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Telomerase activity associated with progression of cervical lesions in a group of Colombian patients

Associação da atividade da telomerase com a progressão de lesões cervicais em um grupo de pacientes da Colômbia

Original Article

Abstract

PURPOSE: To analyze the relation between the cytological findings and telomerase activity (TA). **METHODS:** Cervical samples were evaluated and classified according to the Bethesda System. Telomerase activity was measured total product generated values (TPG) using the TRAP assay (telomeric repeat amplification protocol); data were analyzed statistically using the χ^2 test, with the level of significance set at $p<0.05$. **RESULTS:** The study was conducted on 102 patients. Of these, 3.9% showed normal cytological findings, 8.8% showed cervicitis; 2% showed Atypical Squamous Cells of Undetermined Significance (ASCUS); 67.6% showed Low Grade Squamous Intraepithelial Lesion (LSIL); 11.8% showed High Grade Squamous Intraepithelial Lesion (H-SIL) and 5.9% showed Squamous Carcinoma. Among telomerase-positive samples, the TPG values were cervicitis<normal<ASCUS<L-SIL<H-SIL<Carcinoma. **CONCLUSION:** Results show increased telomerase activity with increasing severity of lesion, supporting the association between TA and type of lesion.

Keywords
Telomere
Telomerase
Cervix uteri
Preneoplastic conditions
Squamous intraepithelial lesions

Resumo

OBJETIVO: Analisar a relação entre os achados citológicos e atividade da telomerase (AT). **MÉTODOS:** Amostras cervicais foram avaliadas e classificadas pelo sistema Bethesda. A AT foi medida como valores de produto total gerado (PTG), utilizando o protocolo de amplificação repetida da telomerase (TRAP); os dados foram analisados estatisticamente usando o teste do χ^2, com nível de significância de $p<0,05$. **RESULTADOS:** Cento e dois pacientes foram analisados: 3,9% com achados citológicos normais, 8,8% com cervicite, 2% com células escamosas atípicas de significado indeterminado (ASCUS), 67,6% com lesão escamosa intraepitelial baixo grau (LEI-BG), 11,8 % com lesão intraepitelial escamosa alto grau (LEI-AG) e 5,9% com carcinoma escamoso. Valores PTG para amostras positivas AT foram: cervicite<normal<ASCUS<LEI-BG<LEI-AG<Carcinoma. **CONCLUSÃO:** Os resultados mostram um aumento na AT com o aumento da lesão, sustentando a associação entre a AT e o tipo de lesão.

Palavras-chave
Telômeros
Telomerase
Colo do útero
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Lesões intraepiteliais escamosas

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Introduction

Telomeres, which form a protective cap on chromosome ends, are usually composed of short G-rich Deoxyribonucleic acid (DNA) repeats (TTAGGG) complexed with proteins[1,2] with estimated lengths of 5-15 kb in humans[3]. Their main function is to protect chromosomes from incomplete replication, nuclease degradation, and end-to-end fusion during replication[3].

During DNA replication, telomeric DNA shortens progressively, mainly due to the end-replication problem, that is, the inability of the DNA replication machinery to fully replicate DNA ends[2,3]. That is why eukaryotic cells have evolved with a specialized reverse transcriptase enzyme, called telomerase, that is responsible for the new telomere extension in most adult tissues[2,3]. Telomerase is a ribonucleic acid (RNA)-dependent polymerase that synthesizes telomeric DNA sequences and provides the molecular basis for unlimited proliferative potential[2-4]. Telomerase contains a template region that is complementary to the telomeric DNA repeat (TTAGGG), to counteract the continuous degradation of telomeres by adding the telomeric DNA repeats to the 3'-ends of chromosomes[2,3].

In certain multicellular organisms, including humans, telomerase is strongly repressed in normal somatic tissues, but expressed in highly proliferative cells, including ovaries, testes and hematopoietic tissues[2], Continued proliferation of human cells requires maintenance of telomere length, usually accomplished by telomerase[1,2]. Telomerase activity can also be detected in most primary human tumor specimens and tumor-derived cell lines, and its activity gradually increases with the progression of cancer[4-6]. In fact, telomerase activity is detected in 85 to 95% of the most common cancers, such as prostate, breast, colon, lung, neuroblastoma, pancreatic, uterine and liver cancer[4-7]. This association between telomerase (specifically the telomerase reverse transcriptase subunit) and cancer has opened new paths for cancer and anticancer therapy research[7,8].

Cervical cancer develops from precursor lesions[9], which can be classified by the Bethesda System[10,11]. This classification system is used for reporting cytological findings during a cervical cytology procedure. Cytological findings are classified as normal/benign reactive changes (as cervicitis), squamous cell abnormalities, and glandular cell abnormalities. Squamous cell abnormalities include: atypical squamous cells of undetermined significance (ASCUS); atypical squamous cells which cannot exclude high-grade intraepithelial lesions (ASC-H); low grade squamous intraepithelial lesions (L-SILs), encompassing evidence of human papilloma virus (HPV) infection and/or mild dysplasia; and high grade intraepithelial lesions (H-SILs), including moderate dysplasia, Cervical Intraepithelial Neoplasia grade II (CINII) and Cervical Intraepithelial Neoplasia grade III (CINIII)[11]. Thus, in the natural process of cervical carcinogenesis, lesions can be classified into three grades: low-grade squamous intraepithelial lesion (L-SIL), high-grade squamous intraepithelial lesions (H-SIL), and cervical carcinoma[10]. The Bethesda system for cervical cytology reporting should be used universally as it provides a standardized interpretation[10,11].

Telomerase has also shown to be reactivated in premalignant lesions such as SILs and other tumors[6,12]. Some studies have shown that telomerase activity is higher in tumor tissue compared with normal tissue, but its role in precancerous lesions and its relation with disease progression are yet to be defined[13], although the analysis of telomerase as a potential biomarker of cervical dysplasia has been the focus of intense study for several years[12-14]. In this way, a rapidly expanding body of data suggests that the detection of telomerase expression could play a useful role as a diagnostic adjunct in the practice of cervical cytopathology. There appears to be a corresponding increase rate of detection of telomerase activity with increasing severity of cytological abnormalities[15]. The induction of telomerase expression in normal human epithelial cells and fibroblasts resulting in infinite replication and telomerase activity is evident in approximately 90% of human solid tumors, suggesting that the expression of telomerase has a role in malignant transformation[4,6].

Telomerase activity can be measured *in vitro* by using the telomeric repeat amplification protocol[16]. This assay has been used to test telomerase activity in numerous cancer specimens, and in uncultured and cultured samples of normal tissue from many cell types[16].

The aim of the present study was to analyze the relation between cytological reports and telomerase activity using samples from women with a cytological abnormality in a Colombian population.

Methods

Patients and cytological samples

This was a descriptive study with patients from the Quindío University Health Center. Women with previous abnormal cytological reports were enrolled; no patients were excluded. However, when including them into the study, a new cytological evaluation was performed, ultimately using this report for the investigation. The Institutional Bioethical Committee approved the research. Informed consents were provided to all patients.

After written consent was obtained, the cervix of voluntary women was scraped with an endocervical brush. Cervical scrapings were evaluated by a pathologist through a pap smear staining procedure in order to classify them according to the Bethesda system. The scraped cells were then suspended in saline solution at room temperature, and finally frozen and stored at -20ºC until used for the telomerase activity assessment by means of the TRAP assay. Telomerase activity assays and cytological/histological examinations were performed independently in a blinded manner.

For the TRAP assay, the biopsy samples were centrifuged, and thus concentrated. Pellets were homogenized in 100 µL of ice-cold lysis buffer — 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol (Chemicon International). After 30 min of incubation on ice, the lysate was centrifuged at 13,000 rpm. for 30 min at 4ºC, and the supernatant was frozen and stored at -80ºC. The protein concentration in the extract was measured by Bicinchoninic Acid assay[17]. For the TRAP assay, 0.5 µg of protein were used. Each extract was assayed in 25 µL of reaction mixture containing 5X TRAP reaction mix containing TS primer (5'-AATCCGTCGAGCAGAGTT-3'), and a reverse primer (RP) with a modified sequence (instead of a CX primer), 2U Taq Polymerase, dH_2O, and tissue extract according to the manufacturer. Each reaction mixture contained an Internal Telomerase Assay Standard for the quantitative estimation of telomerase activity levels used for the identification of false negative tumor samples containing Taq inhibitors[18]. All samples had an additional negative control consisting of RNAse treatment for each one before the performance of the PCR step. Besides, two positive controls were included per each PCR setting, which were: synthetic oligonucleotide with 8 telomeric repeats (TSR8) serving as a template of the PCR reaction, and telomerase-positive cell extract provided in the kit. PCR cycles consisted of two steps: a 30 min incubation step at 30ºC for telomerase-mediated extension of the TS primer, and then 33 cycles at 94ºC for 30s, and at 55ºC for 30s. PCR products were electrophoresed on a 10% polyacrylamide gel and visualized by silver staining[19].

The presence of telomerase activity was established by inspecting the films for the presence of the characteristic 6-base pair increment ladder, as seen in Figure 1. The level of telomerase activity is expressed as an absolute value[20] and called TPG (total product generated) value. One unit of TPG was defined as 0.001 mole, or 600 molecules, of TS primer extended for at least three telomeric repeats by telomerase present in the extract[20]. One TPG corresponds approximately to telomerase activity from one immortal cell and is determined by the formula TPG=(Telomerase sample – RNAse treated sample)/Internal control of sample (TSR8-negative control)/(Internal control of TSR8) x 100[21].

Statistical analysis was performed using the χ^2 test to evaluate significant differences. Findings with $p<0.05$ were considered statistically significant. All analyses were performed using the SPSS statistical analysis program, version 14.0.

Results

This study included 102 patients. The age range was between 17 and 71 years. Four of them had normal cytological findings (3.9% of all the cases), while 96.1% had some abnormal cytological findings described as follows: 9 cases of cervicitis (8.8%); 2 cases of Atypical Squamous Cells of undetermined significance (2%); 69 cases of low grade squamous intraepithelial lesion 69 (67.6%); 12 cases of high grade squamous intraepithelial lesion (11.8%); and 6 cases of squamous carcinoma (5.9%). Thus, L-SIL was the more frequent lesion in our sample, followed by H-SIL, cervicitis, carcinoma and ASC-US.

Table 1 shows the cytological results, final histological diagnosis for the cervical scraping and telomerase activity. The higher frequencies (100%) of positivity for telomerase activity were found in normal (4/4), ASCUS (2/2), H-SIL (12/12) and carcinoma (6/6) reports, followed by L-SIL with a 75.4% of positivity (52/69), and cervicitis reports with 55.6% (5/9).

Telomerase activity was determined as the total product generated value (TPG). Population TPG average was 31.6 with values ranging from zero, in those cases

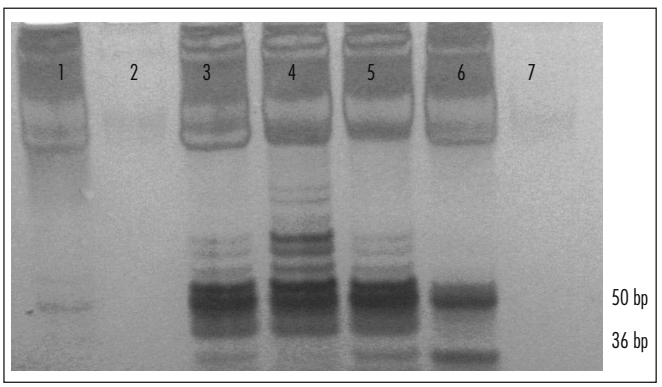


Figure 1. Telomerase activity in human cervical cancer using the TRAP assay (Silver staining). (1) negative sample; (2) sample treated with Proteinase K; (3) sample with increased activity; (4) Telomerase Substrate template oligonucleotide (TSR8) Quantification Control; (5) positive cells provided by the kit; (6) sample with decreased activity; (7) positive cells treated with proteinase K

with telomerase activity absence, to 133.3 in carcinoma. Telomerase activity measured in TPGs showed a differential tendency ($p<0.05$) according to cytological reports (Figure 2).

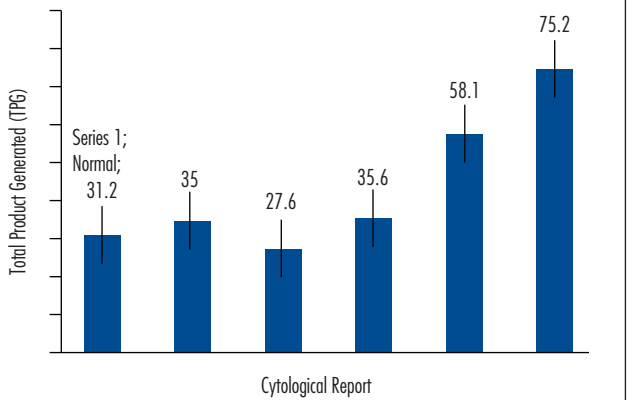
Among telomerase-positive cases, the lower TPG values were found in cervicitis with a minimum value of 11.1 and a maximum value of 50 TPGs. Then, following cervicitis cases, the increase of TPG values is followed by normal, ASC-US, L-SIL, H-SIL, and carcinoma findings. Carcinoma showed the higher values of telomerase activity as shown in Figure 2.

There were significant differences in telomerase activity levels among the groups ($p<0.05$; L-SIL *versus* H-SIL, L-SIL *versus* carcinoma, H-SIL *versus* carcinoma). However, when comparing patients with normal reports with carcinoma, the differences seem to be stronger ($p<0.005$).

Table 1. Cytology results of cervical scraping and telomerase activity

Cytological report	n	Telomerase activity		
		Positives n	Negatives n	Positivity %
Normal	4	4	0	100
Cervicitis	9	5	4	55.6
ASCUS*	2	2	0	100
L-SIL**	69	52	17	75.4
H-SIL***	12	12	0	100
Carcinoma	6	6	0	100
Total	102	81	21	79.4

*ASCUS: Atypical Squamous Cells of Undetermined Significance; **L-SIL: Low grade Squamous Intraephitelial Lesion; ***H-SIL: High grade Squamous Intraephitelial Lesion.



ASCUS: Atypical Squamous Cell of Undetermined Significance; LSIL: Low grade Squamous Intraephitelial Lesion; HSIL: High grade Squamous Intraephitelial Lesion. There were significant differences between TPG values of each cytological report ($p<0.05$).

Figure 2. Telomerase activity (in TPG unites) in different cervical lesions.

Discussion

Telomerase activity has been demonstrated in a wide variety of human tissues, through malignant tumors or precancerous cells, such as cervical lesion[12-15]. However, we found negative telomerase activity in 20.6% of the samples of this study. Related literature describes negative telomerase activity despite the presence of cervical lesions[14,22-24]. Accordingly, a study on a Chinese population, found a negativity percentage close to that obtained for our population[22]. However, 52.9% of negativity was found in a German population[14]; while in two Japanese populations, the negativity percentage was 50 and 54.9%, respectively[18,23]. On the other hand, negativity rates reached 75% for a Venezuelan population[24]. The development of a malignant phenotype among certain types of cancers is independent of telomerase, and telomerase activation may not be a strict requirement of carcinogenesis for all cervical cancers[24], which seems to be the argument that explains these negativity percentages.

Related literature illustrates that about 10 to 15% of cancers and cancer-derived cell lines are telomerase negative but instead maintain telomere length by a homologous recombination-based pathway called alternative lengthening of telomeres (ALT)[25,26]. Likewise, it is also possible that samples might be contaminated with excessive blood or necrotic cells, possibly including telomerase inhibitors, leading to false negative results. Studies suggest that the inhibitory effects observed in the TRAP assay were due to the Taq inhibitors used[27].

A correlation between telomerase positivity and cytological reports could not be found. However, when quantifying telomerase activity, it became significantly higher according to cervical lesion reported. These results support previous reports, in which telomerase activity levels seem to be related to the pathologic stages of different types of cancers including hepatocellular carcinoma (HCC)[28], where human telomerase reverse transcriptase (hTER) RNA expression showed a statistically significant relationship with tumor size in HCC patients regarding a cervical lesion[24,29]. A correlation between telomerase activity or hTER expression and the varying degrees of cervical lesions was observed regarding potentially malignant oral lesions. A correlation in telomerase activity and severity of the lesion[30] was also found.

Furthermore, our study results are not only in accordance with those of several other groups who have identified telomerase activity in the majority of malignant tumors, but also in line with those who have identified telomerase activity in proliferative diseases, predisposing the development of malignant tumors[28-32].

Studies reporting a positive correlation between telomerase activity and pathologic degree of tumor[29-30] suggest that the cells with higher telomerase activity

levels have a selective growth advantage due to improved stability of chromosomes with restored telomeres.

The fact that samples with normal findings showed telomerase activity was not expected, since normal samples have not shown positivity in other populations, such as Japan[23] Venezuela[24], and India[33] among others. Nevertheless, all the normal samples in our study were telomerase-positive. By checking each normal sample individually, it was found that, in previous cytological reports from those women (dated 6 to 12 months before the study), cytological findings were abnormal. One of them showed L-SIL while the others exhibited ASC-US findings. It is possible that, for this study, normal reports rendered a false-negative result for cervical lesion in the screening report. This observation could be a first explanation as to why telomerase activity was found in samples with normal reports. Nonetheless, in some studies, telomerase activity has also been detected on other populations with normal reports[14,22]. This has been attributed to the sensibility of the technique[22], or the presence of activated lymphocytes or granulocytes, which can also induce a positive result since both have active telomerase[14].

Given that predicting the evolution of ASCUS into another type of injury remains a controversial issue, it is not easy to explain the cause of telomerase positivity in samples with normal findings. However, according to some authors, the emergence of HSIL is common in cases where changes that define the ASCUS on immature squamous cells occurred[10,11].

Through the analysis of both samples with ASCUS reports included in this study, it was evident that one of them presumed metaplasia. Thus, it can be said that detecting telomerase activity, in cases with ASCUS reports, is not an eventuality, since cells could be showing a metaplastic abnormality. However, there is not enough evidence for this affirmation. As far as we know, this is the first study about telomerase activity and cervical cytology report in Colombia.

Our study shows increased activity with increasing severity of lesion, supporting the association between telomerase activity and type of lesion, but more studies are needed to strengthen the telomerase activity assay as a potential marker of pre-neoplastic cervical lesions.

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