The p53 Codon 72 arg/arg Homozygous Women in Central Italy are at Increased Risk for HPV Infections

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Abstract. *Background: The oncoprotein E6 binds to and degrades the p53 tumor suppressor protein, with different efficacy depending on the p53 codon 72 (arg/pro) polymorphism. The arg/arg allele has been shown to increase the risk for cervical cancer. Materials and Methods: Fifty-eight women infected with HPV and 32 normal controls were analyzed by restriction fragment length polymorphism to detect arg/arg or arg/pro alleles. Results: The allele frequencies in HPV-positive women were: arg/arg 47/58 (81%); arg/pro 9/58 (15.5%) and pro/pro 2/58 (3.4%), while those in controls were: arg/arg 19/32 (59%); arg/pro 10/32 (31.2%) and pro/pro 3/32 (9.3%) (Fisher's exact test, p=0.068). The risk of having HSIL in arg/arg homozygous patients had odds ratio (OR)=1.33 (95%CI 1.12-1.58, p=0.628). Women with the arg/arg phenotype were at significantly increased risk for HPV infection; OR=2.93 (95%CI 1.11-7.66, p=0.028). Being homozygous arg/arg also substantially increased the risk of HR-HPV infection, with OR=3.84 (95%CI 0.71-20.57, p=0.128), whereas heterozygosity for arg/pro was protective against HR-HPV; OR=0.186 (95%CI 0.03-1.04, p=0.074). Allele frequencies in women with different HPV types were not significantly different, however (p=0.174). Conclusion: These data suggest that arg/arg homozygous patients are at increased risk for HR-HPV infections.*

High-risk human papillomaviruses (HPV) are responsible for almost all cervical cancers. In contrast, the low-risk types are mainly involved in the pathogenesis of benign lesions (condyloma and warts) (1). The high-risk HPVs encode two major oncoproteins, E6 and E7. The E6 protein binds the cellular tumor-suppressor protein p53 and induces its degradation through the ubiquitin pathway (2, 3), while the E7 protein binds to and inactivates pRb (4). These events alter the mechanisms of cell cycle control and favor the progression towards a neoplastic phenotype. Storey *et al.* (5) showed that the degradation of p53 by E6 differs according to the polymorphism of the wild-type p53 in codon 72.

The homozygous arg/arg allele is more susceptible to degradation than the heterozygous arg/pro or homozygous pro/pro forms. This finding was correlated with an increased susceptibility to HPV infections and their associated tumors. The allelic analysis of patients affected by cervical cancer showed an over-representation of the homozygous arginine 72 allele compared with the normal population. However, this observation was confirmed only in some cases (6, 7) and many other studies (7-11) failed to confirm such an association. These discordant results could be related to the different methodologies used in different laboratories or to the study design (case-control study). Furthermore, ethnic differences were observed with regard to the p53 polymorphisms (12).

The incidence of p53 codon 72 polymorphisms among Central Italian women presenting with abnormal Pap smears and shown to be infected with HPV, compared with HPV-negative control women with normal cytology were evaluated.

Materials and Methods

Study group. The material of this study included a series of 58 women admitted to Colposcopy Clinic at "Sandro Pertini" Hospital and Tor Vergata University Hospital, from June 2002-June 2005. All the women underwent a conventional Pap smear and colposcopy was performed at the first or subsequent visit. The mean age of the patients was 37.8 years (range 18-68; SD±11.9).

Exo- and endocervical cells were sampled from each patient for Pap test and HPV-DNA screening. Punch biopsies were taken from all women with abnormal cytology. According to the Bethesda

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Table I. *p53 codon 72 polymorphisms related to PAP smear atypia and HPV types.*

	arg/arg		arg/pro		pro/pro		*Significance
PAP Smear							
Normal	12	75%	3	18.8%	1	6.3%	
ASC-US	7	87.5%	1	12.5%	0	0.0%	
LSIL	14	70.0%	5	25.0%	1	5.0%	
HSIL	2	100.0%	0	0.0%	0	0.0%	p=0.571
Grade**							
High-grade	2	100.0%	0	0.0%	0	0.0%	
Low-grade	33	75.0%	9	20.5%	2	4.5%	p=0.571
HPV Type							
HPV6	1	100.0%	0	0.0%	0	0.0%	
HPV16	29	85.3%	3	8.8%	2	5.9%	
HPV18	3	100.0%	0	0.0%	0	0.0%	
HPV31	5	100.0%	0	0.0%	0	0.0%	
HPV52	3	100.0%	0	0.0%	0	0.0%	
HPV53	1	33.3%	2	66.7%	0	0.0%	
HPV54	1	50.0%	1	50.0%	0	0.0%	
HPV58	0	0.0%	1	100.0%	0	0.0%	
HPV61	1	100.0%	0	0.0%	0	0.0%	
HPV66	1	33.3%	2	66.7%	0	0.0%	
HPV84	2	100.0%	0	0.0%	0	0.0%	
HPV-Negative	19	59.4%	10	31.3%	3	9.4%	p=0.174
HPV Types							
Low-risk	4	57.1%	3	42.9%	0	0.0%	
High-risk	41	83.7%	6	12.2%	2	4.1%	p=0.134

*Fischer's exact test; **HSIL cut-off.

system, the lesions were classified as ASCUS (atypical squamous cells of undetermined significance), L-SIL (CIN1), and H-SIL (CIN2/3). As a control group, 32 women with normal cytology were examined and were shown to be negative for HPV-DNA. Informed consensus was obtained from all women enrolled in the study.

HPV screening and genotyping

a) Polymerase chain reaction. MY09/11 degenerate primers, which amplify a 450-bp fragment of the L1 region of HPV, were used to detect HPV-DNA (13). Amplification was carried out in 50 μl of reaction mixture (1 X PCR buffer, 200 μM dNTPs, 2 mM $MgCl_2$, 40 pmol of each primer, 1 U of Taq Gold polymerase and 5 μl of DNA) with 35 cycles of amplification. Each cycle included a denaturation step at 94˚C for 1 min, an annealing step at 55˚C for 1 min and a chain elongation step at 72˚C for 1 min using a 9700 GeneAmp instrument (Applied Biosystem, CA, USA). The quality of the DNA was tested with primers amplifying the beta-actin gene (14).

The PCR products were analyzed on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Their molecular weight was determined by comparison with a 100-bp DNA ladder.

b) HPV typing. The 450-bp amplified fragment of the L1 gene was used in a sequencing reaction to determine the HPV type. The sequencing reaction was performed using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, CA, USA). After column purification (Millipore, MA, USA), the product was run on a Beckman Coulture CEQ2000XL sequence analyzer. The obtained sequence was submitted to the Genebank and matched against all deposited sequences. The HPV genotype was assigned based on ≥98% sequence homology over 450 nucleotides (http://www.ncbi.nlm.nih.gov/BLAST).

c) Detection of p53 codon 72 polymorphisms. Polymorphism of the p53 gene was evaluated by the PCR-based restriction fragment length polymorphism method, using primers encompassing the polymorphic site at codon 72 (15). The amplified product was digested with 10 *U* of the restriction enzyme *BsTUI* for 2 h at 60˚C and run on a 2% agarose gel. Homozygotes for pro were represented by a fragment of 199 bp, whereas arg homozygotes generated two DNA bands of 133 bp and 86 bp. Heterozygotes showed a combination of both alleles (199, 113 and 86 bp).

Results

The allele frequencies in HPV-positive women were as follows: arg/arg 47/58 (81%), arg/pro 9/58 (15.5%) and pro/pro 2/58 (3.4%). The corresponding values in the controls were: arg/arg 19/32 (59%), arg/pro 10/32 (31.2%), and pro/pro 3/32 (9.3%) and this difference was borderline significant (Fisher's exact test, p=0.068).

Allele frequencies are related to PAP smear abnormalities and HPV types as depicted in Table I. The allele frequencies are practically identical between the different categories of PAP smear abnormalities ($p=0.978$). When HSIL cytology was used as a cut-off, the distribution was still not significant ($p=0.571$), but all the HSIL cases were arg/arg homozygous. The risk of having HSIL in arg/arg homozyous cases had an odds ratio (OR)=1.33 (95%CI 1.12-1.58, $p=0.628$). Similarly, LSIL were more common among arg/pro heterozygotes; OR=1.25 (95%CI 1.08-1.46, $p=0.345$).

The allele frequencies in women with different HPV types were not significantly different ($p=0.174$) (Table I). When HPV-positive patients were compared with HPV-negative controls, the difference was of borderline significance ($p=0.068$); arg/pro and pro/pro were more frequent in the HPV-negative group, while 81.0% of the HPV-positive women were of the arg/arg type. The risk estimates for HPV-positivity reached significance for arg/arg phenotype; OR=2.93 (95%CI 1.11-7.66, $p=0.028$), while being pro/pro or arg/pro were not significantly associated with HPV status; OR=0.355 (95%CI 0.05-2.18, $p=0.343$) and OR=0.404 (95%CI 0.14-1.13, $p=0.106$), respectively.

When stratified according to low-risk (LR) and high-risk (HR) types, there was no significant difference in allele frequencies ($p=0.134$) (Table I). Arg/arg homozygousity substantially increased the risk of HR-HPV infection, with an OR=3.84 (95%CI 0.71-20.57, $p=0.128$), whereas heterozygosity for arg/pro was protective against HR-HPV; OR=0.186 (95%CI 0.03-1.04, $p=0.074$). HR-HPV was a 100% sensitive indicator of HSIL, showing 16.7% specificity, 5% PPV and 100% NPV (OR was not computable; all HSIL cases were HR-HPV-positive).

Discussion

Storey *et al.* (5) first reported that the presence of the homozygous arg/arg allele at the p53 codon 72 to increased the risk of cervical cancer among women infected with high-risk HPVs, prompting a number of studies in different geographic regions, with conflicting results. The reasons for these conflicting data were attributed to the different methodologies used (16) as well as to ethnic differences (17, 18). For instance, in our hands, p53 allele-specific PCR did not give reliable results when checked by sequencing (data not shown). This problem could lead to a misclassification of p53 polymorhisms.

In addition to genetic factors, HPV type and type variation also seem to confer a different susceptibility to cancer progression. A study by van Duin *et al.* showed that in Dutch women with the p53 arg/arg allele, infection with the HPV16 350T variant confers a higher risk for developing cervical cancer (19), while Zehbe *et al.* found a higher incidence of the HPV16 350G in Swedish women with cervical cancer (20). In contrast, a study carried out on German women did not show any significant correlation between HPV16 variants and dysplastic or neoplastic cervical lesions (21). To elucidate these controversies, it is still important to consider the role of p53 codon 72 polymorphism in cervical carcinogenesis.

The results of our study indicate that the allele frequencies among the different categories of PAP smear abnormalities were basically identical, as were the allele frequencies in women with different HPV types ($p=0.174$). However, when HPV-positive women were compared with HPV-negative women, we found that 81% of the HPV-positive women were of the arg/arg type, while the arg/pro and the pro/pro alleles were more frequent among HPV-negative women. Similarly, arg/arg homozygosity increased the risk for HR-HPV infection, while the arg/pro and pro/pro alleles seem to be protective against HR-HPV infection.

Unlike two previous Italian studies (22, 23) carried out on women from North Italy, our study performed on women from Central Italy seems to confirm the role of p53 polymorphism in cervical carcinogenesis. These discordant results could be attributed to geographic variation of the female population inside the country. Thus, a combination of factors, geographic area, HPV type and type variants, p53 polymorphisms, as well as immunological status, could be important determinants of the development of cervical cancer precursors.

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