as high activity in neuroblastomas [30]. To our knowledge, no data has been reported in the English literature about a cut-off value to differentiate gynecological malignancies from benign disorders and normal tissues by using the telomerase PCR-ELISA assay.

In this study the false-negative rate was 6.3% and false positive rate was 13.7% by PCR-ELISA. The telomerase negativity of malignant tumor samples was attributed to inactivation of the enzyme during freezing and thawing of samples by Zheng *et al.* [6]. Some immortal cell lines were shown to have undetectable or very low telomerase activity, so it is thought that these cells can maintain their telomeres by telomerase-independent mechanisms known as alternative lengthening of telomeres [31]. Another explanation for the very weak activity of malignant tumor samples may be possible contamination with hematopoietic stem cells and inflammatory cells, since these cells express low levels of the enzyme which may increase upon proliferation [23].

Telomerase activity was found to be significantly correlated with advanced surgical stage and with pelvic lymph node metastasis in endometrial cancer [32, 33]. The method of quantitative determination of telomerase activity used in this study may be further studied for its prognostic value in larger future studies.

A highly sensitive and specific method for determining telomerase activity was used in this study, and a threshold value was found to differentiate malignant tissues. Experimental anticancer therapy targeting telomerase RNA is currently being investigated [34-37]. Therefore telomerase is a promising marker in the prognosis and follow-up of gynecological malignancies. Quantitative detection of telomerase in the peritoneal fluid of gynecological malignancies is an interesting area of future research. Another interesting area is the differential diagnosis of malignant and benign smooth muscle tumors of the uterus using Telomerase PCR Elisa assay.

Conclusion

In this preliminary study, malignant gynecological tissues could be differentiated from benign tissues by measuring telomerase activity with a high sensitivity and specificity. There is ongoing research for the differentiation of cellular leiomyoma from sarcoma using telomerase activity by the same group.

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