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p53 Codon 72 Polymorphism as a Risk Factor in the Development of HPV-Associated Cervical Cancer

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Human papilloma virus (HPV) has been implicated in cervical carcinoma, and the p53 gene is polymorphic at amino acid 72 of the protein that it encodes. The association between p53 polymorphisms and risk for HPV-associated cervical cancer has been examined, but the results have been conflicting. It has been reported that patients with the arginine form have a higher risk of developing cervical cancer than those with the proline form. The purpose of this study was to examine whether p53 Arg at the polymorphic position 72 could represent a risk factor for women with high-risk HPV-associated premalignant and malignant cervical lesions. The study was carried out on 60 smears from patients with high-risk HPV-related cervical lesions. Also, 74 HPV-negative normal smears and 61 normal blood samples were used as controls. HPV-18 was the most frequent type. There was a difference in the distribution of p53 genotypes between high-risk HPV-cervical lesions and the normal samples. The allele frequency of p53 Arg/Arg was much higher than the normal samples (46.5% versus 20.5% in HPV-negative normal smears and 20% in blood samples). Based on the findings of this study, it is suggested that p53 Arg homozygosity could possibly represent a potential risk factor for the tumorigenesis of the cervix. Statistically significant correlation was not observed between the presence of Arg/Pro homozygosity or Arg/Pro heterozygosity and HPV typing. © 2000 Academic Press

Key Words: **human papilloma viruses; *p53* gene; codon 72; cervical smears.**

Human papilloma virus (HPV) has been implicated in cervical carcinoma (1, 2). The role of HPV in the development of anogenital cancers has been widely studied, and the current evidence shows that HPV infection and persistence are necessary for the evolution and maintenance of cervical cancer (3). Specific viral genes (E6 and E7) of high risk HPVs (types 16, 18, 33 and others) act as oncogenes. High risk E6 and E7 bind and functionally inactivate tumor suppressor proteins p53 and Rb, respectively, and both disrupt the G1 arrest in response to DNA damage.

In human populations, the p53 gene is polymorphic at amino acid 72 of the protein that it encodes (4–6). In the reading frame used, the G or C at the nucleotide residue 347 resulted in an arginine codon (CGC) or proline (CCC) for the amino acid residue. Matlaskewski *et al.* (4), concluded from their observations that p53 with Pro-72 is structurally different from p53 with Arg-72, and this is reflected by its altered electrophoretic mobility. p53 with Arg-72 migrated more rapidly on gels than did p53 with Pro-72 (4). It was also stated that the tumors produced by the Pro-72 p53 containing cells appeared more slowly and were smaller in each case than the Arg-72 p53 tumors, and that both forms of human p53 can increase the tumorigenicity but the Arg-72 form of human p53 is more oncogenic in this respect than the Pro-72 form of human p53.

The association between p53 polymorphisms and HPV-associated cervical cancer risk (7–9) has been examined, but the results were conflicting. Storey *et al.* (8) reveal that the arginine form of p53 is more susceptible to degradation by the HPV E6 protein than the proline form and that patients with the arginine form have a sevenfold higher risk of developing cervical cancer than those with the proline form. However, other studies (10–12) show no evidence of correlation between this polymorphism and the risk of cervical cancer.

We examined whether p53 Arg at the polymorphic position 72 could represent a risk factor for women with high risk HPV-associated premalignant and malignant cervical lesions, in comparison with a normal control groups of women. HPV-18 was the most frequent type among cervical lesions. Twenty-eight of the 60 (46.5%) HPV-associated cervical lesions were found to carry the Arg/Arg polymorphism in p53 codon 12, whereas this genotype appeared only in 20.5% in the normal HPV negative cervical smears and in 20% in the normal blood samples. Therefore, it is suggested

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that p53 Arg homozygosity could represent a potential risk factor for the tumorigenesis of the cervix.

MATERIALS AND METHODS

Subjects and Blood Samples

The study population included 60 patients with high risk HPV-related cervical lesions from Greece. Seventy four HPV negative cervical smears were obtained from normal women. The cervical smears were already fixed and stained to obtain the cytological diagnosis. Peripheral blood was obtained from 61 healthy women aged ≥55 years with no known HPV lesions.

DNA Extraction from Cervical Smears

For each case, one slide with stained cervical smear was used. The slides were soaked for 48 h in xylene, followed by ethanol washes, to remove the coverslip. The cells were scraped into a 1.5 ml Eppendorf tube and DNA extraction was performed under a standard protocol using organic detergents (13).

PCR Amplification for HPV Detection and Typing in Cervical Smears

All specimens were examined for the presence of amplifiable DNA using a set of primers for β-globin gene. For the detection and distinction of the HPV the general primers GP5 and GP6 (14) and specific primers (15) were used to amplify each virus type (HPV 11, 16, 18 and 33) by multiplex PCR, each virus type giving different length of amplified DNA. The extracted DNA (0.5 μl) of each sample was amplified in a volume of 50 μl containing 150–200 μM of each dNTP, 0.5 μM of each primer, 1.5 mM $MgCl_2$ and 1.25 U *Taq* polymerase (Gibco BRL) in its reaction buffer (supplied by Gibco BRL). In each PCR reaction two blank samples were employed as negative controls to ensure that no contaminants were introduced. The mixture was heated for 1 min at 95°C and samples were subjected to 35 cycles of amplification at 94°C for 50 s, at 52°C for 45 s and at 72°C for 45 s (HPV), followed by elongation at 72°C for 5 min. PCR products were analyzed on a 2% agarose gel and photographed on a UV light transilluminator.

Multiplex PCR

Amplification was carried out at 94°C for 1 min, at 55°C for 50 s and at 72°C for 50 s, followed by elongation at 72°C for 5 min. To establish type specificity of primer-directed amplification, each set of primers was tested with template plasmid DNA of the four HPV types (11, 16, 18 and 33). PCR products were analyzed on a 3% agarose gel and photographed on a UV light transilluminator.

PCR Amplification of p53 Polymorphic Sequences

The polymorphic region of the p53 gene was PCR-amplified from the genomic DNA of both cervical smears (normal and HPV related) and blood samples for the amplification of the Pro allele using primer pairs p53Pro+/p53− and p53+/Arg− for the amplification of the Arg allele (8). In each PCR reaction two blank samples were employed as negative controls to ensure that no contaminants were introduced. The mixture was heated for 1 min at 95°C and samples were subjected to 30 cycles of amplification at 94°C for 40 s, at 60°C for 40 s and at 72°C for 30 s (p53+/Arg−), at 94°C for 40 s, at 54°C for 40 s and at 72°C for 30 s (p53Pro+/p53−).

Elongation was at 72°C for 5 min.

PCR products were analyzed on a 2% agarose gel and photographed on a UV light transilluminator.

Statistical Analysis

Statistical analysis of the results was performed with the package SPSS 6.0 (for Windows). Statistical significance was set at $P < 0.05$.

RESULTS

Histological Data of the Specimens

The presence of amplifiable DNA, using primers for a fragment of β-globin gene, was confirmed in all the 60 high risk HPV-related stained smears, the 74 HPV negative normal stained smears and the 61 blood samples examined (data not shown).

Cases were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization (16) as follows: There were 22 cervical intraepithelial neoplasias (CIN I, II: 8; CIN III: 14) and 38 squamous cell carcinomas of the cervix (Table I).

HPV Typing in Cervical Smears

Normal cervical smears were confirmed to be HPV negative. HPV-18 was the most frequent type with an incidence of 81.5% in the high risk HPV positive samples derived from cervical lesions. HPV-16 and HPV-33 were observed at 11.5% and 7% respectively (Fig. 1). HPV-11 was not detected in any lesion (Table I).

p53 Codon 72 Polymorphism

To analyze the codon-72 polymorphism, we used a PCR-based assay that specifically detects either the p53 Pro or the p53 Arg allele. The primer pair p53+/Arg− gives a PCR product of 141 bp of the Arg allele (Fig. 2) whereas the Pro+/p53− primer pair gives a PCR product of 177-bp fragment of the proline allele (Fig. 3).

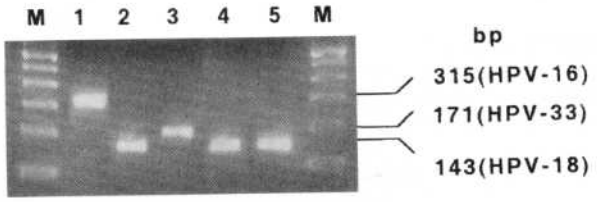

FIG. 1. Type distinction of HPV employing a multiplex PCR. PCR products were electrophoresed through a 3% agarose gel. Lanes 2, 4, and 5, samples positive for HPV-18 (143 bp); lane 3, sample positive for HPV-33 (171 bp); lane 1, sample positive for HPV-16 (315 bp); lanes M, 100-bp molecular weight marker.

The results of the p53 polymorphism distribution of the 60 high risk HPV-related female genital lesions and also the distribution of the 74 normal HPV negative cervical samples and the 61 normal samples used as a control are summarized in Table II. There was a difference in the distribution of p53 genotypes between high risk HPV-cervical lesions and both types of normal samples. The allele frequency of p53 Arg/Arg was much higher (46.5%) than the normal samples (20.5% cervical and 20% blood). The Arg/Pro heterozygosity frequency was 43% in HPV-associated cervical lesions as compared to 62% in HPV negative cervical samples and 67% in blood samples. The Pro/Pro frequency was low both in HPV-associated cervical lesions (8%) and in control groups (16% in HPV negative cervical samples and 10% in blood samples).

Statistically significant correlation was not observed between the presence of Arg/Pro homozygosity or Arg/Pro heterozygosity and HPV typing.

DISCUSSION

HPV infection and persistence are necessary for the evolution and maintenance of cervical cancer (3). Specific viral genes (E6 and E7) of high risk HPVs (types 16, 18, 33 and others) act as oncogenes. HPV oncoprotein E6, was shown to interact with cellular p53 (17). After interaction with E6, p53 was found to be degraded (18).

TABLE I

HPV Typing in Human Genital Lesions by Multiplex PCR Analysis

Histological diagnosis	HPV-18	HPV-16	HPV-33	HPV-11
Cervix of the uterus				
CIN I, II	7	1	—	—
CIN III	11	2	1	—
Squamous cell Ca	31	4	3	—
Total (%):	49 (81.5)	7 (11.5)	4 (7)	—

Note. Ca, carcinoma.

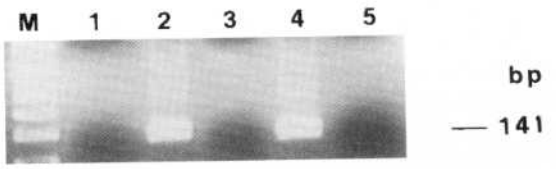

FIG. 2. p53-72 Arg allele amplification products (141 bp) employing a PCR. PCR products were electrophoresed through a 2% agarose gel. Lanes 2 and 4, positive samples; lanes 1, 3, and 5, negative samples; lane M, 100-bp molecular weight marker.

P53 is polymorphic at amino acid 72 of the protein that it encodes, thus p53 may contain either a proline or an arginine residue at this position. Storey *et al.* (8) concluded that patients, with HPV-associated cervical cancer, with two copies of the arginine form have a sevenfold higher risk of developing cervical cancer than those with the proline form. However, other studies (10–12) were not able to confirm this.

We found a much higher rate of infection by HPV-18 in comparison with HPV-16 and HPV-33. A possible explanation for the over-representation of HPV 18 in the Greek population could be attributed to ethnic variations of HPV types. Previous studies regarding the molecular evolution of HPV-18 have shown that HPV-18 has an ancient phylogenetic root in Africa (19), which is located in the near vicinity of our study population. HPV-18 usually exhibits a more aggressive biologic behavior and have a poorer prognosis (20). HPV-18 is associated predominantly with adenocarcinomas and adenosquamous carcinomas while HPV-16 is associated predominantly with squamous cell carcinomas which have better prognosis.

Our results confirm the difference in the Arg/Arg genotype between HPV-infected cervical lesions (46.5%) and our controls (20.5% in HPV negative cervical samples and 20% in blood samples). In our control groups the frequency of p53 Arg/Pro heterozygosity is 62% in HPV negative cervical samples and 67% in blood samples whereas there seems to be some, but not extensive, difference in the prevalence of p53 Pro (16% in HPV negative cervical samples, 10% in blood samples) and p53 Arg (20.5% in HPV negative cervical samples, 20% in blood samples) homozygosity. It has been postulated that the frequencies of p53 codon-72 genotypes vary according to the ethnic group. The fre-

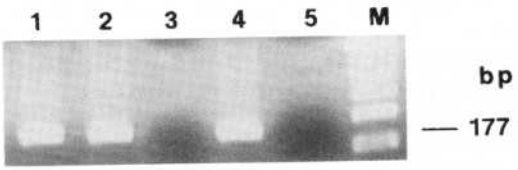

FIG. 3. p53-72 Pro allele amplification products (177 bp) employing a PCR. PCR products were electrophoresed through a 2% agarose gel. Lanes 1, 2, and 4, positive samples; lanes 3 and 5, negative samples; lane M, 100-bp molecular weight marker.

TABLE II

Frequencies of Codon 72 Polymorphism

Histological diagnosis	Number of samples	Arginine	Arginine/proline	Proline	Other
Blood samples					
Total (%)	61	12 (20)	41 (67)	6 (10)	2 (3)
Cervix of the uterus					
Normal samples: Total (%)	74	15 (20.5)	46 (62)	12 (16)	1 (1.5)
Cervix of the uterus					
CIN I, II	8	2	4	2	—
CIN III	14	5	8	1	—
Squamous cell Ca	38	21	14	2	1
Total (%)	60	28 (46.5)	26 (43)	5 (8)	1 (2.5)

Note. Ca, carcinoma.

quency of p53 Arg homozygosity in our control group is lower than that found in a Japanese (7) and in a Norwegian study (10).

However, in our high risk HPV-associated cervical lesions there was a significant over-representation of p53 Arg homozygosity (46.5%) compared to the p53 Pro homozygosity (8%). The frequency of p53 Arg/Pro heterozygotes was 43%.

Our results indicate that p53 Arg homozygosity may represent a possible risk factor for high risk HPV-associated cervical tumorigenesis. In the cervical lesions studied, there was an over-representation of homozygous p53 Arg compared with heterozygous or homozygous Pro alleles.

A small number of high risk HPV-associated cervical tumors and control samples where found to carry no Arg or Pro in the amino acid residue 72 of p53. This can be due to a deletion of that coding sequence of the p53 gene or as Matlashewski *et al.* (4) have stated, further alleles, such as Cys, may exist at position 72 of the p53 protein.

In conclusion, our results indicate that p53 Arg homozygosity is correlated with cervical cancer in high risk HPV-associated lesions (mostly HPV-18 infections) and could represent a potential risk factor for tumorigenesis of the cervix. Further investigation is needed in different ethnic groups and also to determine the influence of this p53 polymorphism on HPV-associated carcinogenesis.

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