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Application of hTERC in thinprep samples with mild cytologic abnormality and HR-HPV positive

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ABSTRACT

Objective. Amplification of hTERC is found to be an important genetic event in the progression from cervical dysplasia to cervical cancer. The hTERC value in predicting high-grade cervical intraepithelial neoplasia (CIN) or squamous cell carcinoma (SCC), in high-risk HPV (HR-HPV) positive thinprep samples with atypical squamous cells (ASC) or a low-grade squamous intraepithelial lesion (LSIL) was explored in this study.

Methods. A total of 300 thinprep cytology specimens (129 of ASC-US, 82 of LSIL, and 89 of ASC-H) with positive HR-HPV DNA was detected by a two-probe dual-color FISH panel, targeting hTERC and the centromeric region of chromosome 3 (CSP3). Using >2 signals for hTERC together with ≥2 signals for CSP3 to define abnormal nucleus, and the cutoff value was set at 6.5 per random 200 nuclei displayed increased hTERC signals and/or tumor ploidy. Statistical analyses were based on histologic findings of colposcopy biopsies, allowing CIN2 or worse (CIN2+) as the positive criterion.

Results. The FISH results were systematically analyzed among groups, based on histologic diagnosis, cytologic finding, HR-HPV viral load, and age status. hTERC presented good consistency with histology, and had satisfactory sensitivity, specificity, and accuracy among different groups, with less difference intergroup. The individual hTERC positive nuclei ratio was generally increased with severity of the cervical lesions.

Conclusions. hTERC could be a stable predictor in assuring the risk of high-grade CIN in women with mild cytologic abnormality and positive HR-HPV, and the individual positive nuclei ratio of it might be helpful in identifying morbid grade for cervical lesions.

Introduction

Cervical cancer used to be one of the leading causes of cancer death among women in the world. However, national screening programs have markedly reduced its incidence and mortality [1]. Cytology is the most widely applied screening method for cervical cancer worldwide, especially in developing countries. Recently, the application of thinprep cytology test (TCT) provides alternative technique to conventional Pap test, and several studies have proved that it yields more favorable detection rates for low-grade cytologic abnormalities [2–4]. According to the Bethesda System (TBS, 2001) [5], the grading of TCT can be classified as diagnoses of negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells (ASC), which can be further divided into atypical squamous cells of undetermined significance (ASC-US) and atypical squamous cells not excluded a high-grade squamous intraepithelial lesion (ASC-H), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and squamous cell carcinoma (SCC). Among these, the management strategy for ASC and LSIL remains controversial up to date.

It has been considered that women with ASC or LSIL are at increased risk for developing high-grade cervical intraepithelial neoplasia (CIN). And there is unequivocal indication that those women most likely harbor a high-risk human papilloma virus (HR-HPV) infection, which is the most important known factor in the pathogenesis of cervical cancer [6], therefore they should receive intensive screening attention. The etiogenic relationship between HR-HPV infection and cervical cancer makes HR-HPV DNA test a very attractive first screen to identify the risk of developing cervical cancer in ASC/LSIL, with high sensitivity plus ease and reproducibility of analysis [7]. A negative HR-HPV test implies extremely low risk for CIN3 or cancer [8–11], whereas in positive results it is hard to predict. It has been well accepted that only persistent infections by HR-HPV genotypes may lead to cervical carcinogenesis [12], and the clearance of HPV infection can be expected in most (especially young) women with mild HPV-related cervical abnormalities [13,14]. However, the problem remains that it is hard for us to distinguish who would be persistently infected and likely to progress to severe dysplasia and cancer, and who have merely transient infections,

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among those with ASC/LSIL cytology and positive HR-HPV testing. In such a case, colposcopy is an option up to date. But controversy still exists in colposcopic management, for our clinical practice indicated that at least half of those women would get negative histologic findings, therefore colposcopy probably means an overshoot diagnostic procedure. So, it should be necessary to find an adjunctive marker to help to identify and evaluate the risk for high-grade CIN, of those with mild cytologic abnormality and positive HR-HPV.

Genetic events are essential for cervical precancerous lesions to progress to malignancy [15], and 3q gain is the most consistent chromosomal aberration [16]. The human telomerase RNA gene (hTERC) is located in the chromosome 3q26 region, and it is the main subunit of telomerase, which is involved in the maintenance of chromosome length and stability [17]. Studies in both tumor cell lines and human tumor specimens have shown that telomerase activity is increased in most malignant cells, in contrast to its inactive in normal somatic cells. So the upregulation of telomerase is in general associated with tumorigenesis [18], and hTERC probably plays the key role in this process. A recent research [19] showed that high-grade lesions of cervix almost invariably had hTERC amplification, and hTERC might be a potential marker for disease progression. Meanwhile, a previous study by our group suggested that hTERC detection could be a useful and specific screening method in cervical cancer and precancerous lesions [20]. So in this study, we applied a dual-color fluorescence in situ hybridization (FISH) panel in detection of hTERC, assessed the value of hTERC as a predictor for identifying high-grade CIN or cervical cancer in mild cytologic abnormality with HR-HPV positive, and systematically analyzed FISH results among different groups, based on histologic diagnosis, cytologic finding, HR-HPV viral load, and age status. The aim was to further characterize the amplification of hTERC and evaluate its significance as an adjunctive tool.

Patients and methods

Cytologic specimen and histologic classification

In this study, we consecutively enrolled 300 women with mild cytologic abnormality and positive HR-HPV DNA test during March 2009 to March 2010, with a mean age of 39.3 years (range, 20–71 years), from the outpatient of Women's Hospital, Zhejiang University School of Medicine, China. Women were excluded from the study if they had a previous history of CIN or cervical, vaginal or vulvar cancer, as well as immuno-suppression or pregnant. None of these patients had received cytology test within 6 months or cervical therapy before. The liquid-based specimens were processed with the Cytyc T2000 Processor (Cytyc Inc., Marlborough, MA), and final cytologic diagnoses were issued using TBS 2001, which were classified as ASC-US ($n=129$), LSIL ($n=82$), and ASC-H ($n=89$). Residual TCT material that remained in the patient's collection vial was stored at 4 °C available for FISH analysis.

All the abnormal cytology diagnoses were histologically confirmed by directed colposcope biopsies. CIN was diagnosed and graded according to international criteria [21], and cervical neoplasia was defined by the most severe lesion found on histologic examination. Normal, inflammation, or wart, which cannot be diagnosed as CIN1 may belong to the group of CIN0, with no dysplasia. This study was carried out in accordance with local ethical guidelines and all patients gave informed consent.

High-risk HPV DNA test

HR-HPV DNA test was performed at the time of the study on liquid cytology specimens, using the Hybrid Capture 2 (HC2) system (Digene Corporation, Gaithersburg, MD). The samples were tested for 13 HR-HPV viral types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The criterion for considering positive was at the relative light unit/cutoff (RLU/CO) ratio of 1.0 pg/ml, as per the manufacturer's guidelines. The amount of light produced by the HC2 method is in proportion to the amount of target DNA in each specimen, so results of the RLU/CO ratio can also be considered as reflective of HPV viral load in the specimens [22,23].

FISH detection

FISH was performed by an expert blinded to the cytologic and histologic findings, and the procedures were briefly as follows. Centrifugated the TCT suspension and digested the residual in a collagenase B solution (1 mg/ml; pH 7.0) at 37 °C for half an hour. After hypotonicity at 37 °C deionized water for at least 40 min, the specimens were fixed with FISH fixative (3:1 methanol/acetic acid) twice for 10 min each. Then processed cervical cells were dropped onto clean slides and stored at room temperature overnight for maturing. The fixed slides were placed in 2× sodium saline citrate (SSC; pH 7.0), 0.1 M HCl successively at room temperature for 5 min each, and then incubated with pepsin in 0.01 M HCl solution at 37 °C for 5 min. Following pepsin treatment, the slides were placed in a graded series of concentrations of ethanol for dehydration and allowed to air dry. Applied 10 μl of probe onto each slide then coverslipped and sealed with rubber cement. Then the target DNA and probe were denatured simultaneously on an 80 °C preheated plate for 5 min and hybridized at 40 °C in a thermotank overnight.

The dual-color fluorescent probe panel (GP Medical, Ltd., Beijing, China) consisted of two probes: a Rhodamine-labeled 3q26 containing the human telomerase gene (hTERC, red), and a fluorescein isothiocyanate (FITC) labeled centromere enumeration probe for chromosome 3 (CSP3, green) as the control. After 16–18 h of hybridization, slides were washed out of direct light in 0.1%NP40/2× SSC and 2× SSC for 5 min each, air-dried then counterstained with 15 μl of 4′6-diamidino-2-phenylindole (DAPI) and coverslipped. Stained slides were scanned using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) at 1000×. Image acquisitions and analyses were performed by software Video Test 2.0 (NatureGene Corp., Medford, NJ). Scoring of FISH result was done without knowledge of cytologic or histologic evaluation.

Statistical analysis

Data analyses were performed using SPSS, version 16.0 (SPSS Inc, Chicago, IL). All statistical tests were two-sided and considered to be significant at $p<0.05$. The receiver operating characteristic (ROC) curve analysis was applied in determination of a cutoff value for hTERC. The area under the curve and the 95% confidence intervals (CI) was calculated. Based on the cutoff value, FISH results were classified as either positive or negative. Nonparametric quantitative comparison was performed using Kruskal–Wallis analysis in individual hTERC amplification ratio. The chi-square tests were used for multigroup, qualitative comparisons. The adjusted odds ratios (OR) with 95% CI were calculated about hTERC and age for CIN2 or worse (CIN2+).

Results

hTERC positive nuclei and cutoff value

Usually a nucleus was scored as positive for hTERC signals greater than two and CSP3 signals no less than two (Fig. 1). The patterns might either show unequal hTERC signals relative to CSP3 signals, or they were in equality, which can be called a tumor ploidy. The distance between any two signals had to be at least the diameter of one signal to avoid counting split signals as two signals. In each case, a totally random 200 nuclei were evaluated. Four quarters were separated on hybridization area, and in each quarter 50 nuclei were