

HPV types and E6/E7 mRNA expression in cervical samples from Turkish women with abnormal cytology in Ankara, Turkey

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Received: 31.08.2015 • Accepted/Published Online: 05.06.2016 • Final Version: 27.02.2017

Background/aim: Human papillomaviruses have been established as a risk factor for invasive carcinoma of the uterine cervix. HPV E6/E7 oncogene expression has recently emerged as a promising biomarker to determine the risk for progression to high-grade cervical lesions. The aim of this study was to evaluate HPV mRNA and DNA detection in samples with abnormal cytology.

Materials and methods: Cervical specimens were obtained at the Department of Obstetrics and Gynecology via cervical brushes during January–October 2011. Liquid-based cytology slides were evaluated according to the 2001 Bethesda System. Cytology specimens from a total of 81 women with abnormal cytology were included. Real-time PCR and NASBA assays were performed to detect HPV DNA and E6/E7 mRNA, respectively.

Results: HPV DNA was identified in 73 samples (90.1%). HPV E6/E7 mRNA expression was observed in 45 samples (55.6%). A statistically significant difference was observed among cytological diagnosis groups. In 25 patients, a biopsy was performed during the follow-up. HPV DNA was detected in all of these patients. HPV E6/E7 expression was present only in CIN I–III diagnosed patients.

Conclusion: The E6/E7 mRNA test is a robust indicator of cytological atypia and correlates better with progressive lesions than DNA assays.

Key words: Cervical cancer, cytology, HPV DNA test, E6/E7 mRNA

1. Introduction

Human papillomaviruses (HPVs) are a large and heterogeneous group of DNA viruses belonging to the family *Papillomaviridae*, comprising about 100 well-characterized genotypes and more than 100 putative new types (1,2). Infections with HPVs have been established as a risk factor for invasive carcinoma of the uterine cervix by epidemiological and laboratory data (1,3). Based on the potential risk to induce invasive cancer, HPV types that infect the anogenital tract are currently grouped into high, potentially high, and low risk types, with HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 regarded as high risk; 26, 53, and 66 as potentially high risk; and 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108 as low risk (4).

The majority of HPV infections are transient and approximately 90% of infections will normally be cleared within 2 years (5,6). However, persistence of high-risk HPV types is associated with the development of high-grade squamous intraepithelial lesions and progression to cervical cancer (6,7). Cytology testing for detection of intraepithelial lesions has low sensitivity (8). HPV DNA testing has been suggested for screening of women over 30 or women with equivocal cytology results (9,10). However, since the overall life-long risk for HPV infection is 80%, only a fraction of these women with detectable DNA are likely to progress to malignant transformation (5,11). Thus, a more reliable viral marker for predicting HPV persistence and initiation of cellular transformation is required to predict progression to cervical neoplasia.

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E6 and E7 oncoproteins of HPV contribute significantly to the malignant transformation of persistently infected cells, by inactivation of the p53 and pRb tumor suppressor proteins (12). Viral E6/E7 proteins are consistently expressed in malignant tissues and upregulated expression of E6/E7 is considered necessary for the initiation and progression of cervical neoplasia. The detection of E6/E7 mRNA transcripts may therefore be superior to HPV DNA detection as an indicator of HPV infection associated with increased risk of progression to neoplasia (13,14). Recently, standardized commercial assays based on nucleic acid sequence-based amplification (NASBA) technology have been available for detecting HPV E6/E7 mRNA expression from the five most prevalent HPV types in cervical cancer. Previous reports on the performance of HPV E6/E7 mRNA detection demonstrated a higher clinical specificity for detecting high-grade histological lesions compared to DNA-based tests, but a lower clinical sensitivity (15). The aim of the present study was to evaluate HPV mRNA and DNA detection in samples with abnormal cytology.

2. Materials and methods

2.1. Study design and cervical sampling

The study was conducted at Hacettepe University Hospital, a tertiary care hospital and a major reference center in Ankara Province, Turkey. The study protocol was approved by the local ethics board (13.07.11/0426).

Liquid-based (SurePath) cervical specimens were obtained at the outpatient clinics of the Department of Obstetrics and Gynecology via cervical brushes during January–October 2011 after informed consent was obtained. The brushes were immediately placed into a BD SurePath Preservative Fluid (TriPath Imaging, Burlington, NC, USA) vial and sent to the Department of Pathology, Cytology division. The specimens were processed for thin layer cytology slides in the laboratory within 1 to 3 days of the date of collection. After slide preparation, all residual samples were transported to Hacettepe University Department of Medical Microbiology, Virology Unit and kept frozen at -70°C until further analyses. The slides were evaluated by experienced cytotechnologists and pathologists under light microscopy. Cytological evaluations were reported according to the 2001 Bethesda System (16). Repeat samples from the same patient were excluded. If a biopsy was performed during the follow-up of the patient, the specimens were fixed routinely in formalin, embedded in paraffin, and stained with hematoxylin–eosin for histopathological diagnosis. The microbiologists from Hacettepe and Gazi Universities were blinded to the cytology results of the patients.

2.2. Detection of HPV E6/E7 mRNA

All nucleic acid extraction and E6/E7 mRNA detections were performed in Hacettepe University Department

of Medical Microbiology, Virology Unit. The residual specimens that had abnormal cytology results were centrifuged for 5 min at 6000 rpm, washed three times, resuspended in 500 μL of Tris-EDTA, and subjected to total nucleic acid extraction via a magnetic silica-based automated commercial system (NucliSENS easyMAG, bioMérieux, France). E6/E7 mRNA detection was performed using a commercial NASBA assay, the NucliSENS EasyQ HPV v1.1 (bioMérieux, France), according to the manufacturer's instructions. The system incorporates 5 molecular beacons to identify and differentiate E6/E7 mRNA expression in HPV types 16, 18, 31, 33, and 45. Concurrent amplification and detection of small nuclear ribonucleoprotein specific protein A (U1A) are also used as an internal control in the system.

2.3. Detection and typing of HPV DNA

HPV detection and typing were performed in Gazi University Department of Medical Microbiology. A real-time PCR-based commercial system (Heliosis Human Papilloma Virus LC PCR Kit, Metis Biotechnology, Turkey) was employed for HPV DNA detection and HPV-16 typing via melting curve analysis. The amplifications were carried out in PTC-200 Thermal Cycler (MJ Research, USA) and LightCycler 2.0 (Roche Diagnostics, Germany) instruments. Amplification of the internal control was visualized at a melting temperature (T_m) of 90°C ($\pm 3.5^{\circ}\text{C}$), whereas amplifications of HPV-16 and other HPV types were interpreted by melting curves at 69°C ($\pm 1.0^{\circ}\text{C}$) and 82°C ($\pm 3.5^{\circ}\text{C}$), respectively. The analytical sensitivity of the system is given as 20 copies/ μL for HPV types 16 and 18.

2.4. Statistical analysis

Statistical analysis of collected data was performed in Hacettepe University Department of Biostatistics. Descriptive parameters of the study group, HPV types, and the presence E6/E7 mRNA expression were assessed via statistical tests. Student's t and extension of Fisher's exact test to rxc tables were employed wherever appropriate. Statistical significance was considered as $P < 0.05$ level. All statistical analyses were performed by SPSS version 15.0 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Cytological and histological diagnoses in cervical samples

A total of 81 women (age: 22–89 years, mean: 42.49, median: 40, standard deviation: 13.37) with suitable specimen conditions and reactive internal control signals were evaluated for HPV DNA and mRNA expression after informed consent was obtained. The cytological diagnoses were atypical squamous cells of undetermined significance (ASC-US) in 25 samples (30.9%), atypical

squamous cells - cannot rule out high-grade squamous intraepithelial lesion (ASC-H) in 3 samples (3.7%), atypical glandular cells (AGC) in 4 samples (4.9%), atrophic cells in 2 samples (2.5%), low-grade squamous intraepithelial lesion (LSIL) in 35 samples (43.2%), high-grade squamous intraepithelial lesion (HSIL) in 9 samples (11.1%), and adenocarcinoma in 3 samples (3.7%) (Table 1). Biopsy results were available for 25 individuals (25/81, 30.9%) and they revealed the presence of chronic cervicitis in 7 (28%), cervical intraepithelial neoplasia grade I (CIN I) in 8 (32%), cervical intraepithelial neoplasia grade III (CIN III) in 7 (28%), and adenocarcinoma/anaplastic carcinoma or carcinosarcoma in 3 samples (12%).

3.2. HPV DNA and E6/E7 mRNA expression in cervical samples

HPV DNA was identified in 73 samples (73/81, 90.1%), consisting of HPV-16 in 46 samples (63.1%), HPV other than 16 in 15 samples (20.5%), and mixed HPV infections in 12 samples (16.4%) (Table 2).

HPV DNA positivity was not associated with patient age ($P = 0.939$) and did not demonstrate a statistically significant difference according to the cytological diagnosis ($P = 0.35$). Variations in HPV type distribution in samples with different cytological lesions, as determined via DNA assay, were also not statistically significant ($P = 0.464$) (Table 1).

HPV E6/E7 mRNA expression was observed in 45 samples (45/81, 55.6%) (Table 1). Expression from HPV-16 was identified in 26 samples (57.8%), followed by HPV-18 (2/45, 4.4%) and HPV-31 (1/45, 2.2%), whereas mRNA from multiple HPV types was revealed in 16 (35.6%) (Table 2). A significant age difference was observed in patients with and without E6/E7 expression, wherein individuals with positive mRNA results were younger (mean age: 39.56 versus 46.17, $P = 0.026$). Moreover, a statistically significant difference in E6/E7 expression among the various cytological diagnosis groups was noted ($P = 0.025$).

Distribution of HPV DNA types and mRNA expression according to the cytological evaluation results is provided in Table 2.

3.3. Comparison of HPV DNA and mRNA expression

Overall, the detection rates of HPV DNA and E6/E7 mRNA in samples were statistically significant (90.1% versus 55.6%, $P = 0.002$). However, no significant difference in mRNA expression among HPV types (as interpreted as type 16, other than 16, and mixed infections) via DNA assay was noted ($P = 0.95$).

A total of 8 samples (8/81, 9.9%) were negative in both assays, which comprised 2 cervical atrophy, 3 ASCUS, and 3 LSIL specimens. All samples with detectable mRNA were positive for HPV DNA. However, expression of E6/E7 mRNA was absent in 28 samples (28/73, 38.4%) with detectable DNA (Table 3). Expression of mRNA was identified in 28 samples (28/46, 60.9%) with positive HPV-16 DNA, in 7 samples (7/46, 15.2%) with HPV types other than 16, and in 10 samples (10/12, 83.3%) with mixed HPV types (Table 3). Expression patterns inconsistent with DNA typing were obtained in 3 samples (3/45, 6.7%), which included HPV-16 mRNA detected in 2 non-HPV-16 DNA samples and HPV-31 expression in a sample with HPV-16 DNA (Table 3). In 4 samples with only HPV-16 DNA (4/46, 8.7%), expression of other HPV types (18 and 45) was also detected. Expression from HPV-16 was observed in 3 samples (3/15, 20%), interpreted as non-HPV-16 according to the DNA assay. Expression from HPV-16 or HPV-18 was detected in 2 samples (2/12, 16.7%) with mixed HPV types (Table 3).

In patients with available biopsy results, HPV DNA was detected in all cytology samples. HPV E6/E7 expression was present in all individuals with a diagnosis of CIN I–III ($n: 17$), while it was only detected in 2 out of 7 individuals with chronic cervicitis (Table 4).

Table 1. Detection of HPV DNA and E6/E7 mRNA expression according to the cytological diagnosis.

	HPV DNA		HPV mRNA	
	Positive (#/%)	Negative (#/%)	Positive (#/%)	Negative (#/%)
ASCUS (n: 25)	22 (88)	3 (12)	12 (48)	13 (52)
ASC-H (n: 3)	3 (100)	0 (0)	1 (33.3)	2 (66.7)
AGC (n: 4)	4 (100)	0 (0)	3 (75)	1 (25)
Atrophy (n: 2)	0 (0)	2 (100)	0 (0)	2 (100)
LSIL (n: 35)	32 (91.4)	3 (8.6)	18 (51.4)	17 (48.6)
HSIL (n: 9)	9 (100)	0 (0)	9 (100)	0 (0)
Adenocarcinoma (n: 3)	3 (100)	0 (0)	2 (66.7)	1 (33.3)
Total	73 (90.1)	8 (9.9)	45 (55.6)	36 (44.4)

Table 2. Distribution of HPV DNA types and mRNA expression according to the cytological diagnosis.

	HPV DNA			HPV mRNA ^a							
	Type 16	OT-16 ^b	Mixed	Type 16	Type 18	Type 31	16, 18	16, 45	18, 31	16, 18, 31	16, 18, 45
ASCUS	15	4	3	8	0	1	0	0	0	2	1
ASC-H	1	1	1	0	1	0	0	0	0	0	0
AGC	2	1	1	2	0	0	0	0	0	1	0
LSIL	20	7	5	8	1	0	4	1	0	3	1
HSIL	6	2	1	7	0	0	0	0	1	1	0
AC ^c	2	0	1	1	0	0	0	0	0	1	0
Total	46	15	12	26	2	1	4	1	1	8	2

^a Expression from undetected HPV types and/or combinations are not depicted in the table

^b Other than 16

^c Adenocarcinoma

Table 3. HPV E6/E7 mRNA expression according to DNA typing.

	HPV mRNA ^a									
	Negative	Type 16	Type 18	Type 31	16, 18	16, 45	18, 31	16, 18, 31	16, 18, 45	Total
HPV DNA										
Negative 8	8	0	0	0	0	0	0	0	0	8
HPV-16	18	23	0	1	2	0	0	0	2	46
OT-16 ^b	8	2	1	0	2	0	1	1	0	15
Mixed	2	1	1	0	0	1	0	7	0	12

^a Expression from undetected HPV types and/or combinations are not depicted in the table

^b Other than 16

4. Discussion

Persistent infections with high-risk HPV genotypes constitute a preventable risk factor for the development of cervical intraepithelial lesions and cervical cancer (1,3). HPV DNA detection, along with Pap test and colposcopy, provides an efficient method of screening to facilitate identification of high-risk HPV infections, which may provide adequate follow-up and treatment on an individual basis, and thus reduced incidence and mortality due to cervical carcinoma (17). Recently, HPV E6/E7 oncogene expression emerged as a promising biomarker to determine the risk for the progression to high-grade cervical lesions, since unregulated and prolonged expression of these viral oncogenes is better correlated with progression of cervical lesions to cancer (18,19).

In our study, HPV DNA and E6/E7 mRNA expression were investigated in a cohort of 81 individuals with cytological diagnoses of ASC-US (30.9%), LSIL (43.2%), and HSIL (11.1%). The biopsy results of 25 patients with

were classified as chronic cervicitis (28%), CIN I (32%), CIN III (28%), and adenocarcinoma/anaplastic carcinoma/carcinosarcoma according to their histological diagnosis. However, due to the lack of patients with a diagnosis of CIN II, HPV DNA and E6/E7 mRNA results of that group of patients could not be compared.

In women with abnormal cytology, HPV DNA was detected at a rate of 90.1%. Global HPV prevalence in invasive cervical cancer is reported to be about 92.4% (20). Based on a study in Greece, the rate of HPV DNA and high-risk HPV DNA in patients with abnormal cytology and/or colposcopy was identified as 55.5% and 50.6%, respectively (21). In Brazil, 93% HPV DNA positivity in women with CIN II biopsy was demonstrated and 90% of them were high-risk HPV genotypes (19). In the present study, high-risk HPV genotypes could only be differentiated as type 16 and non-16 due to the detection methods employed. Therefore, 90.1% HPV reactivity reflects all HPV types in the study group, and is slightly higher compared to the

Table 4. HPV types based on the DNA assay and E6/E7 mRNA expression in patients with cytology and biopsy results.

No.	Cytology	Pathology	HPV DNA typing	HPV mRNA expression
1.	ASCUS	Chronic cervicitis	OT 16 ^a	Negative
2.	ASCUS	Chronic cervicitis	OT 16	Negative
3.	ASCUS	Chronic cervicitis	16	Negative
4.	ASCUS	CIN I	Mixed	16, 18, 31
5.	ASC-H	Chronic cervicitis	OT 16	Negative
6.	ASC-H	Chronic cervicitis	Mixed	18
7.	LSIL	Chronic cervicitis	Mixed	Negative
8.	LSIL	Chronic cervicitis	Mixed	16, 45
9.	LSIL	CIN I	16	16, 18
10.	LSIL	CIN I	16	16
11.	LSIL	CIN I	16	16
12.	LSIL	CIN I	OT 16	16, 18
13.	LSIL	CIN I	OT 16	16, 18, 31
14.	LSIL	CIN I	16	16
15.	LSIL	CIN I	OT 16	16, 18
16.	LSIL	CIN III	Mixed	16, 18, 31
17.	LSIL	CIN III	16	16
18.	LSIL	CIN III	16	16
19.	HSIL	CIN III	16	16
20.	HSIL	CIN III	16	16
21.	HSIL	CIN III	OT 16	16
22.	HSIL	CIN III	Mixed	16, 18, 31
23.	Carcinoma	Anaplastic carcinoma	16	16
24.	Carcinoma	Carcinosarcoma	Mixed	16, 18, 31
25.	Carcinoma	Adenocarcinoma	16	Negative

^aOther than 16

high-risk HPV prevalence around the world. Nevertheless, HPV type 16 emerged as the most frequently detected genotype among all, which is in concordance with various previous reports (13,20).

HPV DNA other than type 16 as well as multiple HPV infections were determined as 20.5% and 16.4%, respectively, in the present study. In another study performed in Turkey, type 16 HPV DNA was detected in 20.8% of the samples and non-16 HPV constituted 79.2% (22). These variations are likely to reflect the difference in study groups where the latter also included samples with normal cytology.

Expression of HPV E6/E7 mRNA was identified in 55.6% of the samples where HPV type 16 constituted 57.8%,

followed by type 18 (4.4%) and type 31 (2.2%). Furthermore, expression from multiple HPV types was revealed in 35.6%. A study by Broccolo et al. in Italy reported HPV E6/E7 mRNA expression was present in 7% of women with normal cytology, in 20.3% with ASC-US, in 37% with LSIL, and in 70.9% with HSIL (23). Discacciati et al. found mRNA expression in 49% of patients with CIN2 grade lesions and with HPV DNA 16/18/31/33/45 (19). Sixty percent of them were type 16, 30% of them were type 33, 15% of them were type 31, and 5% of them were types 18 and 45. Castro et al. detected 55.7% E6/E7 mRNA in patients with ASC-US/LSIL and 77.5% in patients with HSIL (24).

Although there was not a statistically significant difference between HPV DNA positivity and patient age,

an association was observed between E6/E7 oncogene expression and age where E6/E7 mRNA expression was correlated with younger age. This is likely to represent active and transient infections in young adults. In a study by Frega et al., frequency of HSIL/LSIL in mRNA positive patients older than 35 was significantly higher than in mRNA negative patients (25). However, this difference was not detected in patients younger than 35.

When the pathology results of biopsy material is accepted as the gold standard, chronic cervicitis was detected in 7 patients, CIN I was detected in 8 patients, and CIN III was detected in 7 patients. Regarding the 7 chronic cervicitis patients, 3 of them were diagnosed with ASCUS, 2 of them with ASC-H, and 2 of them with LSIL cytologically. Only two of the chronic cervicitis patients were positive for E6/E7 mRNA, while all of them positive for HPV DNA. One of the mRNA positive patients was diagnosed with ASC-H and the other with LSIL by cytology. For CIN I and III patients both mRNA and DNA tests were positive. Thus, mRNA test results seem more correlated with pathology results than cytology and DNA tests especially for nonprecancerous lesions for this study group. The significant difference in E6/E7 expression detected among various cytological diagnosis groups definitely requires further investigation in larger cohorts.

In the present study, a statistically significant difference was observed between HPV DNA (90.1%) and E6/E7 mRNA (55.6%) results. Similar to our study, Tezcan et al. found a significant difference between HPV DNA 16/18/31/45 (7.9%) and E6/E7 mRNA (2.5%) (22). Although integration of some HR-HPV types is not always necessary for progression of squamous intraepithelial lesions, mRNA testing is useful to predict the progression of these lesions (26). mRNA expression could not be detected in 28 samples (38.4%) with HPV DNA. It can be hypothesized that mRNA testing reflects the active replication of HR HPV. The risk of development of CIN2 and higher grades is five times higher in mRNA positive women than in mRNA negative women (18). Rossi et al. found 74.1% HPV DNA and 23.6% mRNA and only 3 HR-HPV DNA negatives were mRNA positive (18). In the study by Spathis et al., HPV DNA and mRNA were detected as 55.5% and 29.7%, respectively, and consistency between the 2 methods was 71.6% (21). Persson et al. stated that mRNA testing has the highest sensibility to predict CIN2 and higher pathologies in ASC-US and LSIL groups;

however, they indicated that its specificity is insufficient (<50%) and HPV DNA testing and repeat cytology are more specific than mRNA testing (27).

Sixty percent of the patients with HPV DNA also demonstrated mRNA expression. Expression of mRNA was detected in 15% of other than type 16 HPV positives and 83% of multiple HPV types. There was no significant difference between HPV DNA types and their mRNA expressions. In the study by Discacciati et al., E6/E7 mRNA was detected at 49% rate for the HPV DNA types 16/18/31/33/45 (19). Two cervical atrophy, 3 ASC-US, and 3 LSIL (totally 8) samples were found negative by the two methods. However, false negativity or latent infection possibility should not be ignored. There were 3 samples whose HPV DNA and mRNA typing results were inconsistent. Although HPV 16 DNA was detected in one of them, HPV type 31 was detected by mRNA testing. The other 2 samples were HPV 16 DNA negative, but HPV 16 mRNA expression was detected in these samples. It is possible that some problems in PCR could cause this situation. Castle et al. found negative 3% of mRNA positive samples by PCR (13).

When HPV DNA and E6/E7 mRNA results are compared according to cytology reports, it is seen that DNA positivity increases a little with lesion grades. Rates of mRNA distinctively increase towards ASC-US, LSIL, and HSIL; however, mRNA could not be detected in one of the three DNA positive samples with carcinoma (Table 1). Moreover, in all of the patients with CIN I and CIN III pathology (n: 17), mRNA testing was positive. Castle et al. detected E6/E7 mRNA positivity in 43% of patients with ASC-US, in 71% of patients with LSIL, and in 87% of patients with HSIL (13). Thus, our data reveal mRNA testing to be more relevant to lesion grade than DNA testing and more useful for the diagnosis and follow-up of women with the risk of progressive cervical disease.

Acknowledgments

This study was supported by Hacettepe University Scientific Research Unit Grant No: 5849. The authors would like to thank Mr Levent Öztepe and Mr Can Aydoğan from BioMérieux, Turkey, for technical assistance and support. Preliminary findings of this study were presented as a poster presentation at the 4th National Virology Congress held in İstanbul, Turkey, during 23–26 July 2011, and appear in the abstract book (p. 142).

References

1. Zur Hausen H. Papillomaviruses causing cancer: evasion from host cell control in early events in carcinogenesis. *J Natl Cancer Inst* 2000; 92: 690-698.
2. de Villiers EM, Fauquet C, Broker TR, Bernard HU, Zur Hausen H. Classification of papillomaviruses. *Virology* 2004; 324: 17-27.
3. Schiffman MH, Bauer HM, Hoover RN, Glass AG, Cadell DM, Rush BB, Scott DR, Sherman ME, Kurman RJ, Wacholder S et al. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. *J Natl Cancer Inst* 1993; 85: 958-64.

4. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ, Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med* 2003; 348: 518-527.
5. Woodman CB, Collins S, Winter H, Bailey A, Ellis J, Prior P, Yates M, Rollason TP, Young LS. Natural history of cervical human papillomavirus infection in young women: a longitudinal cohort study. *Lancet* 2001; 357: 1831-1836.
6. Cuschieri KS, Whitley MJ, Cubie HA. Human papillomavirus type specific DNA and RNA persistence-implications for cervical disease progression and monitoring. *J Med Virol* 2004; 6: 1-6.
7. Schlecht NE, Kulaga S, Robitaille J, Ferreira S, Santos M, Miyamura RA, Duarte-Franco E, Rohan TE, Ferenczy A, Villa LL et al. Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia. *JAMA* 2001; 286: 3106-3114.
8. Nalliah S, Karikalan B, Kademane K. Multifaceted usage of HPV related tests and products in the management of cervical cancer – a review. *Asian Pac J Cancer Prev* 2015; 16: 2145-2150.
9. Boulet GA, Horvath CA, Berghmans S, Bogers J. Human papillomavirus in cervical cancer screening: important role as biomarker. *Cancer Epidemiol Biomarkers Prev* 2008; 17: 810-817.
10. Castle PE, Stoler MH, Wright TC, Sharma A, Wright TL, Behrens CM. Performance of carcinogenic human papillomavirus (HPV) testing and HPV16 or HPV18 genotyping for cervical cancer screening of women aged 25 years and older: a subanalysis of the ATHENA study. *Lancet Oncol* 2011; 12: 880-890.
11. Woodman CB, Collins S, Young LS. The natural history of cervical HPV infection: unresolved issues. *Nat Rev Cancer* 2007; 7: 11-22.
12. Zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* 2002; 2: 342-350.
13. Castle PE, Dockter J, Giachetti C, Garcia FAR, McCormick MK, Mitchell AL, Holladay EB, Kolk DP. A cross-sectional study of a prototype carcinogenic human papillomavirus E6/E7 messenger RNA assay for detection of cervical precancer and cancer. *Clin Cancer Res* 2007; 13: 2599-2605.
14. Coquillard G, Palao B, Patterson BK. Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. *Gynecol Oncol* 2011; 120: 89-93.
15. Burger EA, Kornør H, Klemp M, Lauvrak V, Kristiansen IS. HPV mRNA tests for the detection of cervical intraepithelial neoplasia: a systematic review. *Gynecol Oncol* 2011; 120: 430-438.
16. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T Jr et al. Forum Group Members, Bethesda 2001 Workshop: The 2001 Bethesda System: terminology for reporting results of cervical cytology. *JAMA* 2002; 287: 2114-2119.
17. Khunamornpong S, Settakorn J, Sukpan K, Srisomboon J, Suprasert P, Sriaunkgul S. Performance of HPV DNA testing with Hybrid Capture 2 in triaging women with minor cervical cytologic abnormalities (ASC-US/LSIL) in Northern Thailand. *Asian Pac J Cancer Prev* 2014; 15: 10961-10966.
18. Rossi PG, Benevolo M, Vocaturo A, Caraceni D, Ciccocioppo L, Frega A, Terranato I, Zappacosta R, French D, Rosini S. Prognostic value of HPV E6/E7 mRNA assay in women with negative colposcopy or CIN1 histology result: A follow-up study. *PLoS ONE* 2013; 8: e57600. doi:10.1371/journal.pone.0057600.
19. Discacciati MG, Silva ID, Villa LL, Reis L, Hayashi P, Costa MC, Rabelo-Santos SH, Zeferino LC. Prognostic value of DNA and mRNA E6/E7 of human papillomavirus in the evolution of cervical intraepithelial neoplasia grade 2. *Biomarker Insights* 2014; 9: 15-22.
20. Li N, Franceschi S, Howell-Jones R, Snijders PJE, Clifford GM. Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: variation by geographical region, histological type and year of publication. *Int J Cancer* 2011; 128: 927-935.
21. Spathis A, Kottaridi C, Chranioti A, Meristoudis C, Chrelias C, Panayiotides IG, Paraskeviadis E, Karakitsos P. mRNA and DNA detection of human papillomaviruses in women of all ages attending two colposcopy clinics. *PLoS ONE* 2012; 7: e49205. doi:10.1371/journal.pone.0049205.
22. Tezcan S, Ozgur D, Ulger M, Aslan G, Gurses I, Serin MS, Giray BG, Dilek S, Emekdas G. HPV Genotype and E6/E7 oncogene expression in Turkish women with cervical cytology findings. *Asian Pac J Cancer Prev* 2014; 15: 3997-4003.
23. Broccolo F, Fusetti L, Rosini S, Caraceni D, Zappacosta R, Ciccocioppo L, Matteoli B, Halfon P, Malnati MS, Ceccherini-Nelli L. Comparison of oncogenic HPV type-specific viral DNA load and E6/E7 mRNA detection in cervical samples: results from a multicenter study. *J Med Virol* 2013; 85: 472-482.
24. Castro SP, Fernandez AI, Gonzales MJL, Diez MTS, Lama AC, Martin MJA, Mosquera MP, Lopez-Miragaya I, Estevez N, Pinon JT et al. Human papillomavirus (HPV) E6/E7 mRNA as a triage test after detection of HPV 16 and HPV 18 DNA. *J Med Virol* 2013; 85: 1063-1068.
25. Frega A, Lorenzon L, Giovagnoli MR, De Sangtis L, Fabriano V, Lukic A, Moscarini M, Torrisi MR, French D. Prognostic implication of high risk human papillomavirus E6 and E7 mRNA in patients with intraepithelial lesions of the cervix in relationship to age. *Int J Immunopathol Pharmacol* 2011; 24: 461-470.
26. Manavi M, Hudelist G, Fink-Retter A, Gschwantler-Kaulich D, Pischinger K, Czerwenka K. Human papillomavirus DNA integration and messenger RNA transcription in cervical low- and high-risk squamous intraepithelial lesions in Austrian women. *Int J Gynecol Cancer* 2008; 18: 285-294.
27. Persson, Elfström KM, Wendel SB, Weiderpass E, Andersson S. Triage of HR-HPV positive women with minor cytological abnormalities: a comparison of mRNA testing, HPV DNA testing, and repeat cytology using a 4-year follow-up of a population-based study. *PLoS ONE* 2014; 9: e90023. doi:10.1371/journal.pone.0090023.