ORIGINAL CONTRIBUTIONS

TELOMERASE EXPRESSION AND ACTIVITY IN ENDOMETRIAL CANCER

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ЭКСПРЕССИЯ И АКТИВНОСТЬ ТЕЛОМЕРАЗЫ В ОПУХОЛЕВОЙ ТКАНИ ЭНДОМЕТРИЯ

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Telomerase is an enzyme, the activity of which is detectable in 85–90% of human cancers and its activation may be crucial for the growth of cancer cells. Human telomerase consists of catalytic subunit (hTERT), RNA subunit (hTR) and associated proteins. To evaluate telomerase as a diagnostic factor in endometrial cancer, we tested activity of telomerase and the expression of *hTERT* and *hTR* genes. Samples of normal and tumor tissue from endometrial cancer patients were obtained from 40 postmenopausal women. The activity of telomerase was assessed by a PCR-based telomere repeat amplification protocol assay (TRAP). *hTR* and *hTERT* genes expression was evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) with *GAPDH* as an indirect marker of tissue integrity. 39 (97.5%) of 40 cancerous samples were telomerase positive, whereas only 16 (40%) of 40 control samples exhibited telomerase activity. None of the 40 normal samples had high telomerase activity (after 100-fold dilution) and only one of cancer samples exhibited low activity not detected after 10-fold dilution. The distribution of telomerase activity in normal and cancerous endometrium groups differed significantly ($p < 0.001$). hTR expression was found in 37 (93%) of 40 endometrial cancer samples, whereas 33 (83%) of 40 samples displayed expression of hTERT. In normal endometrium these values were 31 (78%) and 9 (22%), respectively. Our results suggest that the appearance of endometrial cancer may be associated with the expression of human telomerase catalytic subunit, hTERT and human telomerase RNA, hTR. Therefore, coupled expression of hTERT and hTR may be considered as a diagnostic marker of endometrial cancer. Endometrial cancer may be also associated with telomerase activation, but low activity of the enzyme may also be detected in non-cancerous endometrium.
Key Words: **telomerase, endometrial cancer, diagnostic marker, telomere repeat amplification protocol (TRAP), reverse transcriptase polymerase chain reaction (RT-PCR).**

В 85–90% опухолей человека выявляют активность теломеразы – фермента, играющего критическую роль в раковых клетках. Теломераза человека состоит из каталитической субъединицы (hTERT), РНК-субъединицы (hTR) и ассоциированных белков. Для оценки теломеразы как диагностического фактора при раке эндометрия было проведено исследование активности теломеразы и экспрессии генов *hTERT* и *hTR* в образцах нормальной и опухолевой тканей 40 женщин в постменопаузальный период. Активность теломеразы оценивали с помощью TRAP-метода, экспрессию генов *hTR* и *hTERT* – в RT-PCR, используя в качестве непрямого маркера целости ткани *GAPDH*. Выявлено, что по признаку активности теломеразы 39 (97,5%) из 40 образцов опухолевой ткани являются положительными, в то время как таковыми были только 16 (40%) из 40 образцов контроля. Ни в одном из 40 образцов нормальной ткани не обнаружено высокой активности теломеразы (наблюдаемой при 100-кратном разведении образца) и только в одном из образцов опухолевой ткани выявлена низкая активность фермента (не регистрируемая после 10-кратного разведения образца). Различия в уровне активности теломеразы между нормальным и трансформированным эндометрием были значительными ($p < 0,001$). Экспрессия генов hTR и hTERT обнаружена в 37 (93%) из 40 и в 33 (83%) из 40 образцах опухолевой ткани соответственно, для образцов нормального эндометрия эти величины составили 31 (78%) и 9 (22%) соответственно. По нашим данным, рак эндометрия может быть ассоциирован с экспрессией каталитический субъединицы теломеразы человека, hTERT, и РНК-теломеразы человека, hTR. Одновременную экспрессию этих генов можно рассматривать как диагностический маркер при раке эндометрия.
Ключевые слова: **теломераза, рак эндометрия, диагностический маркер, TRAP, RT-PCR.**

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Abbreviations used: hTERT — human telomerase reverse transcriptase; hTR — human telomerase RNA; RT–PCR — reverse transcriptase polymerase chain reaction; TRAP — telomere repeat amplification protocol.

Telomerase is a ribonucleoprotein polymerase that synthesizes telomeric repeats at the very end of eukaryotic chromosomes. It is composed of two main parts: the telomerase reverse transcriptase (hTERT), which is the catalytic protein component, and the telomerase RNA (hTR), which provides the template for telomere synthesis [1]. Telomerase is reported to be expressed in most

human tumors and cancer cell lines, yet is not active in most normal diploid human cells [2–7]. It is required in the advancement of the neoplasm to malignancy [5, 6]. Although the correlation between telomerase activity and prognostic factors, like grade and stage of tumor, is not fully established, the activity of the enzyme is useful in early detection of many types of human cancers [8, 9].

Human telomerase RNA component provides the template for the synthesis of the human telomeric repeat $(TTAGGG)_n$. Telomerase enzyme activity and template RNA have a similar distribution and usually are expressed concordantly [10], although hTR expression may occur independently of telomerase activity or may preceed it [11–13]. Normal human diploid cells contain the integral RNA component of telomerase, hTR, but generally lack the mRNA for the catalytic subunit [14]. An increase in the relative levels of hTR in tumor cells compared with those in adjacent tissue indicate that it may be clinically useful in the diagnosis of cancer [10, 15, 16].

Despite to hTR, the expression of hTERT was predominantly reported in cancer tissues with positive telomerase activity [14, 17, 18]. Furthermore, introduction of a gene encoding hTERT into telomerase negative human cells induced the activation of telomerase [19, 20]. Taking together, hTERT is now regarded as the rate–limiting determinant of human telomerase enzyme activity, and up–regulation of hTERT expression is thought to play an important role in human carcinogenesis [21].

Telomerase activity has been reported to occur in normal human endometrium [22]. The activity seemed to be correlated with the phase of menstrual cycle and tightly connected with the proliferative activity of endometrial cells. These findings suggest that telomerase might be a regulated enzyme and can play a significant role in the maintenance of the cyclic renewal of the human endometrium [22]. The basal rather than the functional endometrial lining has been designated as the probable origin of telomerase activity in normal endometrial tissue [23]. A semiquantitative analysis of normal endometrium demonstrated that most samples of the mid and late proliferative phase exhibited high telomerase activity, correlating with the proliferating cell nuclear antigen labelling index of the endometrial glands [22, 24].

Endometrial cancer, which belongs to the most frequent gynaecological malignancy, is frequently curable, but the commonly used test may give relatively high percentage of false–negative results [25]. Therefore, recognition of a reliable marker of endometrial cancer is an imperative task. Such a marker may contribute to diminishing incidence and mortality from this cancer.

In the current study telomerase activity and the expression of its catalytic subunit, hTERT, RNA template, hTR, in normal and cancerous endometrium derived from 40 postmenopausal women were measured using telomeric repeat amplification protocol (TRAP) and reverse transcriptase polymerase chain reaction (RT–PCR), respectively.

MATERIALS AND METHODS

Patients and samples. Samples of normal and tumor tissues were obtained from 40 postmenopausal women with endometrial carcinoma. The patients were 45 to 73 years old with a median age of 58 years. The samples were immediately frozen and stored at –80 °C. The specimens were stained routinely for histological diagnosis.

Telomerase activity assay. The telomeric repeat amplification protocol assay (TRAP) was carried out with a TRAP–eze Telomerase Detection kit (Oncor, Gaithesburg, MD, USA). Samples of frozen normal and cancer tissue of 100 mg weight were washed in ice–cold phosphate–buffered saline. After washing, the buffer was removed and pellets were homogenized in 200 μl ice–cold CHAPS lysis buffer consisting of 10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β–mercaptoethanol, 0.5% CHAPS, 10% glycerol, pH 7.5. After 30 min incubation on ice, lysates were centrifuged at 12,000 *g* for 20 min at 4 °C and 160 ml of each supernatant was retrieved and frozen at –80 °C. Protein concentration of the extracts was determined by the Bradford assay [26] and 1.5 μg of protein was used in each TRAP assay. For estimation of telomerase activity, the extracts were diluted 10– and 100–fold. Each extract was assayed in 25 μl reaction mixture containing 1 × TRAP reaction buffer (20 mM Tris–HCl, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 0.01% bovine serum albumin), 50 μM dNTPs, 0.5 ml TS primer of sequence 5'–AAT CCG TCG AGC AGA GTT–3', 0.5 μM of supplied Primer Mix (reverse primer; RP, K1 primer and TSK1 template for internal control) and 1 unit of Taq DNA polymerase (Qiagen, Hilden, Germany). After 30 min of incubation at 30 °C for telomerase–mediate extension of the TS primer, reaction mixtures were subjected to 32 cycles of 2–step polymerase chain reaction (PCR) in a MJ Research Thermocycler, model PTC–100. The reaction included denaturation at 94 °C for 30 s and annealing and extension at 55 °C for 30 s. The products of the reaction were electrophoresed on 12% polyacrylamide gel and visualized with SYBIR Green dye (Molecular Probes, Eugene, OR, USA).

Expression of hTERT and hTR. Total RNA was isolated from the samples using commercially available RNeasy total RNA kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol and the quality of the product was checked on an agarose gel. Reverse transcriptase polymerase chain reactions (RT–PCRs) were performed using a OneStep RT–PCR Kit (Qiagen) with primers specific for the telomerase catalytic subunit: 5'–CGG AAG AGT GTC TGG AGC AA–3' and 5'–GGA TGA AGC GGA GTC TGGA–3'; for the RNA subunit: 5'–GGG TTG CGG AGG GTG GGC CT–3' and 5'–ACG GGC CAG CAG CTG ACAT–3'. To minimize the possibility of false–negative results, the tumor material was amplified by RT–PCR for *GAPDH* as an indirect marker of tissue integrity. Briefly, a 297–base pair fragment of the human *GAPDH* gene was amplified with primers 5'–CAC CCA TGG CAA ATT CCA TGGC–3' and 5'–GCA TTG CTG ATG ATC TTG AGG CT–3', corresponding to GenBank positions 213 to 234 and 509 to 487 respectively (GenBank accession no. M33197). Only specimens that were positive

for *GAPDH* mRNA were included in the present study. RT reaction proceeded at 50 °C for 30 min followed by inactivation of RT and primary activation of Hot Start Taq DNA polymerase at 95 °C for 15 min. Amplification parameters were: 31 cycles at 94 °C for 45 s, 57 °C for 1 min and 72 °C for 1 min for *hTERT;* 30 cycles at 94 °C for 1 min, 60 °C for 45 s and 72 °C for 45 s for *hTR* with final extension at 72 °C for 10 min. RT–PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with SYBIR Green dye (Molecular Probes, Eugene, OR, USA).

Data analysis. The significance of the differences between the groups of normal and cancerous endometrium was tested using the χ^2 analysis with SigmaStat v. 2.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 80 endometrial samples including 40 cancerous and 40 respective normal tissue sections originating from 40 postmenopausal women were examined in this study. TRAP assay with 1.5 μg protein extracts revealed that 55 of 80 cases (69%) exhibited positive signal of telomerase activity (Table 1). 39 of 40 (97.5%) cancerous samples were telomerase positive, whereas only 16 of 40 (40%) control samples exhibited telomerase activity. Because the signal intensities varied among samples, we tried to distinguish samples of different telomerase activity by dilution TRAP assay using 10–fold (0.15 μg of protein)– and 100–fold (0.015 μg of protein)–diluted extracts [22]. Telomerase activity detected after 100–fold dilution was classified as high, whereas that detected after 10–fold dilution as moderate. Activity that disappeared in dilution extracts was categorized as low. Table 1 shows that none of the normal 40 samples had high telomerase activity and only one of cancer samples exhibited low activity. The distribution of telomerase activity in normal endometrium group differed significantly ($p < 0.01$) from the distribution observed in cancer endometrium.

The results of RT–PCR examination of expression of the telomerase RNA template subunit (hTR) and telomerase catalytic subunit (hTERT) in endometrial carcinoma and normal samples are displayed in Table 2. 93% of the samples showed the expression of hTR and 83% were hTERT–positive. For normal samples these values were 31 (78%) and 9 (22%) respectively.

Table 1. Telomerase activity in normal and cancerous endometria of postmenopausal women[a]

Endometrium	Telomerase activity			
	Negative	Low[b]	Moderate[c]	High[d]
Normal	24	11	5	–
Cancerous	1	5	12	22

[a]n = 40.

[b]Positive using 1.5 μg of extract, but negative using 0.15 μg of extract.

[c]Positive using 1.5 or 0.15 μg of extract, but negative using 0.015 μg of extract.

[d]Positive using 1.5, 0.15 or 0.015 of μg extract.

Table 2. Expression of the telomerase RNA (hTR) and catalytic (hTERT) subunits in normal and cancerous endometria as assayed by RT–PCR[a]

Subunit	Number of positive samples (%)	
	Normal endometrium	Endometrial cancer
hTR	31 (78)	37 (93)
hTERT	9 (22)	33 (83)

[a]n = 40.

DISCUSSION

In this study we have shown that active telomerase can be present in endometrial tissue of postmenopausal women. We have also clearly shown, that telomerase activity in cancerous endometrium is significantly higher than the activity in normal endometrium.

The use of the sensitive TRAP assay has demonstrated that telomerase activity is present in up to 90% of specimens of the major types of cancer but the fact that telomerase is also active in some normal human cell population, notably lymphocytes and endometrial cells, is problematic [27]. The presence of telomerase activity of normal proliferative tissues in tumor sample material may hamper the clinical application of telomerase assay. The detection of telomerase activity in tissues with normally active enzyme, such as in leukemias [28] and lesions of the endometrium [23, 29, 30], put forward a problem in interpretation as an indicator of tumor status. These facts impose the need for improvement of the methods of telomerase detection in order to allow more quantitative analysis while retaining relative ease of using for routine clinical application. That is why we tried to differentiate between telomerase activities in particular samples by using two dilutions of the extracts.

Our results confirm results obtained in other laboratories. It was shown that nineteen of 20 (95%) endometrial carcinomas and 8 of 8 benign endometrial tissues from premenopausal women exhibited strong telomerase activity, whereas 6 of 6 benign endometrial tissues from postmenopausal women showed only weak telomerase activity [23]. The results of those studies indicate that only weak activity of telomerase is detected in endometrial tissues after menopause, but telomerase activity can be strongly reactivated in patients who develop endometrial cancer. In another study 12 of 13 (92%) endometrial cancers were found to be positive for telomerase activity, but it was also noted, that premalignant lesions and some types of benign tumors might also express weak telomerase activity [22].

Our results, as well as the results of studies conducted in other laboratories, indicate that tissues obtained from postmenopausal women with an inactive endometrium featured only weak telomerase expression. This residual telomerase activity in postmenopausal atrophic endometrium may reflect the loss of regenerating function of the basal endometrial lining. Telomerase activity that is low after menopause may be reexpressed at high levels with the development of cancer.

Our results on the expression of the two main telomerase subunits are in agreement with those obtained in other laboratories [31]. On the other hands, it was also shown that some of non–cancer control tissues expressed similar levels of *hTERT* mRNA to those in cancer tissues, which might indicate that some portion of these tissues might contain displatic or hyperplastic epithelia in which *hTERT* was up–regulated [32]. However, high telomerase activity is specific in cancer tissues and up–regulation of *hTERT* expression in the control tissues does not indicate a high level of en–

zyme activity. A discrepancy between the mRNA level and telomerase activity may originate from an increase in the proportion of lymphocytes, which showed telomerase activity, to epithelial cells. This increase could mask a difference in *hTERT* levels between subsets of carcinoma and normal epithelial cells.

It was reported that normal colon and small intenstine tissues express *hTERT* mRNA and this expression can be detected by RT–PCR in many other normal tissues [14, 17]. Because telomerase is not activated in the great majority of normal tissues, the *hTERT* gene product may be modulated during and after the process of translation in these tissues. It is also possible that splicing of *hTERT* mRNA differs between cancer and normal tissues [33]. Epidermal cells in the suprabasal layers of normal cervical tissues are differentiated; therefore telomerase activity can be inhibited, even though *hTERT* mRNA is expressed due to the inhibitory effect of differentiation on the enzyme activity [34]. This may also concern the endometrium.

In conclusion, high telomerase activity may be linked with appearance of endometrial cancer in postmenopausal women, but non–cancerous tissue from the same organ may exhibit low activity. There is an urgent need to develop an accurate and sensitive method to assess telomerase activity and differentiate between cancer and normal tissue. On the other hand, up–regulation of telomerase RNA, hTR, and telomerase catalytic, hTERT, subunits may be early events during endometrial carcinogenesis. Additional regulation, such as modulation of hTERT gene product, may be needed to activate telomerase, as observed in many cancers, but coordinated expression of the genes encoding hTR and hTERT may be considered as an early indicator of endometrial cancer.

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