RESEARCH ARTICLE

PAX1 Methylation Analysis by MS-HRM is Useful in Triage of High-grade Squamous Intraepithelial Lesions

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Abstract

This study is aimed to investigate the role of paired boxed gene 1 (PAX1) methylation analysis by methylation-sensitive high-resolution melting (MS-HRM) in the detection of high grade lesions in atypical squamous cells cannot exclude high-grade squamous intraepithelial lesion (ASC-H) and compared its performance with the Hybrid Capture 2 (HC2) human papillomavirus (HPV) test. In our study, 130 cases with a diagnosis of ASC-H from the cervical cytological screening by Thinprep cytologic test (TCT) technique were selected for triage. Their cervical scrapings were collected and evaluated by using PAX1 methylation analysis (MS-HRM) and high-risk HPV DNA test (HC2), followed by colposcopy and cervical biopsy. Chi-square test were used to test the differences of PAX1 methylation or HPV infection between groups. In the detection of CIN2+, the sensitivity, specificity, the PPV, NPV and the accuracy of PAX1 MS-HRM assay and high-risk HPV (HR-HPV) tests were respectively 80.6% vs 67.7%, 94.9% vs 54.5%, 83.3%, vs 31.8%, 94.0% vs 84.4%, and 91.5% vs 57.7%. The PAX1 MS-HRM assay proved superior to HR-HPV testing in the detection of high grade lesions (CIN2+) in ASC-H. This approach could screen out the majority of high grade lesion cases of ASC-H, and thus could reduce the referral rate to colposcopy.

Keywords: ASC-H - PAX1 methylation - MS-HRM analysis - HPV testing

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Introduction

Optimal treatment option for women with atypical squamous cells -cannot exclude a high-grade squamous intraepithelial lesion (ASC-H) is not well established. Most low-grade lesions regress spontaneously. On the other hand, a small but significant proportion of cases with ASC-H may harbour cervical intraepithelial neoplasia (CIN), or even invasive carcinoma. ASC-H smears need further investigation to identify patients likely to have a high-grade lesion. The most important etiologic factor for cervical cancer is a persistent infection with high-risk HPV (HR-HPV).

In almost every case of invasive cervical cancer and its pre-malignant progenitors, HPV can be recognized (Walboomers et al., 1999; Nambaru et al., 2009; Wang et al., 2013). In consequence, HPV testing has been adopted for the triage of patients with ASC-H (Wu et al., 2006; Bandyopadhyay et al., 2008). But, HR-HPV triage has poor specificity. Only in the older age group, HPV-DNA testing may be of benefit to better identify those women at risk for high grade lesions (Selvaggi , 2013). Thus, there is a need for other markers which have high sensitivity and specificity. Recently, paired boxed gene 1 (PAX1) was reported as a novel methylation-silenced gene in cervical cancer (Lai et al., 2008). Methylation-sensitive high-resolution melting (MS-HRM) analysis has recently been introduced as a rapid and sensitive technique for the quantification of DNA methylation (Wu et al., 2011; Dimitrakopoulos et al., 2012).

In this study, we tested the efficacy of PAX1 gene methylation analysis by MS-HRM for the triage of ASC-H, and compared its performance with HC2 HPV test.

Materials and Methods

Patient Samples

A total of 130 consecutive women [mean age 46 years, range (25-68)] who participated in the cervical cytological screening by Thinprep cytologic test (TCT) technique in the Weifang city people's hospital from September 2011 to September 2013, and with an ASC-H diagnosis based on the screening, were included. The cytological screening was performed using The 2001 Bethesda System (TBS). The exclusion criteria included current pregnancy, history of cancer at other sites, and history of immune compromise diseases. The cervical scrapings of 130 ASC-H cases were evaluated by using PAX1 gene methylation analysis (MS-HRM)and high-risk HPV-DNA test (HC2). These 130 ASC-H cases were admitted for colposcopy and cervical biopsy. Biopsies were cut and stained with hematoxylin and eosin (H&E), read by a pathologist, and subsequently confirmed by a second independent reading.

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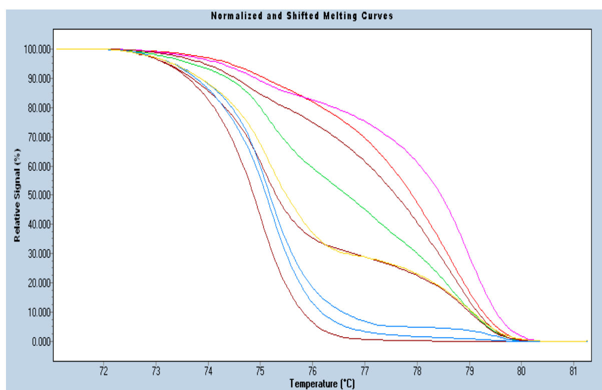

Figure 1. PAX1 MS-HRM Curves Representing the Methylation Status of Three Samples (From left to right ,the curve representing the methylation status: 0, sample1, 10%, sample2, 30%, 50%, 80%, sample3, 100%)

HPV Test

High-risk HPV-DNA (HR-HPV) analysis of the cervical sample was detected by the HC2 test ((Digene Corp., Silver Spring, MD, USA). The DNA in a specimen was denatured and hybridized with a cocktail of RNA probes directed against a panel of 13 HR-HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The RNA-DNA hybrids were captured by hybrid-specific antibodies and detected by alkaline phosphatase-linked second antibody and chemiluminescence.

MS-HRM analysis of PAX1

PCR amplification and HRM were performed using a Roche LightCycler (Roche). DNA was extracted from cervical scrapings using the QIAamp DNA mini kit (Qiagen) and bisulfite converted using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. The bisulfite modified DNA was PCR-amplified with the specific primers (forward, 5'-CGGGAATTAATGAGTTGTTAATT-3'; reverse, 5'-AAACCCAAAATAAACTTCATCC-3') in a HRM Epigenetics PCR Kit (Qiagen). DNA extracted from peripheral blood mononuclear cells of normal individuals was used as unmethylated control DNA. DNA of human peripheral blood mononuclear cells treated with M.SssI methyltransferase served as the fully methylated control. Methylation standards were constructed by diluting 100% methylated bisulfite-modified control DNA in a pool of normal bisulfite-modified DNA at ratios of 10, 30, 50, 80 and 100% . MS-HRM was performed in a total volume of 25μl containing :2μl of modified template DNA, 12.5μl 2×EpiTect HRM PCR Master Mix (HRM Epigenetics PCR Kit, Qiagen), 1.9μl of each primer (10μM), and 6.7μl of PCR grade water. The cycling conditions were: denaturation at 95C for 10 min, followed by 40 cycles of 95°C for 10 s, annealing for 10 s from 62°C to 50°C (decrease 0.5°Cper cycle), extension at 72°C for 10 s; followed by an HRM step of 95°C for 1 min, and 40°C for 1 min, 65°C for 15 s, and continuous acquisition to 95°C at one acquisition per 0.02°C, and then detected using the Roche LightCycler. HRM data were analyzed by use of the dedicated HRM software (LightCycler 480 Gene Scanning Software). All experiments were performed in duplicate. The study was approved by the institutional review board of the Weifang city people's hospital in Shan dong province, PRC, and informed consents were obtained from all participants.

Table 1. The Status of PAX1 Methylation and HR-HPV Infection in Patients with ASC-H

Histopathology	n	PAX1 MS-HRM	HR-HPV
inflammation	39	0% (0/39)	20.5% (8/39)
CIN1	60	8.3% (5/60)	61.7% (37/60)
CIN2	17	70.6% (12/17)	64.7% (11/17)
CIN3	12	91.7% (11/12)	66.7% (8/12)
cervical carcinoma	2	100% (2/2)	100% (2/2)
CIN2 +	31	80.6% (25/31)*[a]	67.7% (21/31)*[b]
CIN2 – (CIN1 or inflammation)	99	5.1%(5/99)	45.5%(45/99)

*comparing CIN2+ group with CIN2- group; [a]P=0.0000 [b]P=0.0303

Statistical analysis

SPSS 13.0 software was used for statistical analyses. Chi-square test were used to test the differences of PAX1 methylation or HPV infection between groups. p values below 0.05 were considered statistically significant.

Results

The sensitivity of the PAX1 MS-HRM assay

The sensitivity of the PAX1 MS-HRM assay was tested using the consistency of normalized melting profiles derived from samples with different ratios of methylated and unmethylated template: 10, 30, 50, 80 and 100% methylated. PAX1 MS-HRM was able to reproducibly detect 10% methylated DNA in a background of unmethylated DNA. At a cutoff value of 10%, In 30 out of 130 ASC-H scrapings, PAX1 methylation was identified. Figure 1 represents the PAX1 MS-HRM results from three representative samples.

The status of PAX1 methylation and HR-HPV infection in Patients With ASC-H

Quantitative assessment of PAX1 methylation by MS-HRM in accordance with disease severity is shown in Table 1. At the cutoff value of 10%, 100% of the cervical carcinoma, 91.7%of the CIN3 and 70.6% of CIN2 specimens were positive for PAX1 methylation compared with 0% for the inflammation, 8.3% for CIN1 specimens (Table 1). At this cut off value, PAX1 methylation of CIN2+ group were more significant than CIN2- group (P <0.001). As a comparison, the HC2 HR-HPV test result was positive in 100% of the cervical carcinoma, 66.7% of the CIN3 and 64.7% of CIN2 specimens. Noteworthily, HR-HPV was present in 61.7% and 20.5% of the benign conditions of CIN1 and the inflammation, respectively, but PAX1 methylation was present in only 8.3% and 0%, respectively (Table 1).

PAX1 MS-HRM assay was as sensitive and much more specific than the HC2 HPV test in detection of CIN2+ in women with a diagnosis of ASC-H

We compared the performance of MS-HRM for PAX1

Table 2. Performance of PAX1 MS-HRM , HC2 Tests in Detection of CIN2+

Methods	Sensitivity	Specificity	PPV	NPV	Accuracy
PAX1 MS-HRM	80.60%	94.90%	83.30%	94.00%	91.50%
HC2 tests	67.70%	54.50%	31.80%	84.40%	57.70%

PPV, positive predictive value; NPV, negative predictive value

methylation testing to the HC2 HPV test in the detection of CIN2+ (Table 2). The sensitivity and specificity of PAX1 MS-HRM assay were 80.6 and 94.9%, compared with 67.7 and 54.5%, respectively, for detection by HPV testing. The PPV, NPV and the accuracy of PAX1 MS-HRM assay and HPV tests were respectively 83.3% vs 31.8%, 94.0% vsvs 84.4%, and 91.5% vs 57.7% (Table 2).

Discussion

Thinprep cytologic test is simple, cost-effective, and accurate tool for the initial screening of cervical pathology. Organized cytological screening resulted in a pronounced decrease in the proportion of women with cervical cancer, and accordingly, a marked increase of the number of ASC-H diagnoses. The ASC-H category denotes a small subset of equivocal cytology that is strongly associated with the risk of an underlying cancer precursor lesion. In this study, we screens out 31 high grade lesions cases in 130 women with ASC-H.

Immediate colposcopic evaluation without HPV testing was recommended for women with ASC-H (Wright et al., 2002). These recommendations were largely based on the very high positive rate for HR-HPV for women with ASC-H in the ASCUS-LSIL Triage Study (ALTS) (Sherman et al., 2001; Wright et al., 2002; The ASCUS-LSIL Triage Study (ALTS) Group, 2003). However, the ALTS was limited by a younger patient age population (median 24 yr) (Sherman et al., 2001). In consequence, HPV testing was adopted for the triage of patients with ASC-H (Wu et al., 2006; Bandyopadhyay et al., 2008). Another study suggests that immediate colposcopy is the appropriate management for young women with ASC-H, whereas HPV testing may be more useful in the evaluation of older women (Sherman et al., 2006).

In this study, we demonstrates that MS-HRM for PAX1 methylation testing is better than HC2 HR-HPV testing in the triage of ASC-H. In the detection of CIN2+ , the sensitivity and specificity of PAX1 MS-HRM assay were 80.6 and 94.9%, compared with 67.7 and 54.5%, for detection by HPV testing. The positive predictive value (PPV), negative predictive value (NPV), and accuracy of PAX1 MS-HRM assay are all superior to those of the HC2 HPV test. Indeed, the diagnosis of CIN2 has been a gray area and is the most difficult for pathologists to reproduce among all cervical smear diagnoses (Carreon et al., 2007). For a long time, CIN2 was considered an intermediate entity that may be over called as CIN3 or under called as CIN1. In the detection of CIN2+, PAX1 MS-HRM assay was as sensitive and much more specific than the HC2 HPV test. The MS-HRM for PAX1 methylation testing can screens out the majority high grade lesions cases of ASC-H , and may avoid unnecessary invasive inspection.

HRM was initially described as a very sensitive technology for detection of single nucleotide variations within the DNA strand (Wittwer et al., 2003). MS-HRM analysis for sensitive and high throughput assessment of DNA methylation, was first described by Wojdacz et al. in 2007 (Wojdacz et al., 2007). The MS-HRM protocol has already been shown to robustly assess methylation in up to 30 year old formalin-fixed paraffin embedded (FFPE) samples (Kristensen et al., 2009). Hence even DNA samples of low quality and retrieved from archival and chemically treated clinical samples do not significantly affect the specificity of the MS-HRM protocol. The MS-HRM protocol is based on DNA melting, which allows the identity of the analyte (in this case, the PCR product) to be confirmed without doubt and therefore contributes to the high specificity of this procedure.The principle of this method is that PCR products generated from bisulfite-treated DNA templates with different contents of methylcytosine show differences in melting temperature (Tm), which can be resolved by melting analysis in a thermal cycler coupled with a fluorometer. Quantification was carried out by interpolation on a standard curve generated with serial dilutions of methylated and unmethylated DNA. In the MS-HRM protocol, methylation status is always investigated by comparison of the result (HRM profile) to the result obtained from methylated and unmethylated controls/references analyzed along with the screened samples in the testing setup. This type of analysis allows high accuracy of the conclusions and hence provides high accuracy of the method.MS-HRM analysis has important advantages over the Methylation-Specific PCR. MS-HRM scans all of the CpGs in the target sequence. It also resolves heterogeneous methylation (Candiloro et al., 2011), allows PCR amplification and methylation analysis in one closed tube. Closed-tube approaches are of particular interest to molecular diagnostics as they minimize the risk of PCR carry-over and cross contamination. Moreover, MS-HRM does not require a reference assay for normalization.

In summary, the PAX1 MS-HRM assay have a better performance than HR-HPV testing in the detection of high grade lesions CIN2+ in ASC-H. This approach could screens out the majority high grade lesions cases of ASC-H, also could reduce the referral rate to colposcopy. Our results may provide a new molecular method of triage for women with ASC-H. Because the number of cases particularly in the CIN3 and carcinoma groups is small, further studies in larger screening populations are warranted to prove its efficacy in screening ASC-H.

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