Integrated human papillomavirus analysis as an adjunct for triage of atypical cervical cytology

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KEY MESSAGES

1. Human papillomavirus (HPV) genotyping, HPV type-specific E6/E7 transcript detection, and hTERT transcript detection could improve the specificity of predicting subsequent development of squamous intraepithelial lesions, compared with cocktail HPV DNA detection alone.
2. The correlation between HPV RNA and hTERT expression supports their interactive role in the development of cervical cancer and precursor lesions.
3. Prophylactic HPV vaccines, besides protecting against cervical cancer, can also reduce the burden of screening for those with atypical squamous cells of undetermined significance.

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Introduction

Cervical cytology screening is effective for prevention of cervical cancer by detecting cervical cancer and its precursors—squamous intraepithelial lesions. The most common abnormal cytological finding encountered is atypical squamous cells of undetermined significance (ASCUS), which is associated with the risk of harbouring cervical cancer and its precursors.[1] The large number of ASCUS cases is a considerable burden for any screening programme.

High-risk human papillomaviruses (HPV) are carcinogens of cervical cancer. For triage of women with ASCUS, the Hybrid Capture 2 test (HC2) is most widely applied. When the ASCUS samples are positive for HPV, the women can be referred for colposcopy directly. When the samples are negative, women can return to cytology screening. This practice enables early referral for colposcopy and earlier diagnosis of serious cervical lesions. To improve the specificity of the HPV cocktail test, HPV genotyping, HPV transcription status, and evaluation of telomerase activity are potential adjunctive indicators.

HPV genotyping can be performed by polymerase chain reaction (PCR) using type-specific or consensus primers and sequencing, HPV DNA chips, or linear blots.[2] In addition, SNIPER HPV Genotyping Biochip Assay (previously named microarray-in-microwell assay) has been developed locally.[3] Active HPV infection can be detected by assessing RNA transcription of two HPV oncogenes—E6 and E7—that interact with two important tumour suppressor genes—p53 and RB—respectively. Among women with ASCUS, HPV RNA transcription may potentially identify high-grade cervical lesions.[4] Telomerase adds telomeric DNA repeats onto the ends of the chromosomes during cell proliferation. Activation of telomerase is important for the sustained proliferation and development of tumours. Telomerase activity may be useful for cervical cancer screening. hTERT is a catalytic protein subunit of the telomerase enzyme complex and is a rate-limiting component of telomerase activity. These assays may be developed as potential adjuncts for cervical cancer screening.

This study aimed to investigate whether one-stop integrated HPV molecular analysis can facilitate efficient triage of women with equivocal cytology diagnoses, and to enhance understanding of HPV genotypes and transcription in relation to telomerase activity in the development of cervical cancer.

Methods

This study was conducted from January 2008 to December 2009. In a screening population of Hong Kong, HPV DNA (HC2)–positive ASCUS was tested for HPV genotypes, HPV type-specific RNA transcription, and hTERT RNA transcription by SNIPER HPV Genotyping Biochip Assay, nested PCR, and real-time PCR. Correlation between these parameters and outcomes of patients was evaluated.

Among 184 419 liquid-based cervical cytology samples,[1] 7286 (4.0%) were diagnosed with ASCUS. Of 3618 cases undergoing HPV DNA testing using HC2, 1984 (54.8%) were positive for high-risk HPV and 1634 (45.2%) were negative. After checking the RNA quality of the cytology residues of 871 HC2-positive ASCUS, 604 cases were used for HPV

genotyping, HPV type-specific and hTERT RNA transcription. For hTERT study, 20 normal cytology samples, 20 low-grade squamous intraepithelial lesions (LSIL), 15 high-grade SILs (HSIL), and 7 squamous cell carcinomas (SCC) were also included.

The subsequent cytological and colposcopic biopsy findings of patients with ASCUS were retrieved. The findings of LSIL (for cytology) or cervical interstitial neoplasia (for colposcopy) or above were used as cut offs.

DNA and RNA were extracted from the cervical cytology residues of the 871 samples. The quality and quantity were checked. Reverse transcription was carried out on extracted RNA using random hexamer with SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). HPV genotyping was performed for 604 samples using the locally developed SNIPER HPV Genotyping Biochip Assay.[3] PCR amplification was carried out using biotin-labelled consensus primers targeting for the HPV polymorphic L1 region together with a combination pool of HPV-specific primers designed to amplify 29 HPV genotypes. Primers targeting beta-globin served as a DNA positive control. The biotin-labelled amplicons were hybridised specifically to the immobilised oligonucleotide probes on a membrane-based biochip when the amplicons contained matched sequences of the complementary probes. Following hybridisation, signal visualisation was achieved through colour complex formation with streptavidin peroxidase conjugate. The image of each biochip in the microplate was captured by an imaging system and analysed automatically.

The HPV E6/E7 transcript of various HPV subtypes was evaluated by type-specific nested PCR.[4] Cervical cancer cell lines HeLa, C4-1, SiHa, ME180, and C33A were used as positive and negative controls. The final products amplified from each HPV type-specific nested PCR was separated by gel electrophoresis and visualised by ultraviolet-transillumination after ethidium bromide staining. Both E6 and E7 viral oncogenes of HPV are transcribed from a common promoter. Full length E6/E7 transcript encodes the E6 and E7 proteins, whereas the spliced variants E6*I and E6*II encode truncated E6 proteins with different roles in the virus life cycle in addition to the E7 protein. The size of the amplified products therefore reflected the expression of the full length E6 or truncated E6 proteins. The HPV E6/E7 transcript level was also measured by quantitative PCR (qPCR) using iQ SYBR Green Supermix kits (Bio-Rad Laboratories, Hercules, CA, USA), and inner primers of nested PCR. The relative level was compared with the $\Delta\Delta C_t$ method using the threshold cycle for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the normalising control.

The level of hTERT in each sample was compared by qPCR using the relative standard curve method. Both custom-made TaqMan Gene Expression Assay (for Taqman qPCR) targeting exon II of hTERT transcript (Life Technologies, Carlsbad, CA, USA) and primers targeting the same region (for SYBR Green qPCR) were used. The TaqMan assay for GAPDH Hs99999905_m1 was used for normalisation of the amount of cDNA used in each reaction. Full-length cDNA of hTERT and GAPDH were serially diluted and included in each plate for the construction of standard curves.

The Chi-squared test (2-tailed) was used to determine whether the distribution of individual HPV genotype or HPV E6/E7 transcript status differed in cases with different follow-up findings. Mann-Whitney *U* test was used to evaluate the correlation between the level of hTERT transcript and the follow-up findings. Receiver operating characteristic curve analysis was used to determine the cut-off value of the hTERT mRNA level, above which was regarded as 'positive'. Sensitivities, specificities, and positive and negative predictive values of these molecular tests were calculated. A P value of <0.05 was considered statistically significant.

+ve	HPV 16	HPV 18	HPV 31	HPV 33	+ve
HPV 35	HPV 39	HPV 45	HPV 51	HPV 33	HPV 56
HPV 58	HPV 59	HPV 68	HPV 6	HPV 11	HPV 26
HPV 40	HPV 42	HPV 43	HPV 44	HPV 53	HPV 54
HPV 55	HPV 57	HPV 66	HPV 67	HPV 69	HPV 73
	HPV 82	HB	HB	-ve	+ve

HPV 58

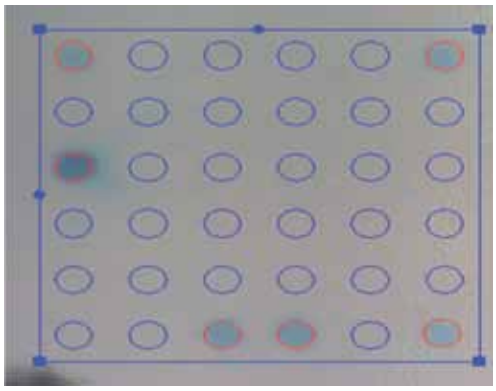

FIG. Human papillomavirus (HPV) genotype HPV58 is identified in a Hybrid Capture 2 test positive sample with atypical squamous cells of undetermined significance using the SNIPER HPV Genotyping Biochip Assay. For control, three spots indicated as positive (+ve) are for colour development control and image alignment. Human beta-globin (HB) serves as an internal control for DNA extraction.

Results

Women with ASCUS who were positive for HPV were more likely to have HSIL (P<0.001, Fisher's exact test) and LSIL or above (P<0.001, Fisher's exact test) detected in follow-up cervical cytology and histology samples.

The HPV genotypes in each ASCUS sample were identified by SNIPER HPV Genotyping Biochip Assay (Fig). The HPV genotype profiles determined by the biochip assay analysis are summarised in Table 1. The five most common high-risk HPV genotypes detected were HPV52 (21.1%), HPV16 (15.5%), HPV58 (15.2%), HPV39 (7.6%), and HPV18 (7.2%). Low-risk HPV genotypes were also detected, but mainly as mixed infection. The six most common types were HPV6 (16.0%), HPV55 (13.8%), HPV44 (12.8%), HPV54 (11.7%), HPV40 (11.7%), and HPV11 (10.6%). This concurred with the focus of HC2 for detection of high-risk HPV. Presence of HPV33 (P=0.001), HPV58 (P=0.024), and HPV68 (P=0.009) correlated with subsequent development of HSIL or above, whereas HPV52 (P=0.025) and HPV31 (P=0.049) correlated with development of LSIL or above. There was no significant difference in outcomes between single or mixed HPV infections.

Full length and spliced variants of E6/E7 transcripts of each HPV genotype could be identified by the size of the amplified products. The distribution of the HPV E6/E7 transcript is summarised in Table 2. To evaluate the significance of HPV oncogenic E6/E7 transcript load, the level of type-specific E6/E7 transcripts was correlated with cytology and biopsy follow-up data. Nested PCR HPV transcription study showed that HPV58 (P=0.023) and HPV59 (P=0.026) mRNA transcripts correlated with subsequent detection of LSIL or above. Correlation between HPV58 mRNA transcripts (P=0.043) and development of HSIL or above was demonstrated by real time PCR.

The hTERT mRNA level significantly increased in each lesion grade (P=0.0129, one-way ANOVA; P=0.0014, test for linear trend). The increase was significant between normal samples and LSIL (P=0.0327), HSIL (P=0.0026), and SCC (P=0.0064), as well as between ASCUS and HSIL (P=0.0401) and SCC (P=0.0319). Among the 600 cases of ASCUS positive for high-risk HPV, the difference was not significant between cases of ASCUS with or without LSIL or above (P=0.696). hTERT/GAPDH level in specific HPV subtype–infected cases was further analysed. In HPV33-infected cases, the hTERT level was significantly higher in cases with a worse follow-up (LSIL or above) [P=0.017]. Among HPV58- (P=0.011), HPV33- (P=0.031), and HPV39- (P=0.045) infected cases, hTERT transcripts correlated with subsequent detection of HSIL or above.

The correlation between E6/E7 mRNA of each HPV genotype and hTERT/GAPDH was conducted using Spearman rho test. Correlation was noted in HPV16- (r=0.486, P<0.000), HPV18- (r=0.557, P<0.000), HPV52- (r=0.704, P<0.000), HPV58- (r=0.451, P=0.003), and HPV33- (r=0.529, P<0.035) infected cases. This indicated that some HPV subtypes may be more capable of inducing oncogenic changes such as telomerase activation.

The sensitivity, specificity, and positive and negative predictive values of HC2 for detecting HSIL or above in this cohort of 3618 cases of ASCUS was 94.3%, 47.6%, 10%, and 99.3%, respectively. Among the HC2-positive ASCUS cases, the specificity and positive predictive value respectively increased to 82.3% and 21.82% after HPV58 genotyping, and to

TABLE 1. Human papillomavirus (HPV) genotypes found in 604 successfully genotyped samples with atypical squamous cells of undetermined significance

HPV genotype	No. (%) of samples with HPV types detected	% of total identifiable HPV genotypes
High risk		
HPV16	112 (15.5)	13.6
HPV18	52 (7.2)	6.3
HPV26	2 (0.3)	0.2
HPV31	23 (3.2)	2.8
HPV33	36 (5.0)	4.4
HPV35	11 (1.5)	1.3
HPV39	55 (7.6)	6.7
HPV45	9 (1.3)	1.1
HPV51	49 (6.8)	5.9
HPV52	152 (21.1)	18.4
HPV53	13 (1.8)	1.6
HPV56	19 (2.6)	2.3
HPV58	110 (15.2)	13.3
HPV59	19 (2.6)	2.3
HPV66	14 (1.9)	1.7
HPV68	38 (5.3)	4.6
82 (MM4)	8 (1.1)	1.0
Subtotal	**722 (100)**	**87.5**
Low risk		
HPV6	15 (16.0)*	1.8
HPV11	10 (10.6)*	1.2
HPV40	11 (11.7)*	1.3
HPV42	6 (6.4)*	0.7
HPV43	9 (9.6)*	1.1
HPV44	12 (12.8)*	1.5
HPV46	1 (1.1)*	0.1
HPV54	11 (11.7)*	1.3
HPV55	13 (13.8)*	1.6
HPV73	6 (6.4)	0.7
Subtotal	**94 (100)**	**11.3**
Indeterminate and other		
HPV67	9 (100)	1.1
Total	**825**	**100**

* Mixed infection

93% and 22.7% after assessment of HPV58 RNA transcript. The corresponding values were 66.0% and 13.4% when hTERT RNA assay alone was used, and 62.8% and 20.0% for hTERT RNA evaluation in HPV58-infected cases.

TABLE 2. Human papillomavirus (HPV) transcripts in cervical cytology samples diagnosed with atypical squamous cells of undetermined significance

HPV genotype (No. evaluated by nested PCR)	No. of samples with either FL or spliced transcript	No. of samples with FL transcript	No. of samples with spliced transcript
HPV16 (n=112)	91/111	90/111	2/111
HPV18 (n=52)	45/52	40/52	5/5
HPV31 (n=23)	21/23	21/23	0/23
HPV33 (n=36)	31/36	31/36	0/36
HPV35 (n=11)	6/11	6/11	1/11
HPV39 (n=55)	37/55	37/55	0/55
HPV45 (n=9)	9/9	9/9	0/9
HPV51 (n=49)	38/49	38/49	0/49
HPV52 (n=152)	141/152	140/152	7/152
HPV58 (n=110)	45/110	40/110	6/110
HPV59 (n=19)	11/19	11/19	0/19
HPV66 (n=14)	12/14	11/14	1/14
HPV68 (n=38)	33/38	33/38	0/38

Discussion

In this study, the five most common high-risk HPV genotypes detected, in decreasing order, were HPV52, HPV16, HPV58, HPV39, and HPV18. These accounted for two-thirds of the HPV genotypes detected. Among ASCUS in this local population, HPV52 and HPV58 were more common than HPV16 and HPV18, with high prevalence in both cervical cancers and precancerous lesions.[5] The distribution of HPV genotypes in different populations varies. This may be due to differences in hospital versus screening populations and the methodology used for HPV genotyping.

About one-quarter of ASCUS in our screening population were HPV negative and did not need colposcopy if HPV vaccine was administered. This may lessen the burden on the screening programme. In addition, HPV33, HPV58, and HPV68 correlated with subsequent detection of HSIL or above, whereas HPV52 correlated with development of LSIL or above. In most studies, HPV16 and/or HPV18 carry prognostic significance. The difference in findings related to disease outcome may be due to the dominant prevalence of HPV58 and HPV52 in this population with ASCUS.

When HPV type–specific RNA transcript status correlated with subsequent follow-up using nested PCR, HPV58 E6/E7 transcription correlated with subsequent development of more serious cervical lesions. Both HPV58 detection and E6/E7 transcription were prominent among the high-risk HPV genotypes.

hTERT transcription levels did not correlate with subsequent detection of serious cervical lesions. Nonetheless, subsequent development of cervical lesions in HPV16- and HPV58-infected cases were noted.

Detection of high-risk HPV by cocktail HC2 achieved a high sensitivity and negative predictive value but moderate specificity and positive predictive value. Additional HPV58 genotyping and RNA transcript assay as well as hTERT assay in HPV58-infected cases enhanced the specificity and positive predictive values. These findings support the use of these adjunct techniques to cytology and HPV DNA testing in triaging women with ASCUS.

Transcription of hTERT correlated with that of HPV16, HPV18, HPV52, HPV58, and HPV33. This suggested significant interaction between HPV and telomerase activity. These HPV genotypes may be more capable of inducing oncogenic changes including telomerase activation. Indeed, HPV oncogenic protein E6 is reported to induce the transcription of hTERT and influence telomerase activity leading to cell immortalisation and cervical carcinogenesis.

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