P53 codon 72 polymorphism as a risk factor in the development of HPV-associated non-melanoma skin cancers in immunocompetent hosts

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Abstract. Human papilloma virus (HPV) has been implicated in skin cancer. Also, in human populations, the p53 gene is polymorphic at amino acid 72 of the protein that it encodes. The association between p53 polymorphisms and HPV-associated skin cancer risk has been examined, but the results were conflicting. It was revealed 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 higher risk of developing 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 patients with high risk HPV-associated malignant skin lesions. The study was conducted on 29 high risk HPV-related skin lesions from Greece. Blood samples from 61 healthy individuals were used as controls. HPV-8 was the most frequent type. There was a difference in the distribution of p53 genotypes between high risk HPV-skin lesions and the controls, and the allele frequency of p53 Arg/Arg was much higher than the controls (65.5% versus 20%). Therefore, it is suggested that p53 Arg homozygosity could represent a potential risk factor for tumorigenesis of the skin.

Introduction

Non-melanoma skin carcinoma (NMSC) is the most frequently occurring malignancy worldwide in the Caucasian population. The ratio of basal cell carcinoma (BCC) to squamous cell carcinoma (SCC) is 5:1 in immunocompetent populations, whereas in immunosuppressed patients the risk for development of SCC is 250 times greater than that in the general population, and the risk for development of BCC is 10 times greater (1). BCC and SCC occur mainly on sun-exposed sites, which implies ultraviolet (UV) radiation as a major environmental cause in their pathogenesis. In addition, the pattern of mutations in the tumor suppressor gene p53 is typical for UV radiation-induced DNA damage (2).

Human papilloma viruses (HPVs) consist of more than 70 different types and are known to be associated with various malignant tumors, including carcinomas of the anogenital region. According to the tissue tropism and oncogenic potential of HPVs, which reflect differences to their nucleic acid composition, they are classified into the following subgroups: cutaneous, cutaneous involved in epidermodysplasia verruciformis (a rare genetic disease), cutaneous and mucosal, and mucosal of low and high risk (2). In cancer of the cervix and in other anogenital lesions, the most commonly found HPV types are high risk HPV-16, HPV-18, HPV-31, HPV-33 and less frequently low risk HPVs such as HPV-6 and HPV-11.

In human populations, the p53 gene is polymorphic at amino acid 72 of the protein that it encodes (3-5). 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 (3), 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 (3). It was also reported 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 (3).

In recent studies employing cervical lesions, Storey et al (6) 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 7-fold higher risk of developing cervical cancer than those with the proline form.

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We examined whether p53 Arg at the polymorphic position 72 could represent a risk factor for patients with HPV-associated malignant skin lesions obtained from immunocompetent hosts, in comparison with a normal control group. HPV-8 was the most frequent type among the 29 skin lesions (62%) HPV-associated skin lesions were found to carry the Arg/Arg polymorphism in p53 codon 72 in 65.5% of the cases whereas this genotype was observed only in 20% of the normal blood samples. Therefore, it is suggested in this study that p53 Arg homozygosity could possibly represent a potential risk factor for the tumorigenesis of the skin.

Materials and methods

Subjects and blood samples. The study population included 29 patients with high risk HPV-related skin lesions (NMSCs and premalignant lesions) treated at the Department of Dermatology, 'A. Sygros' Hospital, Athens. Directly after dissection the specimens were stored at -70°C until DNA extraction. Peripheral blood was obtained from 61 healthy normal people aged ≥55 years with no known HPV lesions, because no normal skin could be obtained for use as control.

DNA extraction from skin lesions and blood samples. DNA extraction from the skin biopsy specimens, and blood samples was performed with standard protocols (7).

PCR amplification for HPV detection and typing in skin lesions. All specimens were examined for the presence of amplifiable DNA using a set of primers for ß-globin gene. For the detection and distinction of the HPV the general primers GP5⁺ and GP6⁺ (8) and specific primers (9) 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. Also, specific primers (10) were used to amplify virus types HPV-2, -5 and -8 by another multiplex PCR, here also each virus type giving a different length of amplified DNA. For the distinction of HPV-1 specific primers were used as previously described (10). 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 the manufacturer). In each PCR reaction two blank samples were employed as negative controls to ensure that no contaminants were introduced. PCR was performed as previously described (8) for the detection of HPV.

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

Multiplex PCR for HPV-11, -16, -18 and -33. Amplification was carried out at 94°C for 1 min, at 55°C for 50 sec and at 72°C for 50 sec. Finally, samples were elongated 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.

Multiplex PCR for HPV-2, -5 and -8. Amplification was at 94°C for 55 min, at 58°C for 45 sec and at 72°C for 45 sec. Finally, samples were elongated at 72°C for 5 min. PCR products were analyzed on a 3% agarose gel and photographed on a UV light transilluminator and for HPV-1 amplification was at 94°C for 55 sec, at 55°C for 45 sec and at 72°C for 45 sec. 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 skin biopsies and blood samples for the amplification of the Pro allele using primers pairs p53 Pro⁺/p53⁻ and p53⁺/Arg⁻ for the amplification of the Arg allele (6).

The mixture was heated for 1 min at 95°C and samples were subjected to 30 cycles of amplification at 94°C for 40 sec, at 60°C for 40 sec and at 72°C for 30 sec (p53⁺/Arg⁻), at 94°C for 40 sec, at 54°C for 40 sec and at 72°C for 30 sec (p53 Pro⁺/p53⁻). Finally, samples were elongated 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

Histology. The presence of amplifiable DNA, using primers for a fragment of ß-globin gene, was confirmed in all the 29 high risk HPV-related skin lesions and the 61 blood samples examined (data not shown).

The histological classification of the samples was 22 basal cell carcinomas (BCC), 3 squamous cell carcinomas (SCC), and 4 specimens with extragenital Bowen's disease.

HPV typing in skin lesions. HPV-8 was the most frequent type with an incidence of 62%. HPV-18, HPV-5 and HPV-11 were found at 48%, 24% and 3.5% respectively (Figs. 1 and 2). HPV-1, -2, -16 and -33 were not detected in any lesion. The results of the HPV type distinction using specific primers for HPV-11, -16, -18 and -33 are summarized in Table I. Also, the results of HPV type distinction using primers for skin specific types 1, 2, 5 and 8 are summarized in Table II.

p53 codon 72 polymorphism. To analyze the codon 72 polymorphism, we used a PCR-based assay that specifically detects either the p53 Pro or p53 Arg allele. The primer pair p53⁺/Arg⁻ gives a PCR product of 141 bp of the Arg allele (Fig. 3) whereas the Pro⁺/p53⁻ primer pair gives a PCR product of 177 bp fragment of the proline allele (Fig. 4).

The results of the p53 polymorphism distribution of the 29 high risk HPV-related skin lesions and also the distribution of the 61 normal samples used as a contol are summarized in Table III. There was a difference in the distribution of p53 genotypes between high risk HPV-skin lesions and the normal samples. The allele frequency of p53 Arg/Arg was much higher than the normal samples (65.5% versus 20%). The Arg/Pro heterozygosity frequency
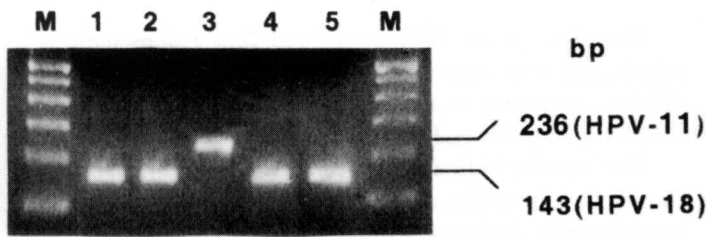
Figure 1. Type distinction of HPV-11, -16, -18 and -33 employing multiplex PCR. PCR products were electophoresed trough a 3% agarose gel. Lanes 1, 2, 4, 5, samples positive for HPV-18 (143 bp); lane 3, sample positive for HPV-11 (236 bp); lanes M, 100 bp molecular weight markers.
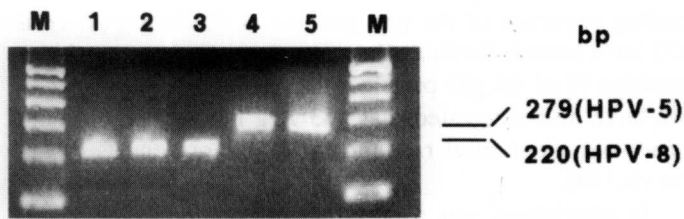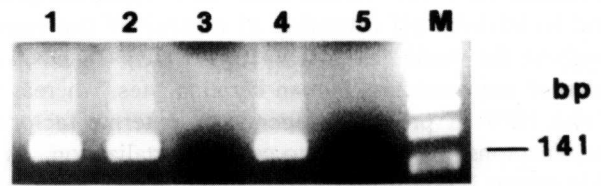

Figure 3. p53-72 Arg allele amplification products (141 bp) employing PCR. PCR products were electophoresed trough a 2% agarose gel. Lanes 1, 2, 4, positive samples; lanes 3 and 5, negative samples; lane M, 100 bp molecular weight marker.
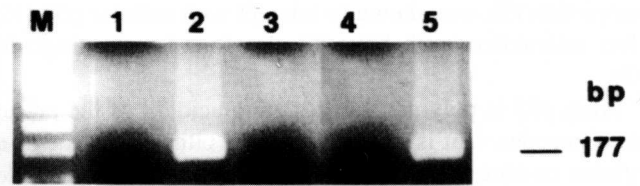

Figure 2. Type distinction of HPV-1, -2, -5 and -8 employing PCR. PCR products were electophoresed trough a 3% agarose gel. Lanes 1-3, samples positive for HPV-8 (220 bp); lanes 4 and 5, samples positive for HPV-5 (279 bp); lanes M, 100 bp molecular weight markers.

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

Table I. HPV typing in human skin lesions by multiplex PCR analysis for HPV-11, -16, -18 and -33.

Histological diagnosis	HPV-11	HPV-16	HPV-18	HPV-33
Basal cell ca.	0	0	10	0
Squamous cell ca.	0	0	1	0
Bowenoid lesions	0	0	2	0
Precancerous	1	0	1	0
Total (%)	1 (3.5)	0	14 (48)	0

ca., carcinoma

Table II. HPV typing in human skin lesions by PCR analysis for HPV-1, -2, -5 and -8.

Histological diagnosis	HPV-1	HPV-2	HPV-5	HPV-8
Skin lesions				
Basal cell ca.	0	0	4	15
Squamous cell ca.	0	0	2	1
Bowenoid lesions	0	0	1	1
Precancerous	0	0	0	1
Total (%)	0	0	7 (24)	18 (62)

Table III. Frequencies of codon 72 polymorphism.

Histological diagnosis	No. of samples	Arginine	Arginine/ proline	Proline	Other
Blood samples					
Total (%)	61	12 (20)	41 (67)	6 (10)	2 (3)
Skin lesions					
Basal cell ca.	22	15	3	3	1
Squamous cell ca.	3	2	1		-
Bowenoid lesions	4	2	1		1
Total (%)	29	19 (65.5)	5 (17)	3 (10.5)	2 (7)

was 17% in HPV-associated skin lesions compared to 67% of the control group. The Pro/Pro frequency was low both in HPV-associated cervical lesions and in the control group (10.5% and 10% respectively).

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

Discussion

Previous studies have confirmed the role of high risk genital HPV-16 and -18 in the development of anogenital

carcinomas, HPV-16, -18 and other HPV types have been found in 95% of all biopsies of cancer of the cervix throughout the world (11). HPV-16 and HPV-18 DNA are capable of immortalizing human keratinocytes, whereas the low risk HPV-11 probably depends on external factors to cause mutations that will lead to cell immortalization. As far as skin cancer is concerned, the majority of the studies were conducted in patients with epidermodysplasia verruciformis as well as in immunosuppressed patients, i.e., renal transplant recipients. Non-melanoma skin carcinoma in epidermodysplasia verruciformis patients contain pre-dominantly HPV-5 and HPV-8 types, which can be considered oncogenic and they simulate the low risk genital HPV types in their mechanism of interaction. Specific viral genes (E6 and E7) of high risk HPVs act as oncogenes. HPV oncoprotein E6, was shown to interact with cellular p53 (12). After interaction with E6, p53 was found to be degraded (13).

Also, p53 is polymorphic at amino acid 72 of the protein that it encodes, that is p53 may contain either a proline or an arginine residue at this position. Storey *et al* (6) concluded that patients, with HPV-associated cervical cancer, carrying two copies of the arginine form have a 7-fold higher risk of developing cervical cancer than those with the proline form. However, this has not been confirmed by other studies (14-16).

HPV types 1 and 2 (usually found in myrmecia and common warts) were undetected in the above lesions. On the other hand, HPV-5 and HPV-8 were found respectively in 24% and 62% of the lesions. HPV-8 was the most common of all types detected and was mainly found in basal cell carcinoma lesions.

In addition, HPV-18 was detected in 48% of the lesions. Dokianakis *et al* (17) have reported a higher rate of HPV-18 infection in Greek women also in lesions of the reproductive tract. HPV-16 and 033 were not detected and HPV-11 was detected in 3.5% of the lesions. The high frequency of mucosal HPV DNA in cutaneous lesions indicates that papilloma viruses are more widespread than previously thought. Another possible explanation is that there is an initial source of anogenital infection and the transmission to the skin lesions occurs through autoinoculation. Moreover, the low frequency of the low risk genital HPV-11 that was found only in pre-cancerous lesions in contrast to the high frequency of the high risk HPV-18 that was detected mainly in BCC lesions, may imply a direct contribution of this oncogenic HPV DNA in the development of non-melanoma skin carcinoma.

In the present study, we analyzed the p53 genotype of skin lesions and control samples using a PCR-based assay. Our results confirm the difference in the Arg/Arg genotype between HPV-infected skin lesions and our control (65.5% versus 20%). In our control group the frequency of p53 Arg/Pro heterozygosity was 67% whereas the prevalence of p53 Pro and p53 Arg homozygosity was 10% and 20% respectively. It has been postulated that the frequencies of p53 codon 72 genotypes vary according to the ethnic group. The frequency of p53 Arg homozygosity in our control group was lower than that found both in a Japanese (18) and in a Norwegian study (14).

However, in our HPV-associated skin lesions there is a significant over-representation of p53 Arg homozygosity (65.5%) compared to the p53 Pro homozygosity (7%). The frequency of p53 Arg/Pro heterozygotes was 20%.

Our results indicate that p53 Arg homozygosity may represent a possible risk factor for HPV-associated skin tumorigenesis. In the skin tumors studied, there was an over-representation of homozygous p53 Arg compared with heterozygous or homozygous Pro alleles and these differences are more significant when compared with our control group.

A small number of HPV-associated skin lesions and control samples where found to carry no Arg or Pro in the amino acid residue 72 of p53. This could be due to a deletion of that coding sequence of the p53 gene or as Matlashewski *et al* (3) have stated, further alleles, such as Cys, may exist at position 72 of the p53 protein. Furthermore, in a recent study we have also suggested that p53 Arg homozygosity could represent a potential risk factor for tumorigenesis of the cervix (19).

In conclusion, our results indicate that p53 Arg homo-zygosity is correlated with skin-cancer in HPV-associated lesions (mostly HPV-8 infections) and could possibly represent a potential risk factor for tumorigenesis of the skin. Further investigation is needed in different ethnic populations and also to determine the influence of this p53 polymorphism on HPV-associated carcinogenesis.

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