Application of TDI-FP for Analysis of A647G Variation in HPV 16 E7 Gene From Cervical Cancer Patients

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Abstract Template direct dye-terminator incorporation with fluorescence-polarization (TDI-FP assay) is a technology for genotyping single nucleotide polymorphisms (SNPs). To apply this method in analyses of A647G variation in human papillomavirus (HPV) 16 E7 gene from HPV 16-positive cervical tissues, a total of 91 and 49 HPV 16-positive DNA samples obtained from women with cervical cancer and normal/inflamed cervices living in Shaanxi in northwest China were subjected to the partial E7 gene PCR with nucleotide (nt) 647 in the products. Then, the oligonucleotide probe designed to anneal immediately to nt 647 was hybridized to the template within the PCR amplicons, and extended specifically by TAMRA-ddTTP or R110-ddCTP directed by the base at nt 647. The increasing FP values were read and the base at nt 647 was identified. The prevalence of nt 647 A→G was 35.71% (50/140). The variation 647G detected in 42.86% (39/91) of women with cervical cancer was significantly higher than 22.45% (11/49) detected in those with normal/inflamed cervices ($x^2 = 5.778$, $P = 0.016$). The odds ratio (*OR*) between these two groups was 2.59 (95% confidence interval=1.17~5.71). The results demonstrate that TDI-FP method can be potentially applied in analysis of interest point mutations in HPVs. The incidence and risk implication of HPV 16 A647G variant infection in Shaanxi, China, displays significant geographic difference from other areas. The HPV 16 with E7 gene A647G point mutation appears to have a higher risk for invasive cervical cancer in women living in Shaanxi.

Key words human papillomavirus type 16, E7 gene, variant, fluorescence polarization, cervical cancer

Cervical cancer is one of the most common cancers for women worldwide. There have been compelling evidences that high-risk human papillomavirus (HPV) play a major causal role in the development of cervical cancer[1]. The E6 and E7 gene products of high-risk HPVs deregulate the host cell growth cycle by binding and inactivating tumor suppressor proteins, cell cyclins, and cyclin- dependent kinases[2], resulting in stimulation of cellular DNA synthesis, cell proliferation and malignant transformation. Especially, the E7 protein is the most potent for transforming activity by interaction with the retinoblastoma gene product, pRb.

HPV type 16 is the prevailing high-risk type, being present in ≈50% of the cervical cancer worldwide[3,4]. Nucleic acid sequencing data show that many natural HPV 16 variations exist[5,6], and specific HPV 16 variations may be associated with increased biological aggression[7]. Nucleotide (nt) 647 A→G nucleotide change (A647G) in HPV 16 E7 gene, from AAT to AGT at codon 29, resulting in an amino acid change from asparagine to serine at amino acid 29, is within the Rb-binding sites for E7 protein. This hot spot variation displays geographic dependence[8,9].

While the general Chinese population is reported to have a low rate of venereal disease, including HPV infections, cervical cancer is relatively common in China, and certain parts of China have among the highest rates of cervical cancer in the world[10,11]. This may reflect a predominance of cervical infection with particularity aggressive HPV variants. However, there are relatively few data on HPV variations among the Chinese population. In this study we adapted template-directed dye-terminator incorporation with fluorescent polarization-detection (TDI-FP), a high throughput genotyping method[12], to detect the E7 A647G variation of HPV 16 gene among cervical cancer patients living in Shanxi, in northwest China.

1 Materials and methods

1.1 Subjects

A total of 140 HPV 16-positive DNA samples from cervical biopsy tissues typed by the method we developed previously[4,13] were included in this study.

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The cervical specimens were obtained from the women visiting the colposcopy clinics at the Second Hospital, Xi'an Jiaotong University, and Xijing Hospital, Fourth Military Medical University, for further management following abnormal Pap smears or cervical cancer. Pathologically, of these, 91 had invasive cervical cancer and 49 had normal/inflamed cervices. The mean age of the population studied was 45 years (range, 25~74), while the mean age of patients with cervical cancer was 46 years and of those with normal/inflamed cervices was 43 years.

1.2 Primers and oligonucleotide probe

The primers of PCR targeting partial HPV 16 E7 gene, sense TTAGATTTGCAACCAGAGACA and anti-sense ACTCTACGCTTCGGTTGTGC, were designed according to the published sequence of the prototype accepted by GenBank[14]. The amplicon is a 168 bp product spanning nt 598~765 and flanking nt 647. The oligonucleotide probe, 5′ TGACAGCTC-AGAGGAGGAG 3′, was designed to anneal immediately upstream of the interest polymorphic site, nt 647. Primers and oligonucleotide probe were synthesized in Sbsbio (Beijing, China).

1.3 PCR condition

All HPV 16-positive DNA samples were subjected to the PCR. Total 25 amplification cycles were run in the PCR processor (Elmerkin, USA) with a 94℃ denaturation step (40 s), a 53℃ annealing step (30 s), and a 72℃ extension step (40 s), including an initial denaturation step of 3 min and a final extension step of 7 min. The 25 μl of PCR volume contained 1 μl DNA extract, 150 μmol/L dNTPs, 2.0 mmol/L $MgCl_2$, 0.5 U of Taq DNA polymerase (Promega, USA), and 0.25 μmol/L each primer. We set up the PCR reactions containing tridistilled sterile water instead of DNA samples, or sequence-verified E7 plasmids of each genotype as blank, negative or positive controls, respectively. PCR products were checked by ethidium bromide agarose gel electrophoresis.

1.4 Primer and dNTP digestion

To digest the excess primers and unincorporated dNTPs in the PCR products which may lessen the specificity of the TDI-FP reaction, 1.8 μl of Clean-up dilution buffer, and 0.2 μl of 10× Clean-up reagent (PerkinElmer, USA) containing shrimp alkaline phosphatase and *E.coli* exonuclease were added to each 5 μl of PCR product and incubated at 37℃ for 2 h, and followed by 95℃ for 20 min for enzyme inactivation.

1.5 Template-directed dye-terminator incorporation reaction

The 7 μl of digested PCR product was added 13 μl of TDI mixture containing 2.0 μl 10× reaction buffer, 0.05 μl acyclopol, 1.0 μl of R110-ddCTP/TAMRA-ddTTP combination (PerkinElmer, USA), 0.5 μl 10 μmol/L oligonucleotide probe, and 9.45 μl water. The solution was then incubated at 95℃ for 3 min, followed by 30 cycles of 94℃ for 15 s and 54℃ for 30 s.

1.6 Florescence polarization determination

After the template-directed dye-terminator incorporation reaction, the solution was transferred to 384-well, black-skirted plates (MJ Research, USA). Florescence polarization (FP) values of both R110 and TAMRA were immediately measured on Fluorescence Polarization-Capable Instrument-Victor^2 (Perkin-Elmer, USA). The base at nt 647 of HPV 16 E7 in each sample was recognized by high FP value of R110 or TAMRA.

1.7 Statistical analysis

x^2 test was used to assess the proportions of HPV 16 prototype and 647G variant among women with cervical cancer and normal/inflamed cervices. Risk association was examined by comparing odds ratio. Difference was regarded as significant when the probability value obtained from the statistical test was less than 0.05 ($P < 0.05$).

2 Results

2.1 Scatter plot of TDI-FP assay

After TDI-FP assay for genotyping the A/G base at nt 647 position of HPV 16 E7, the FP values (measured as mP) clustered into three distinct groups (Figure 1). Blank controls, which lacking DNA, had low FP values for both R110 and TAMRA and were situated in the lower left corner of the plot. Samples that had G at the nt 647 of HPV 16 E7 gene (variant of HPV 16), and the corresponding positive controls had high FP values for R110, consistent with incorporation of R110-ddCTP to the probe, but low FP values for TAMRA. These samples occupied the lower right corner of the plot. Conversely, samples with A at the nt 647 of HPV 16 E7 gene, i.e., with the prototype of HPV 16, and the corresponding negative controls had high FP values for TAMRA, consistent with incorporation of TAMRA-ddTTP to the probe, but low FP values for R110. These samples clustered in the upper left corner of the plot.

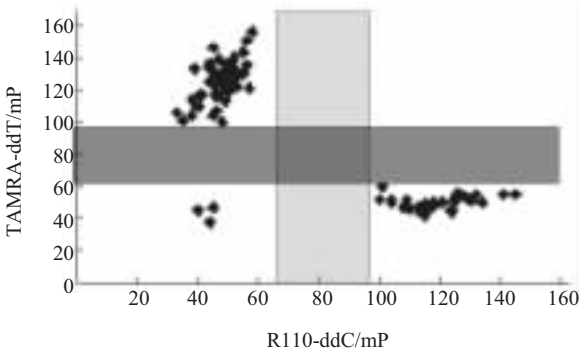

Fig. 1 TDI-FP scatter plot for nt 647 polymorphism of HPV16 E7

Blank controls had low FP values for both R110 and TAMRA and were situated in the lower left corner of the plot. Samples with G at the nt 647 of HPV 16 E7 gene (variant of HPV 16), and the corresponding positive controls had high FP values for R110, but low FP values for TAMRA and occupied the lower right corner of the plot. Conversely, samples with A at the nt 647 of HPV 16 E7 gene, and the corresponding negative controls had high FP values for TAMRA, but low FP values for R110. These samples clustered in the upper left corner of the plot.

2.2 A/G polymorphism at nt 647 in HPV 16 E7 gene determined by TDI-FP

The results of HPV 16 647A/G analysis were shown in Table 1. In women with normal/inflamed cervices, 11 of 49 cases (22.45%) contained the HPV 16 E7 647G variant and 38 of the 49 cases (77.55%) contained the prototype, 647A. Cervical cancer harbored HPV 16 E7 647G variant in 39 of 91 cases (42.86%) and contained the prototype in 52 cases (57.14%). Statistically, the 647 A→G point mutation was significantly more frequent in the patients with cervical cancer than in those with normal/inflamed cervices ($x^2 = 5.778$, $P = 0.016$). The risk association was further examined by comparing the odds ratio for these two groups. The subjects with 647G variant showed a 2.59- fold higher risk (odds ratio 1.17 ~ 5.71) to have cervical cancer than the subjects with 647 A variant.

Table 1 HPV 16 E7 nt 647 polymorphisms in women with normal/inflamed cervices and cervical cancer

HPV 16 647A/G polymorphism	Normal/inflamed cervices *n*/%	Cervical cancer *n*/%	Total *n*/%	OR (95% CI)[1)]
647A (prototype)	38 (77.55)	52 (57.14)	90 (64.29)	2.59 (1.17～5.71)
647G (variant)	11 (22.45)	39 (42.86)	50 (35.71)	

[1)] CI：Confidence interval; OR：Odds ratio.

3 Discussion

Cervical cancer is preceded only by breast cancer as the most common cause of death from cancer in women worldwide. In developing countries, cervical cancer is often the most common cancer in women and may constitute up to 25% of all female cancers [15]. Cervical cancer is also relatively common in China, with the occurrence of 150 000 new cases each year, and certain parts of China, such as Lueyang county in Shaanxi province, have among the highest incidence of cervical cancer in the world [10,11]. As among the non-Chinese populations, HPV 16 is the most frequently identified HPV type among Chinese women, with the prevalence over 50%[4, 13].

A variety of studies have indicated that the E6 and E7 proteins of HPV 16 are transforming proteins, and E7 protein has more transforming potential than E6. It is known that conserved region (CR) 2 of HPV 16 E7 protein binds and inactivates pRb[16]. Especially, amino acids 20~29 in this region contribute strongly to pRb binding. The interaction between E7 and Rb protein is considered to be one of the most important aspects of the mechanism of malignant transformation. Additionally, amino acid 29, the target site of our study, has also been shown to be important for host immune recognition of E7[17].

In vitro mutational analysis has shown that the change of a single amino acid within the CR 2 region of the E7 protein of HPV can affect malignant transformation[18]. A→G substitution at nucleotide 647 in HPV 16, leading to an amino acid change from an asparagine to a serine at residue 29, was the most common variant in E7 gene found in Japanese [19], Korea[8] and Hong Kong China[9], with the prevalence of 60%, 59.7% and 58.0%, respectively. The prevalence of nt 647 A→G mutation was relatively low in other areas in the world: 0.9% for Germany[20], 36.4% for Tanzania[21], 0% for Sweden[22] and 14.3% for Sichuan in central China [23]. In our study, the prevalence of nt 647 A→G mutation in Shaanxi, in northwest China, was 35.71%, being different from elsewhere in the world. As in Korean women[8], this variation was more frequently detected in cervical

cancer lesions than in noncancerous lesions in those enrolled in our study. However, no significant trend for a change in frequencies of this variant was observed among the women with cervical cancerous and noncancerous lesions living in Hong Kong China [9]. One possibility for the discrepant results of studies on this substitution is that the A647G variation may differ on epidemiology and oncogenity among different population. Alternatively, the conflicting observations may be because of the small numbers of cases investigated. Whether this A647G substitution displays a population-dependent risk for cervical cancer would require further investigations, including functional analysis of this E7 variation.

TDI-FP assay has been used in large-scale analysis of human single nucleotide polymorphisms (SNPs)[12,24~26]. In this single base extension technique, an oligonucleotide probe is designed to anneal immediately to a SNP of interest in PCR-amplified product. In the presence of DNA polymerase and fluorescence-labeled dNTPs dictated by the polymorphism site in the target DNA sequence, the probe is annealed to template immediately to the SNP site within the PCR amplicon, and extended specifically by one dye-base directed by the template of the target DNA, resulting in the increasing of the corresponding FP value that could be read by Fluorescence Polarization-Capable Instrument-Victor^2. The dNTP incorporated and assign genotype can then be identified[24].

DNA sequencing is the most common method used in HPV variation analysis. However, it cannot be afforded by most laboratories, especially clinical laboratories due to the cost and equipment requirement. We adapted TDI-FP in our study to identify HPV 16 A647G nucleotide change, the hot spot variation found in E7 gene, as it is simple, sensitive, specific, and suitable for automated genotyping of large number of samples. Before adapting TDI-FP in detecting of HPV 16 A647G variation from clinical samples, we had optimized its conditions using sequence-validated E7 plasmids respectively containing 647A and 647G as templates. These sequence-validated E7 plasmids served as controls of corresponding genotype in later TDI-FP assays. We confirmed TDI-FP genotype results in our study by including sequence-validated controls in each assay. 100% concordance comparing TDI-FP with other methodologies have also been found in our previous studies [4,13] and reported by others [24,25,27]. TDI-FP assay does not require the use of specially modified probes, the conditions can be easily optimized, and FP is detected directly in solution requiring no washing, separation. We believe that this method can also be used in the analysis of other interest point mutations in HPVs, or even in other pathogenic organism.

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应用 TDI-FP 技术分析宫颈癌组织 HPV16 E7 基因 A647G 点突变

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摘要 模板指导的末端碱基掺入反应结合荧光偏振检测技术(template direct dye-terminator incorporation with fluorescence-polarization，TDI-FP) 是 SNP 检测新技术. 应用 TDI-FP 方法分析中国陕西 HPV16 阳性宫颈组织 HPV16 E7 基因第 647 位核苷酸 A→G 热点突变(即 A647G)，首先在 HPV16 阳性的 91 例宫颈癌及 49 例正常 / 宫颈炎妇女宫颈 DNA 标本中，PCR 扩增含 647 位点在内的 HPV16 E7 部分基因, 然后将紧邻 647 位点 5′端的寡核苷酸探针与 PCR 产物内的模板杂交，并延伸一个与 647 位点碱基互补的荧光标记碱基：TAMRA-ddTTP 或 R110-ddCTP. 用荧光偏振仪读取荧光偏振 (*FP*) 值，根据升高的相应 FP值判断 647 位点碱基. 结果表明，宫颈组织 HPV16 E7 A647G 的总体检出率为 35.71% (50/140). 宫颈癌组的 A→G 突变率为 42.86% (39/91)，显著高于正常 / 宫颈炎组 22.45% (11/49) 的突变率 ($x^2 = 5.778$, $P = 0.016$)，两组间的 *OR* 值为 2.59 (95% *CI* = 1.17~5.71). 提示 TDI-FP 可用于 HPV 有意义点突变的分析；我国陕西地区妇女 HPV 16 A647G 突变率及其对宫颈癌的警示性与其他地区相比有明显差异，该地区携带此突变病毒株的妇女患宫颈癌的风险可能较高.

关键词 人乳头瘤病毒 16 型，E7 基因，突变，荧光偏振，宫颈癌

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