

# Telomerase activity in cervical scrapes of women with high-grade cervical disease: A nested case-control study

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**Abstract.** Epidemiological information on telomerase activity (TA) and development of cervical lesions is scarce. A nested case-control study was carried out within a cohort of Colombian women tested for Human Papillomavirus (HPV). Measurement of TA was done in cervical scrapes of 25 women who developed High Grade Squamous Intraepithelial Lesion (HGSIL) during the first 6 years of follow-up and was compared with that of 104 control women who maintained normal cytology during the entire follow-up. TA was measured by a telomerase repeat amplification protocol-ELISA. TA and HPV infections were significantly more frequent in cases than in controls. Likewise, 68% of the cases were positive for both TA and HPV compared with only 7.7% of the controls ( $P<0.0001$ ). Factors independently associated with increased odds of HGSIL included TA, high risk HPV (hrHPV) infections and multiple parities. When restricted to hrHPV positive women, TA was strongly associated with increased odds of HGSIL (adjusted odds ratio=37.94, 95% confidence interval, 1.64-678.1). In addition to an infection with hrHPV, TA appears to be a significant cofactor for HGSIL.

## Introduction

Worldwide, cervical cancer is the third most common cancer found in women. There are 529,000 new cases diagnosed each year (1). In Colombia it has an incidence of 26.1/100,000 and is the most common cause of death from cancer in women (2). High-risk human papillomavirus (hrHPV) are detected in virtually all cervical carcinomas (3,4). However, other host

cell factors are required for progression of hrHPV-induced precancerous lesions to invasive cancer.

Telomeres are specialized structures at the end of linear chromosomes. They consist of non-coding tandem repeated TTAGGG sequences which are involved in chromosomal stability. In somatic cells, during each cell division, telomeres are shortened progressively until reaching cellular senescence (5). Telomerase is a ribonucleoprotein enzyme complex containing several components, between them a catalytic subunit composed of hTERT (human Telomerase Reverse Transcriptase) and a RNA component (hTR). The hTR component acts as a template for elongation of telomeric DNA (6). Normal tissues and human somatic cells show low or undetectable levels of telomerase activity (TA), whereas immortalized cells and tumour cells from a variety of cancers show highly detectable TA (7,8). Telomerase activation, which is very important for cell immortalization, has been proposed as a critical step for the development of different types of cancer including cervical cancer (9).

Identifying molecular markers that will permit diagnosis and prognosis of women who have cervical lesions as well as predicting which precursor lesions are likely to become cancerous will lead to improvements in cervical cancer prevention. Several studies on women with normal cytology and/or different grades of cervical lesions have shown increased TA in higher grade lesions (10-13). Some studies have also shown that infections with HPV 16 and 18 are associated with increased TA (14,15). In addition a few studies have shown that expression of HPV16 E6, along with E6AP can induce hTERT transcription and TA (16-20). Gain of chromosome 3q, containing the sequence for the telomerase RNA component (TERC), and gain of chromosome 5p, containing the TERT gene, have been also associated with CIN2/CIN3+ in cervical tissue (21-23).

To the best of our knowledge, no previous epidemiological analysis has been performed on the association between TA and HPV in cervical scrapings, analyzing the role of TA as a possible risk factor for HGSIL adjusted for different known risk factors. Here we report the results of HPV and TA detection in a nested case control study within a cohort of Colombian women.

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**Key words:** telomerase, papillomavirus infections, risk factors, scraping, cervical intraepithelial neoplasia

## Materials and methods

**Study population.** Between November 1993 and November 1995 the Colombian National Cervical Cancer Institute conducted a population census in four health districts in Bogotá. Two thousand women aged 18-85 years were randomly identified and invited to participate in the cohort study. In order to acquire information on sexually active adolescents, 200 sexually-active women aged 13-17 years, all of whom had come to an adolescent clinic for contraceptive counseling without a doctor's referral, were also invited to participate. The ethics committee of INC approved the protocol. Methods of recruitment and data collection have been described elsewhere (24). Briefly, eligible women were those residing in Bogotá, without history of cervical neoplasia, conisation or hysterectomy willing to participate and who gave written informed consent. At study entry and at each follow-up visit, participants responded to a questionnaire on risk factors for cervical cancer and underwent a gynaecological examination with collection of cervical cells for cytology and HPV DNA detection. Colposcopic examination of the cervix was performed in all women with repeated cytological diagnosis of Low Grade Squamous Intraepithelial Lesions (LGSIL) or with cytological evidence of High Grade Squamous Intraepithelial Lesions (HGSIL). Colposcopically guided cervical biopsies were performed in women with cytological or colposcopic evidence of HGSIL. Follow-up visits were scheduled every 6 months for up to 10 years until March 2004.

The analysis described here is a nested case control study of the study cohort. Cases were defined as any women with cytological HGSIL or biopsy-confirmed CIN2/CIN3+ during the first six years of follow up. Controls were defined as any women that maintained normal cytology results during the entire follow up period.

All cases observed during the first six years of follow up were selected, while controls were randomly chosen from the participants of the study, but assuring a group age match ( $\pm 2$  years) with cases (at diagnosis). To maximize the statistical power of the study sample and considering the small number of cases diagnosed during follow up within the study, a case: control ratio of 1:4 was used.

From the 38 cases observed during follow up, only 25 cases (3 Invasive cancers, 10 CIN3, 5 CIN2 and 7 HGSIL) had available sufficient cervical scrape sample to analyze TA and 104 controls were selected with the defined criteria. HPV infections had previously been tested using GP5+/GP6+ PCR-EIA and genotyped using a Reverse Line Blot assay (RLB), as described previously (25). Samples were analyzed for the presence of 14 high risk HPV types (hrHPV) and 23 low risk HPV types (lrHPV).

**Sample processing for TA.** Samples were processed as described previously (10). Briefly, samples were centrifuged at 1,200 rpm. Pelleted cells were treated with trypsin at 37°C for 5 min to separate cell clusters. Cells were counted with a haemocytometer, washed twice with PBS, and stored as pellets at -80°C until their use in a TRAP (Telomeric Repeat Amplification Protocol) assay.

**TRAP assay.** TA was measured by TRAP assay using a commercially available kit, Telo TAGGG Telomerase PCR

enzyme-linked immunosorbent assay (ELISA) from Roche Applied Science, Mannheim, Germany. Assays were performed according to the manufacturer's specifications. In addition to the kit's positive control, cell extracts from HeLa cells were used as another positive control for each assay. Heat-treated cell extracts at 85°C for 10 min from positive controls and samples were used as negative controls. Final optical Densities (ODs) of samples were obtained by subtracting ODs of negative controls from those of the samples. Samples were regarded as telomerase positive if the difference in absorbance is higher than  $0.2 A_{450nm} - A_{690nm}$  units.

**Statistical analyses.** HPV infection, TA and cofactors related to cervical cancer aetiology (age, parity, smoking status and oral contraceptive use) were included in the analysis. Fisher's exact test and chi-squared test were used to compare HPV detection, TA and cofactors between cases and controls. Association of TA with HGSIL was evaluated using logistic regression analysis. As hrHPV infection is a necessary but not sufficient cause of cervical neoplasia, we also assessed the association of telomerase and HGSIL among women positive for hrHPV. Odds ratios (OR) and 95% confidence intervals (CIs) are reported for crude and adjusted analysis. All statistical analysis was done using STATA software (version 9, StataCorp, College Station, TX, USA).

## Results

**Characteristics of the population.** Characteristics of the cases and controls are summarized in Table I. Women included in this study were aged 14-68 years. While age and education were similar in both groups and no differences were evident in oral contraceptive use and smoking status, parity tended to be higher among cases, but the difference was not statistically significant.

**HPV detection and TA.** Among cases, 84% of the samples were HPV positive; all of these were positive for hrHPV genotypes (100%), predominantly of the alpha 7 and 9 species (Fig. 1). Among controls, 22.1% of the samples were HPV positive and 60.8% of these specimens were hrHPV positive. In this group of women, a mixture of hrHPV and lrHPV types were detected, predominantly of the alpha 3, 7 and 9 species (Fig. 2). HPV was detected in significantly more cases than controls (Table I).

TA was positive in 76% of cases, significantly higher than in controls, of which only 20.2% were positive (Table I).

Of the cases, 17 (68%) were positive for TA and HPV infection concurrently, 2 (8%) were positive only for TA, 4 (16%) had only HPV infection and 2 (8%) were negative for both. Of the controls, 8 (7.7%) were positive for TA and HPV infection simultaneously, 13 (12.5%) were positive only for TA, 15 (14.42%) had only HPV infection and 68 (65.38%) were negative for both (Fig. 3). Detection of concurrent TA and HPV was significantly higher in cases than in controls ( $P < 0.0001$ ) (Table I).

In the seventeen cases that were positive for HPV infection and TA concurrently, the presence of hrHPV types in single and multiples infections was always detected and they were mainly types belonging to the Alpha 9 species. In the eleven cases with

Table I. Main characteristics of cases and controls. (A) The P-value was calculated from Fisher's exact test. (B) The P-value was calculated from Pearson's chi-square tests. hrHPV, high-risk human papillomavirus.

Main characteristics	Cases		Controls		P-value
	n=25	(%)	n=104	(%)	
Education					
None	1	(4)	1	(1)	0.21 <sup>a</sup>
Primary	8	(32)	19	(18)	
Secondary	14	(56)	61	(59)	
College/university	1	(4)	10	(10)	
Missing	1	(4)	13	(12)	
Age (years):					
<20	1	(11)	11	(11)	0.6 <sup>a</sup>
20-29	8	(24)	19	(18)	
30-44	14	(58)	69	(66)	
45-64	1	(4)	3	(3)	
65+	1	(3)	2	(2)	
Parity:					
0-1	3	(12)	35	(34)	0.07 <sup>a</sup>
>2	21	(84)	69	(66)	
Missing	1	(4)			
Oral contraceptives:					
Never	11	(44)	56	(54)	0.49 <sup>b</sup>
Ever	14	(56)	48	(46)	
Smoking status					
Never	18	(72)	72	(69)	0.62 <sup>b</sup>
Ever	7	(28)	32	(31)	
HPV					
Positive	21	(84)	23	(22.1)	<0.0001 <sup>b</sup>
Negative	4	(16)	81	(77.9)	
hrHPV					
Positive	21	(84)	14	(13.5)	<0.0001 <sup>b</sup>
Negative	4	(16)	90	(86.5)	
Telomerase activity (TA)					
Positive	19	(76)	21	(20.2)	<0.0001 <sup>b</sup>
Negative	6	(24)	83	(79.8)	
HPV and TA					
Concurrent	17	(68)	8	(7.7)	<0.0001 <sup>b</sup>
No concurrent	8	(32)	96	(92.3)	

<sup>a</sup>P-value was calculated from Fisher's exact test. <sup>b</sup>P-value was calculated from Pearson's chi-square tests.

single HPV infections, type 58 was found in one sample and HPV 16, 31, 33, 51 and 52 were found in two samples each. In the 6 cases with multiple HPV infections, different combinations were found (HPV 16 and 26, 31 and 33, 58 and 81 in one sample each; and the combination of HPV 31, 33 and 58 was detected in three different samples). In the eight controls that were positive for HPV and TA simultaneously, high and low risk HPV types were detected in single and multiple infections. In the five controls with single HPV infections, HPV 16, 58, 59, 81 and 84 were found in one sample each. In the three controls

with multiple HPV infections, different combinations were found (HPV 39 and 42; HPV 43 and 68; and HPV 35, 52, 59, 40 and 84, in one sample each). There was no statistically significant interaction between TA and hrHPV infection (P=0.76).

**Risk factors for HGSIL.** Significantly increased odds for HGSIL were observed in women positive for hrHPV (OR=26.10, 95% CI 9.10-74.86) or TA (OR=12.51, 95% CI 4.44-35.24), or with multiple parities (OR=3.40, 95% CI 0.92-23.40). These associations decreased, but remained

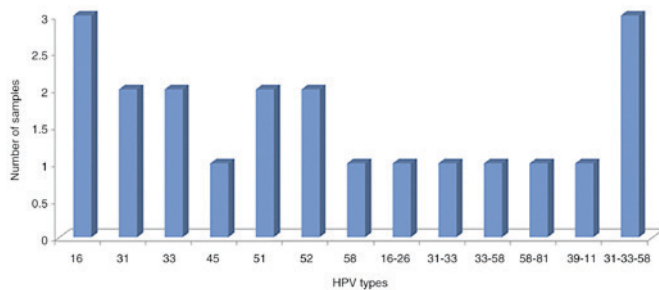


Figure 1. HPV genotypes detected in cases (n=25). Alpha 9 species contains the HPV types 16, 31, 33, 35, 52, 58 and 67. Alpha 7 species contains the HPV types 18, 39, 45, 59, 68 and 70. HPV, human papillomavirus.

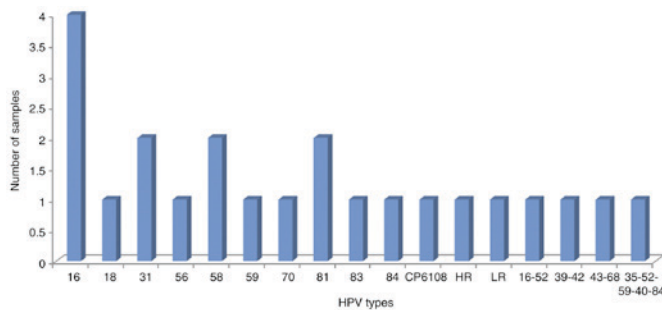


Figure 2. HPV genotypes detected in controls (n=104). Alpha 9 species contains the HPV types 16, 31, 33, 35, 52, 58 and 67. Alpha 7 species contains the HPV types 18, 39, 45, 59, 68 and 70. Alpha 3 species contains the HPV types 61, 72, 81, 83, 84. HR, unidentified high risk HPV genotype(s); LR, unidentified low risk HPV genotype(s); HPV, human papillomavirus.

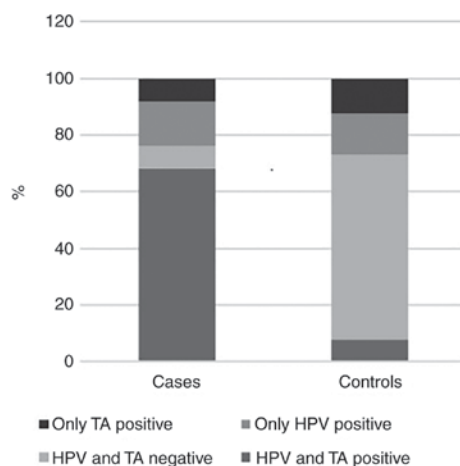


Figure 3. Detection of HPV and Telomerase activity in cases and controls. TA, telomerase activity; HPV, human papillomavirus.

significant, when adjusting for relevant cofactors; (OR=18.26, 95% CI 4.43-76.92), (OR=5.52, 95% CI 1.43-21.30), and (OR=3.50, 95% CI 1.03-10.30), respectively (Table II). When considering only women with hrHPV infection, the association between TA and HGSIL was also significant (OR=7.7, 95% CI 1.63-35.79). When adjusting for age and other cofactors, this association remained significant (OR=37.94, 95% CI 1.64-678.1). No other risk factors were clearly associated with the risk of HGSIL (Table III).

## Discussion

To the best of our knowledge, no epidemiological studies have been performed so far analysing association of TA with HPV in cervical scrapes and its role as a possible risk factor for HGSIL adjusted for different known risk factors. Our results show that TA is a risk factor for high grade cervical lesions. Furthermore, TA was associated with increased odds of high grade cervical lesions in hrHPV infected women. We propose that TA can be added to the list of HPV cofactors previously described (26). Moreover, in addition to the mentioned cofactors, TA can be measured objectively, as can HPV infection.

TA was detected in 76% of the cases studied, and in 21.1% of the controls. These results are in agreement with previous studies which have detected TA in cancer and precancerous lesions while showing undetectable or low TA levels in normal tissue (12,13). Similar results were observed for the detection of HPV DNA. As expected, HPV DNA was detected in 84% of the cases and 22.1% of the controls. Multiple studies have shown a high prevalence of HPV (particularly with high risk genotypes) in women with HGSIL and a low prevalence in women without lesions (27-29).

When both TA and HPV status were considered, we found a higher percentage of TA and HPV in cases (68%) than in controls (7.7%). These results are in agreement with previous studies on cervical biopsies, in which TA and HPV was detected in a higher percentage of cases with CINII or III than in normal cervical specimens (7,15).

Our results indicate increased odds of HGSIL in women positive for hrHPV, in women positive for TA and in women with more than 2 parities. The epidemiological association of HPV infection and parity with HGSIL and cervical cancer have been widely reported (30-33). However epidemiological information on TA with HGSIL is scanty. A systematic review analyzing the accuracy of telomerase assay in cervical lesions showed a positive association between a positive TA test and high grade cervical lesions with a diagnostic odds ratio (DOR) of 5.8, which is similar to our results (34). However in that review the authors did not take into account other important risk factors, such as HPV infection, of pivotal importance for the development of the disease. When only women with hrHPV infection were included in our analysis, TA showed a positive association with HGSIL in both crude and adjusted models. It was observed that the odds ratio increases when TA detection is adjusted to additional cofactors, although it is important to note that the CI is very wide.

Research into the biological relationship between HPV infection and TA are ongoing. Some studies have shown a relationship between the activation of the enzyme and HPV infection, especially HPV 16 and 18 (14,15). Telomerase activation by HPV has been principally attributed to hrHPV E6 oncoprotein because this protein can act as a transcription factor at the hTERT promoter and upregulate its expression (16,19,35,36). The E7 protein and the pRB pathway have been also found to be involved in the maintenance and induction of TA (37). The fact that amongst cases that were positive for TA, different HPV types of the alpha 9 species were detected, i.e. HPV-16, 31, 33, 52, and 58, could suggest that other hrHPV types can activate telomerase either directly or indirectly. Recently one *in vitro* study published

Table II. Crude and adjusted OR and 95% CI of telomerase activity and relevant risk factors.

Risk Factors	Cases n=25	Controls n=104	Crude		Adjusted	
			OR	(95% CI)	OR <sup>a</sup>	(95% CI)
High Risk HPV infection						
No	4	90	1			
Yes	21	14	26.1	(9.10-74.8)	18.2	(4.33–76.9)
Telomerase Activity						
No	6	83	1			
Yes	19	21	12.5	(4.44-35.2)	5.5	(1.43–21.3)
Age (years):						
<30	9	30	1			
≥30	16	74	1.8	(0.70-4.7)	1.2	(0.46–3.5)
Parity:						
0-1	3	35	1			
>2	21	69	3.4	(0.92-23.4)	3.5	(1.03–10.3)
Oral Contraceptives:						
Never	11	56	1			
Ever	14	48	1.5	(0.69-3.5)	1.4	(0.61–3.5)
Smoking status						
Never	18	72	1			
Ever	7	32	0.6	(0.27-1.6)	0.8	(0.33–2.3)

OR, Odds ratio crude; OR<sup>a</sup>, Odd ratio adjusted for age and all cofactors; CI, confidence interval.

Table III. Association of telomerase and main cofactors with HGSIL, in presence HR HPV infection.

HPV HR Cofactors	Controls n=14	Cases n=21	Crude		Adjusted	
			OR	95% CI	OR <sup>a</sup>	95% CI
Viral load (tertiles)						
I	4	1	1		1	
II	2	2	4.0	(0.56-28.3)	0.4	(0.01-20.3)
III	8	18	4.8	(0.84-27.2)	7.0	(0.52-95.7)
Telomerase activity						
No	9	4	1		1	
Yes	5	17	7.7	(1.63-35.7)	37.9	(1.64-678.1)
Parity:						
0-1	8	3	1		1	
>2	8	19	5.0	(1.17-14.6)	2.3	(0.12-50.3)
>3	2	2	3.5	(0.37-32.9)	2.4	(0.11-53.0)
Oral contraceptives:						
Never	8	8	1		1	
Ever	6	13	2.2	(0.54-8.5)	10.1	(0.74-137.5)
Smoking status						
Never	9	16	1		1	
Ever	5	5	0.8	(0.16-3.6)	0.2	(0.02-2.6)

HR HPV, High risk human Papillomavirus; HGSIL, high-grade squamous intraepithelial lesion; OR, crude odds ratio; OR<sup>a</sup>, Odds ratio adjusted for age and all cofactors; CI, confidence interval.



by Schutze *et al*, 2014 showed that cell lines generated using various hrHPV types were able to activate telomerase after a period in culture (38). However more functional assays are needed to establish the mechanism used by these genotypes.

In the present study, we observed samples with HPV infection without TA and samples with TA without HPV infection, and analysis between TA and hrHPV show no statistically significant interaction. This suggests that there are other factors aside from HPV infection that can activate the enzyme. Factors including HPV integration state can influence TA (39). When the viral genome is maintained as an episome, the E2 protein is expressed and can inhibit expression of hTERT. In contrast, when HPV is integrated into the host genome, E6 and E7 expression increases producing the expression of hTERT. Epigenetic regulation of the hTERT gene could also be important to evaluate, as some researchers have shown that methylation play a key role in the regulation of hTERT, although contradictory results have been reported (40-43). Recently we have published an association between type specific HPV infections and hTERT methylation in patients with cervical cancer but additional studies in patients with HGSIL have to be done (44). In addition, lifestyle aspects including diet, exposure to stress, and level of physical activity can also produce changes in TA (45-47). Unfortunately, we did not assess these factors in this study to have a more complete picture of the different mechanisms involved in TA in cases and controls. However our results show clearly that TA is a risk factor for HGSIL, with the strongest association in women who are hrHPV positive.

Our study suggests that detection of both HPV infection and TA at the same time could have a higher predictive value for HGSIL than either HPV detection or TA detection independently. The next step of this study is to evaluate TA in a larger cohort as a diagnostic test in the triage of HPV positive women.

Limitations of this study include low sample size resulting in very wide confidence intervals when adjusting for relevant cofactors.

In summary, this is the first epidemiological study that shows an independent association of TA with HPV as a risk factor for HGSIL when adjusted for different risk factors. TA could be used as an adjunct tool, in addition to HPV DNA detection, for identifying patients with higher risks of progression to cervical neoplasia.

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