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Analysis of E6/E7 mRNA gene expression by means of NucliSENS EasyQ (NASBA) technique in patients suffering from cervical dysplasia with HPV positive

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Summary

Objective: In this study, the authors aimed at examining E6/E7 mRNA gene expression by means of NucliSENS EasyQ (NASBA) in patients in which HPV infection was detected and diagnosed with cervical dysplasia. *Materials and Methods:* This study included 77 patients diagnosed with cervical dysplasia. The patients were grouped based on cervico-vaginal smear anomalies. Digene HC2 DNA Collection Device transport medium was used for taking and keeping cervical samples, QIA symphony SP device, and QIAsymphony DSP AXpH DNA kit were used for HPV DNA extraction from cervical samples kept in transport medium, HPV Q24 complete kit, RotorGene and PyroMark Q24 for detection of HPV-DNA and determination of HPV types and NucliSENS Easy Q Genetic Analyzer technique for determination of E6/E7 gene expression in HPV positive samples. SPSS (Statistical Package for Social Sciences) Version 22.0 was employed in statistical evaluation of the research date. *Results:* The most common HPV was considered as type 16 in this study. When pathology specimens of patients in whom dysplasia was detected in their cervical biopsies were examined in terms of HPV E6/E7 mRNA expression, a statistically significant difference was found between the normal and pathology groups ($p < 0.05$). *Conclusion:* Today, the presence of HPV infection in etiology of cervical smear pathologies and dysplasia is undisputedly accepted. The main determinant factor of HPV virulence is the frequency of E6 and E7 gene expressions existing in the DNA structure and responsible for virulence. NucliSENS EasyQ (NASBA) is a technique employed to analyze mRNA gene expression in an accurate, reliable, and rapid way. Parallel to the conclusions of studies in the literature, frequency of E6/E7 gene expression increases in proportion to the increasing dysplasia degree of cervical pathologies in the present study as well.

Key words: Cervical dysplasia; HPV, NucliSENS EasyQ (NASBA) technique; E6/E7.

Introduction

Cervical cancer is still a major health issue in especially developing countries. Generally, approximately half of patients diagnosed with cervix cancer die due to complications developing based on this disease [1]. The fact that progression of cervical dysplasia to cervix cancer takes longer, concentrated the studies on early detection of cervical cancer, and the cervico-vaginal smear test was for the first time brought up by Papanicolaou. Although reports of smear tests firstly published in 1988, have been revised in 1991 and 2001, the most important revisions have been formulated with modifications made in 2001 [2].

According to 2001 Bethesda system, the definition of atypical squamous cells involves to atypical squamous cells of undetermined significance (ASC-US), and atypical squamous cells where high-grade squamous intraepithelial lesion (HSIL) in samples of smear cannot be excluded (ASC-H). Histopathology of atypical glandular cells is subdivided as atypical glandular cells favoring neoplasia (AGC-favor neoplasia), and atypical glandular cells, not otherwise specified (AGC-NOS). The term of mild dysplasia defined as cervical intraepithelial neoplasia (CIN)1 has been replaced by low-grade squamous intraepithelial lesion (LSIL) in 2001 Bethesda system. Definition of CIN2 and CIN3 has been replaced by high-grade squamous intraepithelial lesion (HSIL) in the Bethesda system [3].

Many etiological factors are blamed for the etiology of cervical dysplasia. These factors include age of sexual debut, number of sexual partners, smoking, and contraceptive methods [4]. However, it is generally acknowledged that human papilloma virus (HPV) is the most important factor in the etiology of cervical cancer and cervical dysplasia. While it is reported that HPV prevalence is in the range of 1.4% and 25.6% around the world [5], today over 100 HPV subtypes have been identified and only about 20 of them have been shown to be associated with cervical cancer [6].

Though genomic structure of HPV is complex, the effect of E6 and E7 proteins in formation of cervical dysplasia and carcinogenesis is undisputedly accepted. These genes inactivate first p53 and then retinoblastoma genes (pRb) - the tumor suppressor genes, prevent apoptosis in cell cycle;

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thus prevent controlled cell death which results in dysplasia and cancer development [7].

In the present study, the authors aimed at investigating the effect of these proteins in the etiopathogenesis of cervical smear abnormality by analyzing E6/E7 gene expression in various HPV subtypes in patients diagnosed with cervical dysplasia.

Materials and Methods

Smear test results of the patients referred to outpatient clinics of Inonu University, Turgut Ozal Medical Center, Department of Obstetrics and Gynecology between November 1, 2010 and July 13, 2012 were evaluated. A small sample of cells from the surface of the cervix was collected using a spatula or cyto-brush. All samples were collected by rotating the device through 360°. The collected material was spread on appropriately labeled slides and immediately sprayed with a fixative. The fixed smears were sent to the cytology laboratory and then examined under a microscope. The smears were stained with a Pap stain and screened by cytologists. All abnormal smears were examined by a pathologist. ASCUS nucleus are approximately two and one-half to three-times the area of the nucleus of a normal intermediate squamous cell or twice the size of a squamous metaplastic cell nucleus.

ASC-H cells usually occur singly or in small groups of less than ten cells. Nuclear to cytoplasmic ratio may approximate that of HSIL. LSIL cells occur singly, in clusters, and sheets. Nuclei are generally hyperchromatic but may be normochromatic. Koilocytosis or perinuclear cavitation consisting of a broad, sharply delineated clear perinuclear zone and a peripheral rim of densely stained cytoplasm is a characteristic viral cytopathic feature but is not required for the interpretation of LSIL. HSIL cells occur singly, in sheets or in syncytial-like aggregates. Nuclear to cytoplasmic ratio is higher in HSIL compared to LSIL. Contour of the nuclear membrane is quite irregular and frequently demonstrates prominent indentations. AGC nucleus enlarges up to three- to five-times the area of normal endocervical nuclei. Mild nuclear hyperchromasia and mild degrees of chromatin irregularity occur.

Cervical biopsies were also evaluated in terms of CIN. Diagnosis of CIN I was made in case neoplastic basaloid cells hold the bottom 1/3 epithelial layer, diagnosis of CIN II in case basaloid cells invade 2/3 of epithelial layer, and diagnosis of CIN III in case entire epithelium is held by neoplastic basaloid cells up to basal layer.

Digene HC2 DNA Collection Device transport medium was used in taking and storing the cervical samples.

For this purpose, HPV Q24 complete kit, RotorGene, and PyroMark Q24 systems were used. This system encompasses the real-time PCR phase (determination of HPV) realized by SybrGreen and phases of determination of HPV types by means of pyrosequencing of PCR products. At real-time PCR phase, each amplification mixture was prepared according to kit procedures. The amplification mixture prepared was made as Real-Time PCR with RotorGene device. For the purpose of identification of HPV types in samples detected as positive in the melting curve analysis, and sequence analysis of PCR products was made with pyrosequencing using HPV Q24 complete kit. This kit is capable of detecting high risk HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 genotypes; medium risk HPV 26, 30, 53, 66, 67, 68, 70, 73, 82, and 85 genotypes and low risk HPV 6, 11, 40, 42, 43, 44, 54, 55, 61, and 69 genotypes. In addition to these, the studies conducted have shown that this system is also capable of defining more different genotypes such as HPV 3, 7, 10, 13, 29, 32, 57, 72, 74, 75, 77, 81, 84, 86, 87, 89, 90, 91, 94, 97, 102, 106, 114, 117, and 125.

Table 1. — *Demographic characteristics of patients in the study.*

Demographic Characteristics	Mean, number of patients, %
Age (years)	
< 35	20 (26 %)
> 35	57 (74 %)
Age (mean ± SD)	43.09 ± 10.36
Gravidity (mean ± SD)	4 ± 2.61
Parity	
Nulliparous	5 (6.5 %)
Parity < 3	34 (44.2 %)
Parity > 3	38 (49.4 %)
Average parity (mean ± SD)	2.74 ± 1.93
Age of sexual debut	
Before age of 20	45 (58.4 %)
After age of 20	32 (41.6 %)
Number of sexual partners	
One partner	72 (93.5 %)
Two partners	5 (6.5 %)
Oral contraceptive (OCC)	
User of OCC	16 (20.8 %)
Non-user of OCC	61 (79.2 %)
Smoking history	
Smoker	57 (74 %)
Non-smoker	20 (26 %)
Education status	
Illiterate or primary school	62 (80.5 %)
Secondary and high school	9 (11.7 %)
University	6 (7.8 %)
Socio-economic Level	
Low	19 (24.7 %)
Middle	52 (67.5 %)
High	6 (7.8 %)
Cancer history in family	
Yes	29 (37.7 %)
No	48 (62.3 %)

Cervical tissue biopsy samples taken for HPV E6/E7 gene expression and stored in RNA Later Solution at -80 degrees were made ready for suitable analysis. Presence of E6 and E7 gene expression in patient samples, the high-risk HPV types of which were detected by fragment analysis method, was investigated by means of NASBA method using NucliSENS Easy Q Genetic Analyzer device.

SPSS (Statistical Package for Social Sciences) Version 22.0 was employed in statistical evaluation of the present research date. Definition of quantitative variables was made with mean ± standard deviation (SD) and definition of qualitative variables as number and percentage. Pearson Chi-Square analysis was employed in statistical evaluation. $P < 0.05$ was accepted as statistically significant.

Results

The present study included 77 patients who applied at the clinic and whose smear results were reported as ASC-US, ASC-H, LSIL, HSIL, and AGC. Demographic characteristics of cases are summarized in Table 1. Of 77 patients in the

Table 2. — *Distribution of abnormal smear results.*

Result of abnormal smear	Number of patients (%)
ASCUS	46 (59.7 %)
LSIL	15 (19.5 %)
ASC-H	8 (10.4 %)
HSIL	5 (6.5 %)
AGC	3 (3.9 %)
Total	77 (100 %)

ASCUS: atypical squamous cells of undetermined significance,
LSIL: low-grade squamous intraepithelial lesion,
ASC-H: atypical squamous cells where high-grade,
HSIL: high-grade squamous intraepithelial lesion,
AGC: atypical glandular cells.

Table 3. — *Distribution of HPV types in patients of HPV positive.*

HPV type	Number of patients (%)
HPV 16	12 (54.54 %)
HPV 18	2 (9.09 %)
HPV 33	1 (4.54 %)
HPV 35	1 (4.54 %)
HPV 39	1 (4.54 %)
HPV 67	2 (9.09 %)
HPV 73	1(4.54 %)
HPV 83	1 (4.54 %)
HPV 16 + HPV 39	1 (4.54 %)
Total	22 (100 %)

Table 4. — *Distribution of HPV DNA positivity by types according to abnormal smear result.*

HPV DNA types	ASCUS n (%)	ASC-H n (%)	LSIL n (%)	HSIL n (%)	AGC n (%)	Total n (%)
HPV 16	4 (8.6 %)	3 (37.5 %)	4 (26.6 %)	1 (20 %)	0	12 (54.5 %)
HPV 18	1 (2.1 %)	0	0	1 (20 %)	0	2 (9.09 %)
HPV 33	0	0	1 (6.6 %)	0	0	1 (4.54 %)
HPV 35	0	1 (12.5%)	0	0	0	1 (4.54 %)
HPV 39	0	0	1 (6.6 %)	0	0	1 (4.54 %)
HPV 67	1(2.1 %)	0	1 (6.6 %)	0	0	2 (9.09 %)
HPV 73	0	0	1 (6.6 %)	0	0	1 (4.54 %)
HPV 83	1(2.1 %)	0	0	0	0	1 (4.54 %)
HPV 16 + HPV 39	1(2.1 %)	0	0	0	0	1 (4.54 %)
Total HPV DNA positivity	8 (17.9 %)	4 (50 %)	8 (53.33 %)	2 (40 %)	0	22 (100 %)

Table 5. — *HPV E6/E7 mRNA positivity according to various HPV sub-types in patients of cervical dysplasia with HPV DNA.*

HPV E6/E7 mRNA types	HPV DNA (+) ASCUS	HPV DNA (+) ASC-H	HPV DNA (+) LSIL	HPV DNA (+) HSIL	Total HPV DNA (+)
HPV mRNA Type-16	2 (25 %)	1 (25%)	3 (37.5 %)	0	6 (27.27 %)
HPV mRNA Type-18	1 (12.5 %)	0	0	0	1 (4.54 %)
HPV mRNA Type-31	0	0	0	0	0
HPV mRNA Type-33	0	0	1 (12.5 %)	0	1 (4.54 %)
HPV mRNA Type-45	0	0	0	0	0
HPV mRNA Type-16 + Type-18	0	1 (25%)	0	1 (50 %)	2 (9.09 %)
HPV mRNA Type-16 + Type-33	0	1 (25 %)	0	0	1 (4.54 %)
Total HPV E6/E7 mRNA positivity	3 (37.5 %)	3 (75 %)	4 (50 %)	1 (50 %)	11 (50 %)

study, ASCUS was detected in 46 (59.7%), LSIL in 15 (19.5%), ASC-H in eight (10.4%), HSIL in five (6.5%), and AGC in three (3.9%) (Table 2). When the HPV results of the patients in whom cervical dysplasia was detected, while HPV DNA positivity was detected in 22 patients (28.6%), HPV DNA negative was identified in 55 patients (71.4%). The most common HPV type in this study was detected as type 16 (Table 3). HPV DNA was detected as positive in eight of the patients who were diagnosed with ASCUS, in four diagnosed with ASC-H, in eight diagnosed with LSIL based on smear, and two diagnosed with HSIL. Distribution of HPV DNA positivity detected in patients, identified with smear abnormality, by HPV subtypes is summarized in Table 4. E6/E7 mRNA expression was studied in 22 cases of HPV DNA positive, and E6/E7 mRNA positivity was detected in three of the patients diagnosed with ASCUS (37.5%), three of the patients diagnosed with ASC-H (75%), four of the patients whose cervico-vaginal smear diagnosis was LSIL (50%), and two diagnosed with HSIL (50%) (Table 5).

Cervical biopsy results of 77 patients in whom cervical dysplasia was detected were also evaluated. Cervical dysplasia was detected in total 17 patients. CIN I was detected in six patients, CIN II in six patients, and CIN III in five patients. When the abnormal cervical biopsy results between HPV DNA positive group and HPV negative group were compared, the frequency of cervical pathology in the HPV DNA positive group was found to be statistically significant ($p < 0.05$) (Table 6). When pathology specimens of

Table 6. — *Distribution of cervical biopsy results by HPV DNA results when classified as mormal - abnormal.*

	Result of cervical biopsy		Total
	Normal	Abnormal (CINI, CINII, CINIII)	
HPV DNA			
Negative	48 (87.2 %)	7 (12.8 %)	55 (100 %)
Positive	12 (54.5 %)	10 (45.5 %)*	22 (100 %)
Total	60 (77.9 %)	17 (22.1)	77 (100 %)

*$p < 0.05$.

Table 7. — *Distribution of cervical biopsy results according to E6/E7 mRNA gene expression when classified as normal - abnormal.*

	Result of cervical biopsy		Total
	Normal	Abnormal (CIN1, CIN2, CIN3)	
E6/E7 mRNA			
Negative	54 (81.8 %)	12 (18.2 %)	66 (100 %)
Positive	6 (54.5 %)	5 (45.5 %)	11 (100 %)
Total	60 (77.9 %)	17 (22.1)	77 (100 %)

$p < 0.05$.

patients in whom dysplasia was detected in their cervical biopsies were examined in terms of HPV E6/E7 mRNA expression, a statistically significant difference was found between the normal and pathology groups ($p < 0.05$) (Table 7).

Discussion

Cervical cancer is among the commonly seen female genital tumors and patients receiving new diagnosis are added to the medical literature each year around the world. This clinical table is alarming in today's world where diagnosis and screening programs are effectively implemented [8]. Though numerous factors have been defined in the etiology of cervical cancer, HPV infection is specifically the most important agent with known and proven high-risk HPV subtypes. Today, more than 100 HPV subtypes have been defined; however, about 15% of them are defined as high-risk subtypes and HPV types 16 and 18 are characterized as the high-risk types most frequently isolated [9].

The E6 and E7 oncoproteins are the primary viral agents and significantly contribute to the oncogenic potential of high-risk HPV. In detail, the high-risk HPV E6 protein predominantly binds to the cellular E3 ubiquitin ligase E6-associated protein (E6AP), which leads to the ubiquitination degradation of tumor suppressor p53, subsequently preventing cell growth arrest and apoptosis. While E7 protein predominantly results in the degradation of retinoblastoma (Rb) family members and in turn regulates the activity of the E2F family of transcription factors, eventually leading to deregulated proliferation of cells. More importantly, these two oncoproteins act cooperatively in promoting cervical carcinogenesis, with the action of one complementing that of the other. However, little is known about the role of HR-HPV E6 and E7 in mediating invasion and metastasis of cervical cancer cells [10].

There are studies showing the presence of E6/E7 mRNA gene expression in the etiology of cervical carcinogenesis, one of which is NucliSENS EasyQ test. The NucliSENS EasyQ HPV uses a nucleic acid sequence–based amplification (NASBA) technique. This assay utilizes molecular beacon probes for real-time detection and typing of E6/E7 mRNA from HPV types. Although there are studies showing the HPV genomic structure in patients diagnosed with cervical dysplasia, the comparative studies suggest that NucliSENS EasyQ test is more advantageous [11, 12].

One study conducted on Pap smear samples of 476 patients diagnosed with cervical dysplasia employed the method used in the present study for detection of different HPV subtypes. HPV positivity is significantly higher in samples in which HSIL was detected as result of smear; yet it was also found as 18.9% in patients with normal cytology detected. While HPV DNA positivity was detected in 7.9% of patients, E6/E7 oncogenic mRNA positivity was detected in only 2.5% [13]. The findings of the present study are in parallel with the results of this research as well.

Another study by Padalko *et al.* included 1,422 samples diagnosed with cervical dysplasia. The failure rate of NucliSENS EasyQ method applied in patients in whom HPV DNA detected was found as 1.4%. At the same time, a higher progression rate was detected in cervical biopsies of patients in whom E6/E7 mRNA gene expression was detected [14]. This study is supportive of the present finding of the statistically significantly higher rate of E6/E7 mRNA gene expression in patients in whom pathology (CINI, CINII, CINIII) was detected in cervical biopsies.

In another study in the literature, 105 patients in whom abnormal smear results were detected and first of all, HPV DNA isolation of the patients was ensured. Then, E6/E7 mRNA positivity was detected by means of NASBA (NucliSENS EasyQ HPV v1.1) technique. As was the case in the present study, it was seen that as the abnormal smear dysplasia intensified, and the percentage of mRNA positivity increased as well [15]. Halfon *et al.* conducted E6/E7 mRNA gene expression analysis by the same method, also utilized in the present study, have used it in another study, in 140 patients in whom cervical dysplasia was detected and likewise, parallel to the present study, they found that mRNA expression in patients diagnosed with high-grade cervical tissue biopsies and advanced dysplasia smear was significantly higher compared to other groups [16].

In conclusion, the role of HPV infection in etiology of cervical smear abnormalities and cervical intraepithelial neoplasia is, without doubt, accepted. However, presence of HPV infection may not always be a sufficient reason for

development of cervical dysplasia. The studies on high-risk HPV subtypes show that increased function of E6 and E7 gene sites in the HPV genome are responsible especially for the pathogenicity. While various techniques are employed for the purpose of evaluating te E6 and E7 mRNA expression, it is considered that NucliSENS EasyQ (NASBA) technique is more advantageous compared to other analysis methods. In the light of studies conducted, isolation incidence of E6/E7 mRNA increases in proportion to the frequency of increasing cervical dysplasia. The present study derived parallel results with the studies available in the literature.

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