# Human telomerase RNA gene (TERC) gain and polysomy of chromosome 3 in cervicovaginal liquid-based pap preparations: a fluorescence in situ hybridization study

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**Summary**

*Purpose*. This study investigated human telomerase RNA gene (TERC) gain and polysomy of chromosome 3 in cervicovaginal liquid-based pap preparations in Guilin, China, and assessed the relationship between FISH findings and clinical diagnoses. *Methods*. Slides prepared from 63 liquid-based preparations with cytologic diagnoses of negative for squamous intraepithelial lesion or malignancy (NILM n = 9), atypical squamous cells of undetermined significance (ASCUS, n = 18), low-grade squamous intraepithelial lesion (LSIL, n = 14), high-grade squamous intraepithelial lesion (HSIL, n = 9), and cervical squamous cell carcinoma (SCCA, n = 13) were analyzed for TERC gain and polysomy of chromosome 3 using a commercially available two-color FISH probe. The results of the cytologic analysis and those of concurrent or subsequent biopsies, when available, were compared with the FISH findings. The Mann-Whitney test was used to assess associations between FISH findings and diagnoses. *Results*. TERC gain and polysomy of chromosome 3 were significantly associated with the cytologic diagnosis ($p$? 0.001). Patients with HSIL or SCCA cytology diagnoses had a significantly higher percentage of cells with TERC gain and polysomy of chromosome 3 than did patients with NILM, ASCUS or LSIL cytologic diagnoses. Those abnormal cases with CIN1 histological diagnosis had a significantly lower percentage of cells with TERC gain and polysomy of chromosome 3 than did patients with a CIN2, CIN3 and SCCA histological diagnosis. *Conclusions*. TERC gain and polysomy of chromosome 3 may be important associated genetic events in cervical intraepithelial neoplasia and carcinoma. FISH is a potential tool for the diagnoses of uterine cervix disease.

*Key words:* FISH; TERC; Chromosome 3; Pap preparations; Uterine cervix disease.

## Introduction

Despite substantial progress in understanding cervical carcinogenesis and the development of advanced preventive measures, cervical carcinoma continues to be a leading cause of cancer death among women worldwide [1]. There are about 510,000 cases of cervical carcinoma reported annually: 68,000 in Africa, 77,000 in Latin America, and 245,000 in Asia [2]; The People's Republic of China has historically been considered at relatively low risk for cervical carcinoma, but nationwide mortality surveys show a variable pattern of risk across the country, which is on the increase among younger women, particularly in urban settings [3].

For the last 50 years, cervical cytology has provided the cornerstone of cervical carcinoma prevention programs, but the limited sensitivity of cervical cytology makes these programs difficult and expensive to maintain [4]. Fortunately, astonishing progress has been made in our understanding of the pathogenesis of cervical carcinoma. For example, infection with human papilloma virus (HPV) is considered to be the initiating factor in the carcinogenesis of uterine cervix, and HPV prevalence in any given population correlates well with cervical carcinoma risk [5, 6]. In addition, numerical chromosomal aberrations, such as aneuploidy and tetraploidy, are the most prevalent genetic changes observed in human solid tumors [7], have been reported in women diagnosed with precancerous and cancerous cervical lesions [8]. Recently, some researches have documented that chromosome arm 3q gain is a common feature of squamous cell carcinoma, with an overlapping area of gain at 3q26 having been reported in squamous cell carcinoma at different anatomic sites, including the lung, head and neck, esophagus, and cervix of the uterus [9]. Interestingly, chromosome arm 3q26 region contains the human telomerase RNA gene (TERC), encoding one main component of telomerase, which is of interest to research because of the correlation between telomerase activity and tumourigenesis in later years [10]. However, a study dealing with these genetic events of cervical squamous cell lesions in different regions worldwide, including both preneoplastic/preinvasive proliferations and early-stage invasive carcinomas of the cervix is still lacking. In this study, we investigated the prevalence of TERC gain and polysomy of chromosome 3, as measured by fluorescence in situ hybridization (FISH), in routine liquidbased cytologic preparations in Guilin, China, and assessed the associations between the FISH finding and clinical diagnoses.

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Table 1. — *Summary of TERC gain according to cytology.*

| Cytology diagnosis | No. of cases | No. of cases with TERC gain | No. of cases with polysomy 3 | Mean of cells with TERC gain (%) | Range of cells with TERC gain (%) | Mean of cells with polysomy 3 (%) | Range of cells with polysomy 3 (%) |
|---|---|---|---|---|---|---|---|
| NILM | 9 | 2 | 0 | 0.3 | 0~2 | 0 | 0 |
| ASC-US | 18 | 10 | 3 | 1.2 | 0~6 | 0.1 | 0~1 |
| LSIL | 14 | 8 | 1 | 1.3 | 0~6 | 0.1 | 0~1 |
| HSIL | 9 | 9 | 9 | 15.6 | 2~40 | 10.4 | 1~27 |
| SCCA | 13 | 13 | 13 | 42.8 | 6~96 | 24.9 | 6~74 |

## Methods and Materials

*Cervicovaginal specimens*

The 63 cases of specimens made from liquid-based preparations and collected from the cervix were confirmed cytology diagnoses and the cases with abnormal cytology results were further confirmed by histological diagnosis. The residual material of liquid-based preparations was prepared for FISH analysis. All these 63 patients were informed of the study's goals and all signed a consent form.

In the performance of cytologic diagnosis, the SUREPATH (TriPath Imaging, Inc., Burlington, NC) liquid-based preparations were stained using the papanicolaou screened by a cytotechnologist, and interpreted according to the Bethesda System [11] as follows: negative for squamous intraepithelial lesion or malignancy (NILM, 9 cases); atypical squamous cells of undetermined significance (ASC-US, 18 cases); low-grade squamous intraepithelial lesion (LSIL, 14 cases); high-grade squamous intraepithelial lesion (HSIL, 9 cases); and cervical squamous cell carcinoma (SCCA, 13 cases). The histologic diagnoses were obtained from the pathology database of Guilin 181st Hospital, China, and categorized as follows: negative for squamous intraepithelial lesion or malignancy (NILM), intraepithelial neoplasia (CIN1, CIN 2, and CIN 3), and SCCA.

*Cervical cell collection and preparation*

The cervicovaginal specimens which were residual material of liquid-based preparations were centrifugated and rinsed in 10 ml physiological saline two times, then digested in 5 ml collagenase at the temperature of 37°C (0.5) for 10 min, and centrifugated again, abandoning the supernate fluid, and hypotension with the solution of KCL (0.075 mol/l) at the temperature of 37°C (0.5) for 20 min, and then centrifugated again, abandoning the supernate fluid, and fixed in 5 ml of amethanol: acetic acid mixture (3:1) two times. After centrifugation, and abandoning the supernate fluid until the volume was 0.2~0.6 ml, the cells were misce bene slightly. At last, the cervical cell suspension was dropped onto clean glass microscope slides, open-air dryed, and baked at 56°C [1] for 30 min.

*FISH and signal enumeration*

FISH analyses were modified in cooperation with the manufacturer of China Medical Technologies, Inc. (Beijing, China). The commercially available two-color FISH probe consists of two probes: 3q26 (Rhodamine, red) covering the whole gene of TERC and the centromeric chromosome 3 (FITC, green). The FISH fixed glass microscope slides were placed in 2 × sodium saline citrate (2 × SSC, pH 7.2) at room temperature for 3 min, digested in a protease solution (20 mg/ml, pH 2.0) for 5-12 min at 37°C, rinsed in 2 × SSC at room temperature for 3 min, and fixed in 1% formaldehyde for 10 min at room temperature. After dehydration in a graded series of concentrations of ethanol, the slides were dried in the open-air. To denature DNA, the slides were placed in 78.5°C preheated 70% formamide/2 × SSC for 8 min and then were dehydrated in a graded series of concentrations of ethanol which were precooled in -20°C. After being dried in the open-air, 10 μl of probe destructured at 75.5°C for 7 min was applied onto each slide, which was then coverslipped and sealed with rubber cement, then hybridized overnight at 42.8°C. After 16-18 h of hybridization, the slides were washed in 46°C preheated post-hybridization buffer (2 × SSC/0.1% sodium dodecyl sulfate) for 5 min and rinsed in 70% ethanol. After air-drying (out of direct light), the slides were counterstained with 15 μl DAPI/anti-fade solution and coverslipped.

FISH analyses were performed by cytotechnologistes who were blinded to the clinical diagnoses at the time of evaluation. The slides were scanned using an OLYMPUS BX51 fluorescent microscope (OLYMPUS BX51, Japan) equipped with a 100-watt mercury lamp and filter set to detect DAPI, FITC (chromosome 3), and Rhodamine (TERC) at 1000×; 100 epithelial cells were noted for the signal numbers of both TERC and centromeric chromosome 3. To avoid counting split signals as two signals, the distance between any two signals had to be at least the diameter of one signal for them to be counted as separate signals. A cell scored as amplification of TERC was defined as a ratio > 1.0 between the TERC and the chromosome 3 copy number; polysomy 3 by the finding of more than two specific signals for chromosome 3.

The Mann-Whitney test was used to assess associations between FISH findings and diagnoses. All computations were carried out using SPSS 13.0 for windows.

## Results

Cervicovaginal specimens were obtained from 63 women, ranging in age from 23 to 63 years (mean age, 42 years). Twenty-seven of the 63 cases had HPV (16 and 18) tested. Of the nine NILM cases, none were HPV positive; of the 18 ASCUS cases, all showed CIN1 on biopsy; of the 14 LSIL cases, 13 showed CIN1 and one case showed CIN2 on biopsy and two cases were HPV positive. Of the nine HSIL cases, three cases showed CIN2, six cases showed CIN3, two cases showed SCCA on biopsy, and two cases were HPV positive. All SCCA cytologic diagnoses were confirmed by biopsy and eight cases were HPV positive.

Representative features of TERC gain and polysomy 3 are depicted in Figure 1. Table 1 summarizes TERC gain and polysomy of chromosome 3 results by FISH. Only two of NILM cases showed one to two epithelial cells with TERC gain, and none of them showed epithelial cells with polysomy of chromosome 3. Ten of ASC-

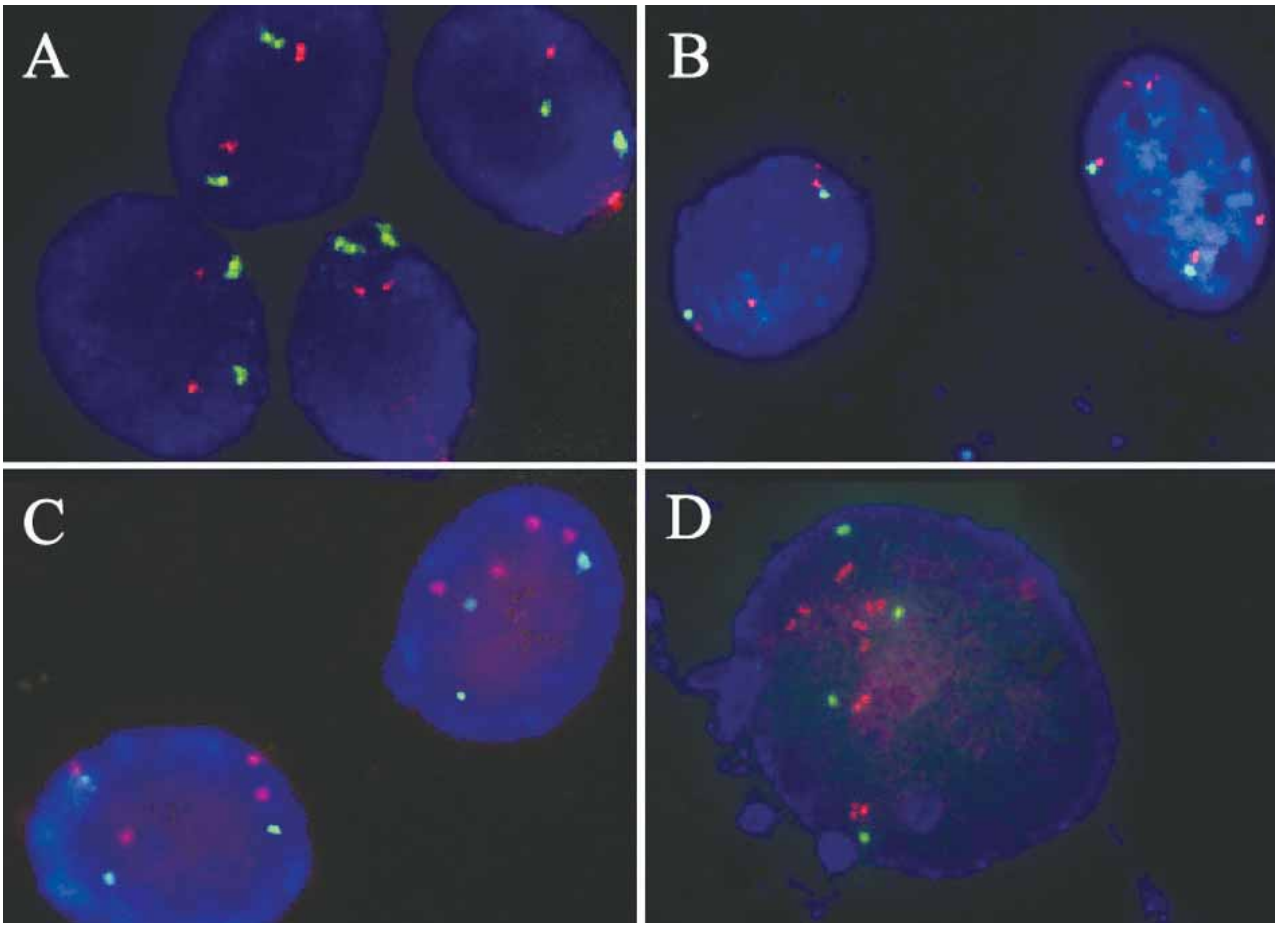


Figure 1. — Fluorescence in situ hybridization showing the positive TERC gain and polysomy 3 in cervicovaginal liquid-based Pap preparations; A) Normal cells; B) Abnormal cells with TERC gain but without polysomy 3; C and D) Abnormal cells with with TERC gain and polysomy 3.

US cases showed one to six epithelial cells with TERC gain, and three cases showed one epithelial cell with polysomy of chromosome 3. Eight of LSIL cases showed one to six epithelial cells with TERC gain, and only one case showed one epithelial cell polysomy of chromosome 3. All HSIL cases showed two to 40 epithelial cells with TERC gain and one to 27 epithelial cells with polysomy of chromosome 3. All of SCCA cases showed three to 96 epithelial cells with TERC gain, and six to 96 epithelial cells with polysomy of chromosome 3.

The FISH finding (TERC gain and polysomy of chromosome 3) were significantly associated with the cytologic diagnosis and histological diagnosis ($p < 0.001$; Table 1). Cases with HSIL or SCCA cytologic diagnoses had a significantly higher percentage of cells with TERC gain and polysomy of chromosome 3 than did patients with a NILM, ASC-US or LSIL cytologic diagnosis. Those abnormal cases with CIN1 histological diagnoses had a significantly lower percentage of cells with TERC gain and polysomy of chromosome 3 than did patients with a CIN2, CIN3 and SCCA histological diagnosis.

## Discussion

The main findings of our investigation can be summarized as follows: (a) TERC gain and polysomy of chromosome 3 may be two early genetic events associated with cervical affection; (b) TERC gain and polysomy of chromosome 3 may be involved in the progression of cervical carcinogenesis, and they are allowed distinction of cervical affection to some extent. (c) FISH may be an adjunct to cytology screening to detect patients with high-grade lesions.

A great deal of evidence has shown that infection with distinct types of HPV is the primary risk factor for the development of cervical carcinoma [12-15]. The most critical molecules in HPV replication are E6 and E7, which functionally inactivate the products of two important tumor suppressor genes, p53 and pRb, respectively. Both oncoproteins induce proliferation, immortalization, and malignant transformation of the infected cells [16]. However, evidence supports the theory that cervical squamous cell carcinoma is not simply caused by a single transforming event, and some resarches have documented that chromosomal instability as manifested by increases

in aneuploidy and structural chromosome aberrations could be found in most cervical carcinomas, especially chromosome 3q which contains the human telomerase RNA gene (TERC) [17-19].

Heselmeyer-Haddad *et al*. used FISH to investigate previously stained papanicolaou tests for the amplification of TERC. They took images of abnormal cells that were situated somewhere in previously stained papanicolaou tests. After FISH detecting (using the same previously stained papanicolaou tests), the same cells were again imaged and analyzed if they were a positive gain of TERC. Their findings constituted a comprehensive retrospective evaluation of papanicolaou tests in an attempt to validate amplification of TERC [17]. Caraway *et al*. performed FISH on cervicovaginal liquid-based reparations to analyze the most atypical 25 cells for abnormal signal numbers with a 3q26 gain, and they found that gain of 3q26 was associated with high-grade squamous intraepithelial lesions and cervical SCCA [18]. Alaharski *et al*. performed FISH to analyze cervical cell samples collected from 143 cases of patients with diagnoses ranging from NILM to HSIL, and found that the frequencies of cells exhibiting either tetrasomy or aneusomy for chromosomes 3 & 17 increased significantly with disease progression [8]. There are also other similar researches [19-21]. In our research, we analyzed abnormal cells on liquid-based cervicovaginal preparations for TERC gain similar to that used by Caraway *et al*. However, our research is not simply repeating their works because we used another probe (centromeric chromosome 3) defining a cell scored as TERC gain by a ratio > 1.0 between the TERC and the chromosome 3 copy number, which could discern gene gain from polysomy. The cells selected for the assessment of TERC gain in this research were different from Caraway *et al*. as well; we chose more cells and analyzed 100 epithelial cells for the assessment of signal numbers of both TERC and centromeric chromosome 3 for each case. In addition, the genetic makeup, lifestyle, and individual sanitation of Chinese people are somewhat different from other races, thus all of these are needed to be further researched. From our data, it can be seen that two cases with NILM had one or two abnormal cells with another copy of TERC. This indicates that evaluation for the threshold of positive TERC gain is needed because those abnormal signals may be caused by the experiment itself or there may be little clinical value with only a small proportion of abnormal cells. In LSIL cases, there were eight cases with abnormal cells of TERC gain, and only one case with polysomy of chromosome 3. It may be that polysomy of chromosome 3 is a later genetic event compared to TERC gain. However this research can not provide enough evidence to support it.

In short, TERC gain and polysomy of chromosome 3 are significantly associated with clinical diagnosis; FISH may be an adjunct to cytology screening to detect high-grade lesions and assess the risk of cervical carcinogenesis. Although this study can not prove the association between multiple HPV infection and findings, our work may help with early detection of cervical carcinogenesis.

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