RESEARCH COMMUNICATION

Clinical Significance of Human Telomerase RNA Gene (hTERC) Amplification in Cervical Squamous Cell Lesions Detected by Fluorescence in Situ Hybridization

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Abstract

Background: Genomic amplification of the human telomerase RNA gene (hTERC), located in the chromosome 3q26 region, has been documented in tumorigenesis. The present study was designed to detect hTERC amplification in cervical lesions and evaluate whether this might serve as a supportive biomarker to cytopathology or histopathology in the diagnosis of cervical lesions. Methods: Liquid-based thin-layer cytopathologic examination and detection of amplification by fluorescence in situ hybridization (FISH) was conducted in 130 women, along with assessment of human papillomavirus DNA, colposcopy with biopsy, and histopathologic examination. Results: In cytopathologic examinations, hTERC amplification rates for negative for intraepithelial lesion or malignancy (NILM),atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and squamous cell carcinoma (SCC) cases were 0% (0/10), 4% (1/25), 20% (6/30), 77% (27/35), and 100% (10/10), respectively. The difference among abnormal cellular change groups was statistically significant (P<0.05). In histopathologic examinations, hTERC amplification rates in normal squamous cell with or without inflammatory, cervical intraepithelial neoplasia 1 (CIN 1), CIN 2, CIN 3 and SCC cases were 3.8% (2/52), 18.2% (6/33), 66.7% (6/9), 84.6% (22/26), 100% (10/10), respectively. There were significant differences among CIN1, CIN2, CIN3 and SCC cases (P<0.05). The hTERC amplification was more specific than HPV positivity in differentiating low-grade from high-grade cervical disorders (specificity: 88.5% vs. 70.8%, P<0.05). Conclusions: FISH detection of hTERC amplification could be an effective adjunct to cytopathologic or histopathologic examination for differential diagnosis of low- and high-grade cervical squamous cell disorders.

Keywords: hTERC - FISH - liquid-based cytology - CIN1, 2, 3 - cervical carcinoma

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Introduction

Cervical squamous cell carcinoma is the second most common cancer in women worldwide (zur Hausen et al., 2002). In recent years, this disease has increased gradually in China; therefore, it is very important to establish early detection of high-grade cervical intraepithelial neoplasia (CIN), which is the precursor of invasive cervical squamous cell carcinoma. It is a major challenge in screening programs for cervical intraepithelial neoplasia, as some lesions may regress spontaneously and may not necessarily need treatment, while others may evolve into high-grade lesions (zur Hausen et al., 2002). Epidemiological and molecular biological studies have shown that infection with the human papillomavirus (HPV) is considered the most significant risk factor in initiating CIN and cervical squamous cell carcinogenesis, but other genetic events are necessary for a cervical precancerous lesion to progress to malignancy (Munger et al., 2004). The application of cytogenetic and molecular cytogenetic techniques to study genomic alterations have identified frequent gains involving the long arm of chromosome 3q26 which contains the human telomerase RNA gene (hTERC) (Heselmeyer et al., 1996). Gains of the 3q26 region (hTERC) have been found both in cervical squamous cell carcinoma and in low- and high-grade preneoplastic lesions, increasing according to the severity of the lesion (Alameda et al., 2009; Caraway et al., 2008). The value of detection of hTERC genomic amplification of 3q as a biomarker for progression during cervical tumorigenesis has been evaluated in several studies (Heselmeyer-Haddad et al., 2003; Heselmeyer-Haddad et al., 2005; Cao et al., 2008; Tu et al., 2009).Study by Heselmeyer-Haddad established the central role of 3q for progression from low-grade dysplastic lesions to higher grades and to invasive carcinomas. However, applying FISH-based analysis in

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liquid-based cytology specimens for hTERC amplification in a clinical setting is challenging, especially in routine cytological screening. Whether hTERC amplification can serve as a supportive biomarker to cytopathology or histopathology for diagnosing precancerous lesion of uterine cervical squamous cell carcinoma, especially for differentiating high-grade squamous intraepithelial lesion from low-grade squamous intraepithelial lesion, so as to improve the cervical screening accuracy and predictability, is still uncertain.

Therefore, the present study was designed to investigate the relationship between hTERC amplification and cervical squamous cell lesions using liquid-based cytology and fluorescence in situ hybridization (FISH) technology, to determine the correlation between hTERC gene amplification and HPV infection and to evaluate whether hTERC amplification can serve as a molecular marker supporting the differential diagnosis of low-grade and high-grade squamous cell lesions of the cervix in routine cytological screening.

Materials and Methods

Patients

One hundred and thirty women visited the Third Affiliated Hospital of Sun Yat-sen University from January 1st, 2009 to December 31st, 2009 were recruited in our study under their consents. The medium age of the patients was 39 years (range: 19~61 years). All of them had liquid-based thin-layer cytopathologic examinations (autocyte cytologic test, LCT) and fluorescence in situ hybridization (FISH) analysis with the hTERC-specific probe in a blind fashion. In addition, HPV DNA testing, colposcopy with biopsy, and histopathologic examinations were also conducted.

Cervical cytopathologic and histopathologic examinations

Cytopathologic examination was performed after standard LCT management of the sample and was reported according to the 2001 revision of the Bethesda system (Solomon et al., 2002): negative for intraepithelial lesion or malignancy (NILM, 30 cases), atypical squamous cells of undetermined significance (ASCUS, 25 cases), low-grade squamous intraepithelial lesion (LSIL, 30 cases), high-grade squamous intraepithelial lesion (HSIL, 35cases), and squamous cell carcinoma (SCC, 10 cases). Colposcopy with biopsy was concurrently performed. The histopathologic diagnoses were made and categorized according to WHO criteria as follows: normal squamous cell with or without inflammatory (normal or inflammatory); cervical intraepithelial neoplasia (CIN) 1, 2, and 3; and SCC. With histopathological evaluation, 52 cases were diagnosed as normal or inflammatory; 33 cases as CIN 1; 9 cases as CIN 2; 26 cases as CIN 3; and 10 cases as SCC.

FISH detection of hTERC amplification

The dual-color FISH probes were produced and provided by GP Medical Technologies Limited Company (Beijing, China), with the hTERC probe labeled in red (rhodamine) and the chromosome 3 centromere-specific probe (CSP 3) labeled in green (fluorescein isothiocyanate).

For FISH hybridization, the slides were washed in 2× saline sodium citrate (SSC) solution (PH 7.0) for 5 minute twice and then soaked in 0.1 mol/L HCl solution for 10 minute under room temperature. After washed in 2× SSC solution for 5 minute twice, the slides were incubated with pepsin in 0.01 mol/L HCl at 37°C for 10 minutes, washed again with 2× SSC for 5 minutes twice, dehydrated with precooled ethanol at 70%, 85%, and 100% in sequence, and air dried.

The slides and probe mixture (including 2μl probe, 7μl hybridizing buffer and 1μl deionized water for each slide) were denatured separately in 70% formamide/2×SSC at (73±1)°C for 5 minutes. After denaturation, the slides were dehydrated with 20°C precooled ethanol at 70%, 85%, and 100% in sequence and air dried. The denatured probe mixture was then dropped onto the slides and hybridized overnight in a wet chamber at 42°C. After hybridization, the slides were washed in formamide/2×SSC solution at 46°C for 10 minute three times, 2×SSC solution for 10 minute, 2×SSC/0.1% NP-40 solution for 5 min and then air dried. Finally, the slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) in dark place for 45-60 minute.

The fluorescence hybridization signals were observed with fluorescence microscope (OLYMPUS BX51). Images were analysised by analysis software of FISH supplied by GP Medical Technologies Limited Company.

Judgement of hTERC amplification

For each specimen, 100 cells were evaluated. In a normal cell, the signal ratio of CSP3 to hTERC is 2:2, whereas in abnormal cells the ratio will be 2:3, 2:4, 2:5, 3:3, 4:4, and so on. Therefore, a cell with three or more hTERC signals, regardless of the signal numbers of CSP3, will be considered as having an abnormal signal pattern. For a positive result with hTERC amplification, the percentage of cells with abnormal signal patterns should be more than the threshold value.

Establishment of the threshold value

Cervical cells from additional 20 normal individuals, with both normal cytopathologic test and negative HPV test, were used to establish the threshold value. The threshold value was mean and three times standard deviation (SD) of the percentage of cells with abnormal signal patterns of these 20 specimens. In this study, the threshold value is 5.3% (Mean = 2.65%, SD = 0.88%).

High-risk HPV DNA test

Cervical cells were collected and used for high-risk HPV detection with the Digene Hybrid Capture 2 method (Qiagen, Gaithersburg, MD) according to the manufacturer's instructions. HPV DNA measured at > 1 pg/mL was regarded as a positive result.

Statistical Analysis

Chi-square test was used to evaluate the relationship between hTERC amplification rate and the cytopathologic or histopathologic findings. The results were considered to

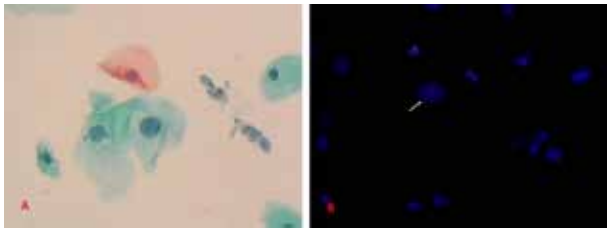

Figure 1. A. Liquid-based Cervicovaginal Preparation from a Case of ASC-US that Shows Comparatively Large Nucelus of Squamous Cells. B. hTERC Detected by Dual-color Fluorescence in Situ Hybridization Probe on the Cervical Smear. Interphase FISH Shows that the CSP3:hTERC Signal Pattern is 2:3 Green:Red (arrow).

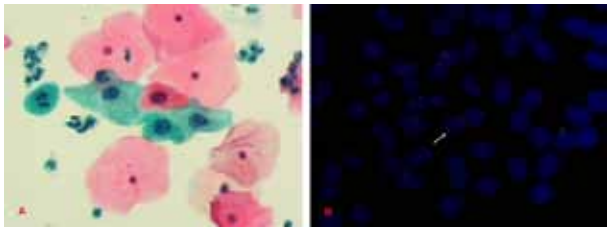

Figure 2. A. Case of LSIL that was Confirmed on Biopsy. B. Interphase FISH Shows the Abnormal CSP3:hTERC Signal Pattern is 2:3 or 3:3 Green:Red (arrow).

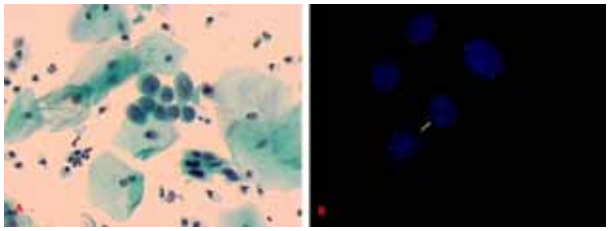

Figure 3. A. Case of HSIL that was Confirmed on Biopsy. B. Interphase FISH Shows that the CSP3:hTERC Signal Pattern is 4:4 or 5:5 Green:Red (arrow).

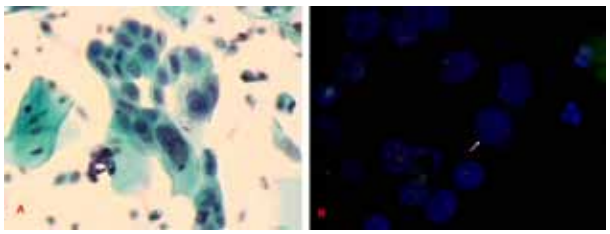

Figure 4. A. Case of SCC Showing Highly Dysplastic Cells and Diathesis in the Background. B. Interphase FISH Shows Higher Signal Patterns. The CSP3:hTERC Signal Pattern is 5:5 or 6:6 (arrow).

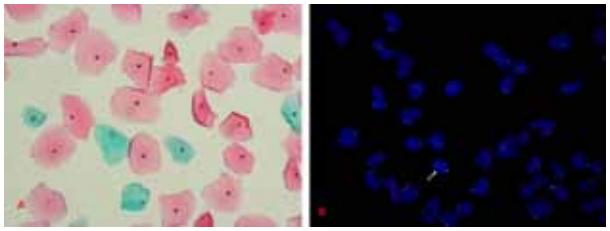

Figure 5. A. Liquid-based Cervicovaginal Preparation from a Case of NILM that Shows Unremarkable Intermediate Squamous Cells. B. Interphase FISH Shows the CSP3:hTERC Signal Pattern is 2:2 Green:Red (arrow). There is No hTERC Gene Amplification in this Case of NILM

be statistically significant at a P-value of less than 0.05. All the P-values presented in the present study are two-sided.

Results

hTERC amplification in association with cytopathologic evaluations (Table1)

FISH results showed that in the group classified as ASC-US there were 1 of 25 (4%) (Figure 1); in the group classified as LSIL 6 of 30 (20%) (Figure 2); in the group classified as HSIL 27 of 35 (77%) case hTERC-positive (Figure 3). SCC was 100% (10/10) hTERC-positive (Figure 4).There were no hTERC amplification in the NILM group (Figure 5).

There was significant difference between NILM group and all abnormal cellular change groups (P<0.05). Among the abnormal cellular change groups, amplification rate of hTERC increased gradually corresponding to the severity of cervical lesions (P<0.05).

Histopathologic findings in comparison with cytopathologic evaluations (see Table 1)

After cervical biopsy, there were 15 cases of chronic cervicitis, 15 cases of normal in 30 cases of NILM. In 25 cases of ASC-US, there were 3 cases of CIN1, 18 cases of chronic cervicitis, 4 cases of normal. All were CIN1 in 30 cases of LSIL. There were 26 cases of CIN3, 9 cases of CIN2 in 35 cases of HSIL. All cases were SCC in 10 cases of SCC.

hTERC amplification in association with histopathologic findings

In histopathologic examinations, hTERC amplification was observed in 2 of 52 (3.8%) normal or inflammatory cases, in 6 of 33 (18.2%) CIN 1 cases, in 6 of 9 (66.7%) CIN 2 cases, in 22 of 26 (84.6%) CIN 3 cases, and in 10 of 10 (100%) SCC cases (Table 2).

There was significant difference between benign cases and all abnormal cases (P<0.05). There were also significant differences among CIN1, CIN2, CIN3 and SCC cases (P<0.05).

Table 1. hTERC Amplification and HPV Positivity in Cytopathologic Specimens

Group	N	hTERC Amplification		HPV Positivity	
		-	+ (%)	-	+ (%)
ASCUS	25	24	1 (4)	17	8 (32)
LSIL	30	24	6 (20)	9	21 (70)
HSIL	35	8	27 (77.1)	3	32 (91.4)
SCC	10	0	10 (100)	0	10 (100)
NILM	30	30	0 (0)	24	6 (20)

There was significant difference of hTERC amplification rate between NILM group and all abnormal cellular change groups (P<0.05). The hTERC amplification rate increased corresponding to the severity of cervical lesions (P<0.05); The hTERC amplification was more specific than HPV positivity in differentiating low-grade cervical disorders from high-grade ones (specificity: 88.5% vs. 70.8%, P<0.05); ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; SCC: squamous cell carcinoma; NILM: negative for intraepithelial lesion or malignancy.

Table 2. hTERC Amplification in Histopathologic Specimens

Group	N	hTERC Amplification	
		-	+ (%)
CIN1	33	27	6 (18.2)
CIN2	9	3	6 (66.7)
CIN3	26	4	22 (84.6)
SCC	10	0	10 (100)
Normal or Inflammatory	52	50	2 (3.8)

There was significant difference of hTERC amplification rate between benign cases and all abnormal cases (P<0.05). There were also significant differences of hTERC amplification rate among CIN1, CIN2, CIN3 and SCC cases (P<0.05); CIN: cervical intraepithelial neoplasia; SCC: squamous cell carcinoma; normal or inflammatory: normal squamous cell with or without inflammatory.

hTERC amplification in comparison with HPV positivity

The percentages of cases positive for high-risk HPV in the NILM, ASCUS, LSIL, HSIL, and SCC group were 20% (6 of 30), 32% (8 of 25), 70% (21 of 30), 91.4% (32 of 35), and 100% (10 of 10), respectively (Table1). The hTERC amplification was thus more specific than HPV positivity in differentiating low-grade cervical disorders from high-grade ones (specificity: 88.5% vs. 70.8%, P<0.05).

hTERC amplification patterns in relation to severity of cervical lesions

The fluorescent signal pattern of CSP3 versus hTERC was 2:2 in normal cells. For the 52 cases classified as normal or inflammatory, there were 69 (2.3%) abnormal cells in total, which exhibited the 2:3 or 2:4 signal pattern. For the 33 CIN1 cases, there were 118 (3.6%) abnormal cells. The signal patterns in abnormal cells were 2:3, 2:4, 2:5, 3:3, 4:4, 5:5, accounting 55.1% (65/118), 32.2% (38/118), 5.1% (6/118), 4.2% (5/118), 2.5% (3/118) , and 0.8% (1/118), respectively. For the 35 CIN2-3 cases, there were 326 (9.3%) abnormal cells. Among them, the 2:3, 2:4, 2:5, 3:3, 4:4, 5:5, 6:6 signal patterns accounted 103 (31.6%), 76 (23.3%), 37 (11.3%), 44 (13.5%), 41 (12.6%) , 18 (5.5%), and 10 (3.1%), respectively. For the 10 SCC cases, there were 199 (19.9%) abnormal cells. Among them, the 2:3 signal pattern was the most common (21.6%), followed by the 2:4 (19.6%), 6:6 (13.1%), 5:5 (12.6%), 3:3 (12.1%), 2:5 (11.6%) and 4:4 (9.4%) patterns.

The numbers of hTERC-amplified cases as well as the complexity of abnormal signal patterns increased with the severity of cervical diseases.

Discussion

In the present study, 100 patients with abnormal cellular changes on liquid-based cervicovaginal preparations and 30 normal patients were analyzed for hTERC amplification using FISH method. The FISH findings were then compared with the HPV DNA testing and the histological examination.

In cytopathologic evaluations, the hTERC amplification rates in NILM, ASCUS, LSIL, HSIL and SCC cases were 0%, 4%, 20%, 77%, and 100%, respectively. In histopathologic examinations, hTERC amplification was observed in 3.8% of normal or inflammatory cases, 18.2% of CIN 1 cases, 66.7% of CIN 2 cases, 84.6% of CIN 3 cases, and 100% of SCC cases. These findings were similar to other studies (Caraway et al., 2008; Heselmeyer-Haddad et al., 2005; Andersson et al., 2006; Tu et al., 2009; de Wilde et al., 2008).

Our findings demonstrated that patients with a cytologic diagnosis of HSIL or SCC had significantly higher percentages of cells with hTERC amplification than patients with an NILM or ASC-US cytologic diagnosis. Statistical analysis showed that there were significant differences between pathological cases and normal cases, and also among LSIL, HSIL and SCC cases. These findings strongly supported the notion that hTERC amplification could be a major, clinically useful genetic marker assisting cytopathologic and histopathologic analysis for the differential diagnosis of low-grade (≤CIN 1) versus high-grade (≥CIN 2) cervical lesions.

On the other hand, our findings that all cases of hTERC amplification were also positive for high-risk HPV infection supported the idea that HPV DNA integrated into human genome and led to amplification of the hTERC gene, which was crucial in the malignant transformation of cells in the cervical region (Nair et al., 2000; Riethdorf et al., 2001; Ferber et al., 2003; Kailash et al., 2006; Oikonomou et al., 2007). It was thought that HPV infection also played an important role in the development of cervical cancer1. Moreover, the hTERC amplification was more specific than HPV positivity in assisting the differential diagnosis of precancerous lesions between low-grade (≤CIN 1) and high-grade (≥CIN 2) cervical disorders. It indicated that hTERC amplification could serve as a useful molecular marker providing the differential diagnosis of low-grade and high-grade lesions of the cervix in routine pathological screening.

Our study on liquid-based preparations also showed that the numbers of hTERC-amplified cases as well as the complexity of abnormal signal patterns increased with the severity of cervical diseases. The percentages of cells with hTERC amplification increased as the disease progressed, and the amplification patterns got more diverse and complex as well. More complicated patterns of hTERC amplification were found only in the high-grade lesions. Therefore, hTERC amplification is not only important for uterine cervical carcinogenesis, but also can indicate the degree of malignancy in CIN. hTERC amplification patterns can be used as an auxiliary diadynamic marker for judging the degree of cervical pathological changes.

From the results, it can be concluded that hTERC gene amplification can serve as a molecular marker supporting the differential diagnosis of low-grade and high-grade lesions of the cervix in routine cytological screening. We suggest that FISH testing for hTERC amplifications on cervical smears as a screening test for patients with a high risk of cervical cancer. FISH detection of hTERC amplification may be a useful adjunct to cytopathologic or histopathologic examinations for differential diagnosis of low- and high-grade cervical squamous cell disorders.

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