Detection of human papillomavirus E6/E7 mRNA in women with high-risk HPV types 16, 18, 31, 33 and 45 which are associated with the development of human cervical cancer

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Summary

Purpose of investigation: The aim of our study was to increase the clinical meaningfulness of the virological data through mRNA E6/E7 oncoprotein identification, and to find a correlation between codon 72 polymorphism of the p53 gene and integration of HPV in host cell genomes. *Methods:* We analyzed 80 cervical samples from women with HPV DNA types 16, 18, 31, 33 and 45. Transcripts of HPV were detected by the NucliSense EasyQ HPV assay and genotyping of the TP53 polymorphism was conducted using a TaqMan assay. *Results:* Twenty percent of 80 tested samples were positive for mRNA Papillomavirus. The frequency of Arg/Pro heterozygotes in controls was over-represented compared with mRNA positive samples while there were no significant differences in the distribution of Pro/Pro and Arg/Arg alleles. *Conclusion:* The introduction of HPV mRNA testing in clinical analysis improved diagnostic accuracy of HPV infections. Our data suggest that a structural difference at codon 72 of the p53 gene may not be a sufficient risk factor for cervical carcinogenesis.

Key words: HR-HPV; E6/E7 mRNA; p53 polymorphisms; Cervical cancer.

Introduction

Human papilloma viruses (HPVs) are the cause of a range of proliferative lesions upon infection of epithelial cells [1].

More than 130 epitheliotropic genotypes belong to the heterogeneous group of papillomaviruses, 16 of these DNA viruses are considered "high-risk" types and are linked with the development of malignant diseases [2]. HPV types 16 and 18 are associated with more than 70% of all cervical cancers [3].

The integration of the viral genome into the host genome disrupts E2, the negative regulator of early gene E6 and E7 transcription, and this results in an overexpression of these oncogenes [4, 5].

The overexpression of E6 and E7 leads to the accumulation of DNA damage and the development of cervical cancer [6]. These oncoproteins bind and functionally inactivate tumor suppressor protein p53 and members of the retinoblastoma (Rb) tumor suppressor family (pRb, p107 and p130), resulting in the dysregulation of critical cellular functions such as cell-cycle control, apoptosis, senescence, DNA repair and maintenance of genomic stability [7-10].

Normally, p53 promotes cell cycle arrest or initiates apoptosis responding to cellular stress. E6 promotes p53 accelerated proteosomal degradation via ubiquitination [11]. The absence of p53 stops cell cycle arrest in response to genetic insult, promoting the accumulation of harmful mutations that may contribute to malignant progression. The oncoprotein E7 promotes cell cycle advancement and entry into the S-phase. The best characterized target of E7 in this regard is the retinoblastoma tumor suppressor protein (pRB), which is linked and destabilized by E7 via ubiquitin-mediated proteosomal degradation [12]. The substitution of arginine (Arg) by proline (Pro) at codon 72 in the p53 gene seems to be a polymorphism that alters the primary structure of the p53 protein [13]. Recently, biochemical and functional differences between the two p53 forms have been identified [14, 15]. It was demonstrated experimentally that the Arg form of the p53 protein was more susceptible than the Pro form to binding and degradation by the HPV-E6 oncoprotein [16]. However, similar studies on cervical and other human cancers have produced contradictory results [17, 18].

The aim of this study was to increase the clinical meaningfulness of the virological data through mRNA E6/E7 oncoprotein identification by using the real-time NASBA method, and to find a possible correlation between codon 72 polymorphism of the p53 gene and the integration of HPV in host cell genomes.

Materials and Methods

In the study we analyzed 80 cervical samples of women aged between 18 and 60 years, with HPV DNA types 16, 18, 31, 33 and 45 from the city of Messina and province. In particular these were taken from patients who, according to the Bethesda System Cytology classification, 57% (46 patients) belonged to category HSIL (high-grade squamous intraepithelial lesion), 25% (20 patients) to category LSIL (low-grade squamous intraepithelial lesions) and 18% (14 patients) had normal cytology. The largest percentage of patients were positive for HPV DNA type 16 (54%; 43 patients), while for other high-risk HPV

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genotypes studied, type 31 was present in 28% (22 patients) of the samples, type 18 in 14% (11 patients) and only 2% (2 patients) of specimens were positive for HPV types 33 and 45. Cervical specimens were tested with the NucliSENS EasyQ HPV assay (bioMérieux Italia S.p.A) according to the manufacturer's instructions. This is a real-time nucleic acid amplification and detection assay for the qualitative determination of E6/E7 mRNA from five carcinogenic HPV types 16, 18, 31, 33 and 45 in cervical scrapes [19, 20]. We have also investigated the p53 codon 72 polymorphism in high-risk HPV positive samples. Our controls were women resulting positive to HPV-DNA testing and negative to NucliSENS EasyQ HPV testing. From whole blood, we performed the extraction of nucleic acids using the Puregene Blood Kit (Gentra, Milano Italy). Genotyping of the TP53 codon 72 (rs1042522) polymorphisms was conducted using a TaqMan assay with an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The polymerase chain reaction (PCR) contained 10 ng of genomic DNA, 1x TaqMan Genotyping Master Mix, and 1x of assay mix (C_2403545_10). PCR was performed using 96 well plates on a thermal cycler (ABI 9700; Applied Biosystems). Reaction conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

Results

The NucliSENS test allowed us to identify the viral mRNA-positive patients and, depending on that, those presenting an integration of the virus into the host genome. Twenty percent (16 specimens) of the 80 tested samples were positive for papillomavirus mRNA. Particularly 12.5% (10 specimens) of mRNA positive samples showed HPV DNA type 16 and the remaining 7.5% (6 specimens) showed HPV DNA type 31 while none of the samples positive for types 18, 33 and 45 had viral mRNA detected. No multiple infections were detected in patients positive to the NucliSENS test while different genotypes of HPV DNA were found in 20 of 46 (43.5%) patients with HSIL, 14 of 20 (70%) patients with LSIL, and six of 14 (43%) patients with normal cytology. Eight of 46 HSIL patients were positive for E6/E7 mRNA, six of 20 LSIL patients showed viral mRNA while two of 14 patients with normal cytology had papillomavirus mRNA. In particular, 14.2% of the total number of samples with normal cytology presented E6/E7 mRNA of HPV type 31, 30% of the LSIL specimens showed mRNA of HPV type 16 while for HSIL specimens the presence was highlighted in amounts equal to 8.65% of mRNA HPV types 16 and 31. Eight of 16 (50%) mRNA positive samples were HSIL patients treated with injury excision which had shown a relapse within the following 12 months. LSIL patients positive for HPV mRNA treated with destructive treatment had not shown a relapse within the following 18 months just like the only two patients with normal cytology positive for mRNA. None of the patients with negative results to the NucliSENS test showed a relapse after treatment. In this study, mRNA testing correlated better to histology grade and seemed to give more prognostic information. Regarding the p53 codon 72 polymorphism in high-risk HPV positive samples, the frequencies of Pro/Pro, Arg/Pro and Arg/Arg in the control group were 11 (7/64), 39 (25/64) and 50% (32/64), respectively. In the mRNA E6/E7 HPV positive samples the allelic frequencies of Pro/Pro, Arg/Pro, Arg/Arg were 18.75 (3/16), 18.75 (3/16) and 62.5% (10/16), respectively. Therefore, our data suggest that the distribution of the Pro/Pro and Arg/Arg alleles of codon 72 did not show significant differences between controls and mRNA E6/E7 positive specimens while the frequency of Arg/Pro heterozygotes in controls was over-represented compared with that of mRNA positive samples.

Discussion

It is widely known that persistent infection by high-risk types of papillomaviruses has been associated with the development of human cervical cancer. Epidemiological and experimental evidence has demonstrated that progression to malignancy requires the loss of expression of the viral E2 gene in cancer cells and the over-expression of E6 and E7 genes in the integrated HPV genome. It follows that the detection of E6/E7 mRNA indicates HPV oncogenic activity and may be used as a clinically predictive marker to identify women at risk of developing high-grade cervical dysplastic lesions and cervical carcinoma.

HPV testing is today widely accepted for reflex testing in case of abnormal Pap smear and screening with HPV may be implemented in the future.

HPV infection is necessary [21] but not sufficient to cause cervical cancer. The prevalence of HPV DNA in cervical smear specimens is very high, and most infections are transient. For these reasons the potential use of HPV DNA testing for early detection of cervical cancer is limited and would only marginally reduce the follow-up colposcopy and histology [22]. Testing for HPV oncogenic activity, rather than for the presence of HPV DNA, may therefore be a more rilevant clinical indicator of the development of cervical lesions and cervical cancer [23, 24].The NucliSENS EasyQ HPV test tries to establish direct detection of the expression of oncogenic risk factors E6 and E7 from the five most prevalent HPV types in cervical cancer worldwide (HPV 16, 18, 31, 33 and 45). NucliSENS EasyQ HPV is a high medical value test for physicians that can significantly contribute to improved patient management due to its capacity to not only detect E6/E7 oncogenic activity, but also provide individual genotypic information for these five high-risk HPV genotypes in cervical specimens. Our data revealed that a significant percentage of patients with normal cytology showed integration of the viral genome, thus highlighting how the introduction of HPV mRNA testing in clinical analysis improved diagnostic accuracy of HPV infections, distinguishing one clinical infection by a transient infection with high risk of clinically active tumorigenic transformation. The oncogenic activity of high-risk HPVs is explained in part by the ability of the viral E6 oncoprotein to target p53 for degradation and thus to inhibit p53-mediated transcription [25]. Several studies

report the p53 dysfunction caused by HPVs depend on the status of a polymorphism at codon 72, Arg or Pro. Although our study was conducted on a heterogeneous group of specimens, data suggested that a structural difference at codon 72 of the p53 gene may not be an obvious and sufficient risk factor for cervical carcinogenesis. In any case the real effect of the p53 codon 72 polymorphism on HPV-associated cervical neoplasia needs to be investigated along with other in vivo and in vitro research.

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