Original contribution

3q26 (hTERC) gain studied by fluorescence in situ hybridization as a persistence-progression indicator in low-grade squamous intraepithelial lesion cases

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Summary Gains of 3q26 chromosome region, where the human telomerase RNA gene (hTERC) is located, have been previously documented in cervical carcinomas and preneoplastic lesions. The aim of our study was to define the value of 3q26 gains related to persistence-progression in cervical specimens with cytologic diagnosis for low-grade squamous intraepithelial lesions, using liquid-based cytology (ThinPrep; Hologic, Marlborough, MA) and fluorescence in situ hybridization. For these purposes, 55 patients were included in the study: 25 cases with a negative cytologic diagnosis for squamous intraepithelial lesion or malignancy (20 premenopausal and 5 postmenopausal women, used as control negative cases) and 30 low-grade squamous intraepithelial lesion cases. The follow-up was performed using cytology at 6, 12, and 24 months after the low-grade squamous intraepithelial lesion diagnosis. When the cytology result showed a high-grade lesion, colposcopy and biopsy were performed. Fluorescence in situ hybridization technique with a 3q26 2-color commercial probe was performed to determine the number of hTERC copies. There were no differences between premenopausal and postmenopausal normal cases. Low-grade squamous intraepithelial lesion cases with regression in the follow-up at 6, 12, and 24 months showed a percentage of cells with 3q26 gains similar to the control cases and lower than low-grade squamous intraepithelial lesion cases with persistence or progression (P < .05). Fluorescence in situ hybridization results were similar in preserved and frozen samples. However, in frozen samples, the number of cells suitable to be evaluated by fluorescence in situ hybridization was lower than in preserved (nonfrozen) cases. In conclusion, the determination by fluorescence in situ hybridization of 3q26 gains in low-grade squamous intraepithelial lesion cases could be useful to predict the persistence-progression of such cervical lesions using both preserved and frozen cervical material.

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1. Introduction

Cervical carcinomas are one of the most common gynecologic tumors worldwide. The early detection of precursors of invasive cervical squamous cell carcinoma represents a major challenge in screening programs, as some mild lesions may regress spontaneously and may not necessarily need treatment, but others may evolve to high-grade lesions [1]. At present, there is no method to detect those low-grade squamous intraepithelial lesion (LSIL) cases that would evolve to high-grade lesions. Infection with human papillomavirus (HPV) is known to play a crucial role in the immortalization of epithelial cells. Nevertheless, high-risk HPV infection is nowadays generally accepted as a necessary but not sufficient cause for the development of a cervical neoplasia [1]. The morphological changes associated to HPV infection as those corresponding to the preneoplastic lesions are well known. High-risk HPV produces the E6 and E7 oncoproteins whose actions drive the infected cells to a continuous proliferating state without checking the DNA and a consequently increased risk of mutations. Moreover, a high number of chromosomal alterations have been described related to dysplastic lesions of the uterine cervix. Among them, the most frequent are 3q gains, specifically those involving 3q26 chromosome bands, where human telomerase RNA gene (hTERC) is located. hTERC gene encodes for an RNA unit of telomerase that maintains the length of telomeres through cellular divisions. When this gene is overexpressed, the cells avoid undergoing apoptosis, potentially leading to tumorigenesis. Gains of hTERC have been frequently found both in uterine cervical squamous cell carcinoma [2-5] and in low- and high-grade preneoplastic lesions, increasing according to the severity of the lesion [4,6,7]. Moreover, in high-grade lesions, the correlation between progression and 3q26 gains has been shown [4,6].

The aim of the study was to determine the value of 3q26 (hTERC) gains using liquid-based cytology (ThinPrep; Hologic, Marlborough, MA) and fluorescence in situ hybridization (FISH) technology related to persistence-progression in LSIL cases. In addition, the correlation between 3q26 gains and HPV viral load in the same sample was evaluated. Finally, we compared the results of 3q26 gain determination in preserved and preserved and subsequently frozen liquid-based cytology samples (ThinPrep).

2. Materials and methods

2.1. Cytologic specimens

Fifty-five samples were included in the study, corresponding to 30 consecutively diagnosed LSIL cases and 25 consecutive cervical samples from healthy women. All cases were studied in liquid-based cytology (ThinPrep) and were selected accordingly to have adequate material to study. All LSIL cases were followed with a cytologic control 6 months after the diagnosis of LSIL, and half of them were followed for 24 months. Seven cytologic specimens from LSIL cases were frozen before the 3q26 study was performed.

The study was conducted with the approval of the local ethics committee and in accordance with the guidelines of the Declaration of Helsinki.

The frozen specimens were centrifuged at 2500 rpm for 10 minutes. The pellet was placed in an Eppendorf with 0.5 mL of PreservCyt (Hologic) and was frozen and preserved in a freezer at −75°C. To defreeze the specimen, we took it out of the freezer, placed it at room temperature (RT) for 2 hours, and resuspended it in 30 mL of PreservCyt (Hologic). All cases were processed in a T-3000 automated processor (Hologic) obtaining a slide for 3q26 study.

2.2. Fluorescence in situ hybridization

FISH analysis was performed in all patients on liquid cytology-based smears. The probe solution contained 2 DNA probes corresponding to hTERC gene (3q26) labeled with ULS-Rhodamine and the centromeric region of chromosome 7 labeled with fluorescein isothiocyanate (FITC), as a hybridization control (Vitro SA, Seville, Spain). Slides were incubated for 3 to 4 days in xylene to allow the removal of the coverslips. Then, slides were incubated in ethanol 100% for at least 1 hour and in Carnoy’s solution for 10 minutes at RT. Later on, slides were incubated for 40 minutes with a 60% acetic acid solution in a hot plate at 45°C. Then, they were refixed in Carnoy’s solution for 10 minutes at RT. After that, slides were incubated in a Coplin jar with a pepsin solution (P-6887; Sigma-Aldrich Quimica SA, Madrid, Spain) at 0.02% in HCl 0.01 mol/L for 50 minutes at 37°C. Slides were then washed twice with a 1× phosphate-buffered saline solution at RT for 1 minute each; dehydrated in 70%, 80%, and 100% ethanol series; and allowed to air dry. The probe solution was preheated at 96°C for 5 minutes. Five microliters of the probe were added to each slide, which was covered by a coverslip. The preparations were placed in a hot plate (Thermobrite; Abbott Molecular, Abbott Park, IL), denatured at 80°C for 2 minutes, and then hybridized at 37°C overnight. Posthybridization washes consisted three 50% formamide solution washes, one 2× SSC wash, and one 2× SSC 0.1% NP-40 wash of 5 minutes each, at 45°C. A minimum of 400 nuclei per case were scored, taking into account the number of hybridization signals for hTERC locus: 1, 2, 3, 4, or more than 4 hybridization signals.

2.3. HPV detection

In 19 of 35 LSIL cases, the HPV test was performed using the Hybrid Capture II technique (Hybrid Capture II; Digene, Gaithersburg, MD). Previously, the samples were treated with the Conversion Kit following the manufacturer instructions (Digene). Briefly, the method consists a liquid medium
hybridization with RNA probes producing a quimioluminiscen
t reaction. The amount of light produced is captured by a
luminometer using relative light units (RLU). The light
amount produced is proportional to the viral copy number
(viral load) measured as picograms per milliliter. This method
allows to detect 13 high-risk HPV viral types (16, 18, 31, 33,
35, 39, 45, 51, 52, 56, 58, 59, and 68), and the cutoff for
considering positive cases was 1 pg/mL.

2.4. Statistic analysis

Quantitative variables were described as mean and ranges.
Qualitative variables were described as frequency and per-
centage. To compare these variables, we applied the U
Mann-Whitney test. Statistic computations were performed
using the SPSS v.15 software (SPSS Inc, Chicago, IL). A
P value of .05 or less was considered statistically significant.

3. Results

3.1. Cytologic and histologic characteristics

The specimens analyzed were obtained from 55 women.
Of them, 25 cases were diagnosed as normal and used as
control cases. Among the control cases, 20 were premeno-
pausal women with a mean age of 35 years (range, 27-50
years) and 5 were postmenopausal, with a mean age of 58
years (range, 51-63 years). The previous cytologic studies of
these patients, followed for 24 months, were normal. The
remaining 30 analyzed cases were considered as problem
cases, with a mean age of 36 years (range, 22-53 years). All
of them were HIV negative. Twenty patients had not had any
lesion 24 months before the LSIL diagnosis, and 10 had a
lesion previously diagnosed (1 atypical squamous cells of
undetermined significance [ASCUS], 6 LSILs, and 3 high-
grade squamous intraepithelial lesions [HSILs]).

3.2. Follow-up studies (Table 2)

The patients’ follow-up was performed 6, 12, and 24
months after the diagnosis using cytology. When the cytology
showed the presence of a high-grade lesion, the colposcopy
and, eventually, the biopsy were performed.

The first follow-up study (6 months) was performed in all
30 cases. Thirteen patients (mean age, 34 years; range, 22-49
years) showed the lesion cytologically vanished. Seventeen
patients (mean age, 39 years; range, 22-53 years) showed a
cervical lesion (1 ASCUS, 12 LSILs, and 4 HSILs). Of the
4 HSILs cases to be submitted to the colposcopy, 2 were
missing, and in the remaining 2 cases, the colposcopy showed
major changes. In such cases, the biopsy was performed
demonstrating severe dysplasia (CIN-III). The patients were
submitted for the surgical conization. The posterior cytologic
controls were negative.

The second follow-up study (12 months) was performed
in 26 cases (4 cases were missing: 2 HSILs, 1 LSIL, and 1
negative with respect to 6 months of follow-up study). Of
these 26 cases, 8 showed some kind of pathology (2 ASCUS,
4 LSILs, and 2 HSILs) and 18 cases were cytologically
negative. The colposcopy in both HSIL cases showed major
changes. In these cases, the biopsy was performed
demonstrating severe dysplasia. The patients were submitted
for surgical conization. The following cytologic controls
were negative.

The third follow-up study (24 months) was performed in
21 cases (5 cases were missing with respect to the 12-month
follow-up study, and the rest showed no cytologic lesion). Of
these 21 cases, 13 showed no lesions in the cytologic study
and 8 showed some kind of pathology: 2 ASCUS, 4 LSILs,
and 2 HSILs. The colposcopy in both HSIL cases showed
major changes. In these cases, the biopsy was performed
demonstrating severe dysplasia. The patients were submitted
for surgical conization. The following cytologic controls were negative.

### 3.3. 3q26 (hTERC) amplification and persistence-progression in LSIL cases

The cytologically negative cases showed a mean of 5.1 cells containing only one 3q26 copy, a mean of 264 cells containing two 3q26 copies, and a mean of 4.4 cells (1.6%) (range, 0-15) containing 3 of more 3q26 copies. There were no differences between cases corresponding to premenopausal and postmenopausal women (Table 1). None of the 30 LSIL cases showed any cell with only one 3q26 (hTERC) copy. The mean of cells with more than two 3q26 copies in LSIL-follow-up negative cases was 3.6 (range, 1-10) (1.6%), and a mean of cells with more than two 3q26 copies in LSIL-follow-up positive cases was 13 (range, 1-52) (4.3%) ($P < .05$). The follow-up study at 6 months showed a mean cell number with 3 or more hTERC copies of 3.6 (range, 0-10) (1.3%) in the 13 cases with regression and 13 (range, 2-52) (4.3%) in the 17 cases with persistence-progression ($P < .05$). The follow-up study at 12 months showed a mean cell number with 3 or more hTERC copies of 5.1 (range, 0.21) (1.67%) in the 18 cases with regression and 11.9 (range, 2-41) (4.3%) in the 8 cases with persistence-progression ($P > .05$). The follow-up study at 24 months showed a mean cell number with 3 or more hTERC copies of 5.3 (range, 0-21) (1.67%) in the 13 cases with progression and 11.9 (range, 2-41) (4.3%) in the 9 cases with persistence-progression ($P > .05$) (Table 2).

The mean number of cells with 3 of more copies in cases with progression to HSIL were 12.6 (range, 1-52) and in cases persisting LSIL or ASCUS were 11.4 (range, 1-41). There were no differences between these 2 groups.

Establishing a cutoff in 1.6% (the percentage of normal cases) of cells showing 3 or more hTERC copies, the sensitivity at 6 months of follow-up was 80%, the specificity was 53%, the negative predictive value was 73%, and the positive predictive value was 63% (Table 3A). The sensitivity and the specificity were similar considering the follow-up at 12 and 24 months, but the negative predictive value increased to 91% and the positive predictive value decreased to 43% (Table 3B).

### 3.4. HPV detection (Table 5)

The HPV test was performed in 17 of 35 LSIL cases (7 showing regression and 10 showing progression). A comparative analysis between 3q26 and RLU showed increase of mean RLU with increasing positive cases across time and decreasing RLU in negative ones, but the differences are not statistically significative ($P > .05$).

### Discussion

Our results regarding the number of hTERC copies in normal cells are similar to those reported in the literature. Heselmeyer-Haddad [7] reported a mean of 5.5 cells with more than 2 copies, whereas our mean number was 4.4 cells (4.1 cells in premenopausal women samples and 5.2 cells in postmenopausal women ones). Our results showed a variable number of cells with only one 3q26 copy in normal cases, greater in postmenopausal women samples than in premenopausal women ones. Among the LSIL cases and referring to the mean number of cells with more than 2 hTERC copies,

### Table 3A

<table>
<thead>
<tr>
<th>Status</th>
<th>&gt;1.6%</th>
<th>&lt;1.6%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per/pro</td>
<td>12</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Regr</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>11</td>
<td>30</td>
</tr>
</tbody>
</table>

Abbreviations: Per/pro: persist or progress; regr, regress.

NOTE. Sensitivity, 80%; specificity, 53%; negative predictive value (NPV), 73%; positive predictive value (PPV), 63%. <1.6% indicates less than 1.6% of cells with more than 2 hTERC copies; >1.6%, more than 1.6% of cells with more than 2 hTERC copies.

### Table 3B

<table>
<thead>
<tr>
<th>Status</th>
<th>&gt;1.6%</th>
<th>&lt;1.6%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per/pro</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Regr</td>
<td>8</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>12</td>
<td>26</td>
</tr>
</tbody>
</table>

Abbreviations: Per/pro: persist or progress; regr, regress.

NOTE. Sensitivity, 75%; specificity, 53%; NPV, 91%; PPV, 43%. <1.6% indicates less than 1.6% of cells with more than 2 hTERC copies; >1.6%, more than 1.6% of cells with more than 2 hTERC copies.

### Table 4

<table>
<thead>
<tr>
<th>Diagnostic</th>
<th>Cases</th>
<th>Frozen</th>
<th>Total cell</th>
<th>&gt;2C hTERC</th>
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</thead>
<tbody>
<tr>
<td>LSIL (−)</td>
<td>11</td>
<td>No</td>
<td>400</td>
<td>4.9 (1.3%)</td>
</tr>
<tr>
<td>LSIL (−)</td>
<td>4</td>
<td>Yes</td>
<td>60</td>
<td>1.0 (1.6%)</td>
</tr>
<tr>
<td>LSIL (+)</td>
<td>14</td>
<td>No</td>
<td>400</td>
<td>15 (4.3%)</td>
</tr>
<tr>
<td>LSIL (+)</td>
<td>3</td>
<td>Yes</td>
<td>60</td>
<td>2 (3.7%)</td>
</tr>
</tbody>
</table>

Abbreviations: LSIL (−), LSIL, regress; LSIL (+), LSIL, persist-progression; >2C hTERC, number of cells (mean and percentage) with more than 2 hTERC copies.

NOTE. Comparative study between frozen and nonfrozen material.
Our results showed statistically significative differences between cases with persistence or progression and cases with regression at first control (13 versus 3.6 cells, \( P < .05 \)). The mean number of cells with more than 2 \( hTERC \) copies in cases with regression was similar to the mean number of cells with more than 2 \( hTERC \) copies in normal cases.

These differences are maintained at 12 and 24-month controls, but the differences are not statistically significant, probably because of missing cases and the fact that, on some patients, the lesion regresses between 6 and 12 months. Furthermore, the mean number of cells with 3 or more \( 3q26 \) copies in cases with persistence-progression was similar when we considered the results at 12 and 24 months. In addition, the mean number of cells with 3 or more \( 3q26 \) copies was independent of the lesion detected in the follow-up. The mean number of \( 3q26 \) copies cells is similar in cases with progression of HSIL than in cases with persistence of LSIL or ASCUS. As a consequence, the mean number of more than two \( 3q26 \) copies cells is not useful to know if the lesion will persist or will progress.

If we compare the results with those in frozen material, the rates are maintained if the number of assessed cells is lower than nonfrozen samples. Furthermore, in our LSIL cases, we could not find cells with only one \( hTERC \) copy.

Among follow-up data, negative cases showed an increasing number of cells that contained more than two \( 3q26 \) copies according to follow-up time but were similar to the number of cells in negative cases. The positive follow-up cases showed a similar number in the 3 follow-up controls.

In the comparative analysis with HPV detection and viral load, a regression was observed in all negative HPV cases. The differences between preserved and preserved and subsequently frozen liquid-based cytology samples (Thin-Prep) are based on the fact that it is possible to study a higher number of cells in nonfrozen cases than in frozen material because the process of freezing and defreezing can damage the cells. However, in frozen cases, the preserved cells are suitable to be studied properly.

In conclusion, \( hTERC \) gains studied by FISH can be useful as a predictive factor for the persistence-progression of LSIL. Both frozen and nonfrozen cytologic specimens can be used.

More studies are necessary to validate the predictive value of \( hTERC \), counting a limited number of cells for practical purposes because the technical and the lecture difficulties are limiting factors for the daily routine application of this technique. Furthermore, the cost that FISH adds to the liquid-based cytology is 5 times more than the cost of the liquid-based cytology. Obviously, a cost-effectiveness study must be performed before introducing this technique in the daily routine, and this study must contain the psychologic patient “cost.”

### Acknowledgment

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### References


<table>
<thead>
<tr>
<th>Table 5</th>
<th>Relationship between ( hTERC ) and viral load (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up</td>
<td>Positive/ Negative</td>
</tr>
<tr>
<td>6 mo</td>
<td>Positive</td>
</tr>
<tr>
<td>6 mo</td>
<td>Negative</td>
</tr>
<tr>
<td>12 mo</td>
<td>Positive</td>
</tr>
<tr>
<td>12 mo</td>
<td>Negative</td>
</tr>
<tr>
<td>24 mo</td>
<td>Positive</td>
</tr>
<tr>
<td>24 mo</td>
<td>Negative</td>
</tr>
</tbody>
</table>