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p53 codon 72 polymorphism and its association with bladder cancer

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Abstract

p53 codon 72 Arg homozygosity has been associated with increased risk of developing cervical cancer. This association has been tested in various human cancers with controversial results. In the present study we investigated the impact of this polymorphism in a population-based case-control study of bladder cancer. Using allele-specific polymerase chain reaction to detect the *p53* codon 72 polymorphism, we tested peripheral blood samples from 50 patients with bladder cancer and 99 healthy individuals of similar age and from the same geographical region. Tumor specimens from all bladder cancer patients were examined for the presence of human papilloma virus (HPV). The distribution of *p53* alleles in bladder cancer patients and in controls was statistically significant ($P < 0.002$; odds ratio, 2.67; 95% confidence interval, 1.38–5.20), and homozygosity for arginine at residue 72 was associated with an increased risk for bladder cancer ($P < 0.00002$; odds ratio, 4.69; 95% confidence interval, 2.13–10.41). The presence of HPV was found in six of the 50 patients (12%). This is the first study correlating *p53* codon 72 polymorphism with bladder cancer. Our results provide evidence that this *p53* polymorphism is implicated in bladder carcinogenesis and that individuals harboring the Arg/Arg genotype have an increased risk of developing bladder cancer.

Keywords: *p53*; Codon 72 polymorphism; Bladder cancer; Human papilloma virus

1. Introduction

Bladder cancer is the sixth most common cancer in the USA with an estimated 54,300 new diagnoses and 12,400 tumor-associated deaths for the year 2001 [1]. Factors reported to be causally related to the disease include occupational exposure to chemicals, cigarette smoking, coffee drinking, artificial sweeteners and bacterial, parasitic or viral infections [2]. Several oncogenes and tumor suppressor genes, such as *p16*, $p21^{WAF1/CIP1}$, *Rb1*, *ras*, *c-erbB-1* and *p53* are involved in the development and progression of bladder cancer [3].

The human papilloma virus (HPV) family has been implicated in the development of bladder cancer. HPV genome consists of 7200–8000 bp and encodes six early (E1–E7) and two late proteins (L1 and L2) [4]. The early proteins E6 and E7, especially of high-risk HPVs, are capable of interacting with cellular proteins involved in the control of cell proliferation and in the prevention of cell immortalization [5]. E6, in particular, binds the cellular tumor-suppressor protein *p53* and promotes its degradation with the E6-AP protein

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through the ubiquitin-dependent proteolytic pathway [6,7]. The presence of HPV DNA in squamous and transitional cell carcinoma of the bladder has been detected in 0–80% of tumors examined [8]. Nevertheless, most studies report low HPV numbers (<10%) [9–11], suggesting that HPV plays an important role in a small portion of bladder cancer cases.

The human *p53* tumor suppressor gene encodes a 393 amino acid, 53-kDa nuclear phosphoprotein [12], which is a central element in fundamental cellular processes, including induction of cell cycle arrest, gene transcription, DNA repair and apoptosis [13]. The *p53* gene is mutated in 20–60% of bladder tumors [14], with codons 280 and 285 being mutational hotspots [15]. These alterations seem to be more frequent in invasive than in superficial tumors [16], and are usually correlated with worse clinical outcome [17].

p53 codon 72 polymorphism, encoding either arginine or proline [18], has been proposed to affect the susceptibility of *p53* protein to HPV E6-mediated degradation in vitro. Moreover, the frequency of the Arg/Arg genotype was found to be significantly higher in cervical cancer patients compared to the general population, indicating that individuals homozygous for Arg are seven times more susceptible to HPV-associated tumorigenesis than heterozygotes [19]. Similar studies have been conducted in several human tumors, such as cervical [20–22], head and neck [23], esophageal [24], lung [25], breast [26], skin [27] or laryngeal cancer [28], but the results were controversial. Despite the great variety of human tumors that have been already examined for *p53* codon 72 polymorphism, no study on bladder cancer has been carried out yet.

In the present study, we conducted a case-control study in a geographically homogeneous Greek population, to examine the genotypic frequency of the *p53* codon 72 polymorphism and the presence of HPV in 50 bladder cancer patients compared to matched healthy controls, in order to determine any association to bladder carcinogenesis.

2. Materials and methods

2.1. Blood and tumor specimens

Fifty tumor specimens were obtained from patients with histologically confirmed bladder cancer from the Department of Urology, University General Hospital of Heraklion, Greece. The specimens were stored at −80°C immediately after surgical removal, until DNA extraction. In all cases, fresh patient blood

Table 1
Primer sequences and length of PCR products

Primers	Sequence (5′– 3′)	PCR product (bp)
GP5	TTT GTT ACT GTG GTA GAT AC	139–145
GP6	GAA AAA TAA ACT GTA AAT CA	
HPV18F	AAA CTA ACT AAC ACT GGG TTA TAC A	143
HPV18R	ATG GCA CTG GCC TCT ATA GT	
HPV16F	CTG CAA GCA ACA GTT ACT GCG ACG	315
HPV16R	CAT ACA TCG ACC GGT CCA CC	
HPV11F	TGT GTG GCG AGA CAA CTT TCC CTT	236
HPV11R	TGG TTA TTT AGT TTT ATG AAG CGT GCC TTT CCC	
HPV33F	AAC AGT TAA AAA ACC TTT AAA	171
HPV33R	AGT TTC TCT ACG TCG GGA CCT C	
ArgF	TCC CCC TTG CCG TCC CAA	141
ArgR	CTG GTG CAG GGG CCA CGC	
ProF	GCC AGA GGC TGC TCC CCC	177
ProR	CGT GCA AGT CAC AGA CTT	

was collected in tubes containing ethylenediaminetetra-acetic acid (EDTA) and was stored at 4°C, to serve as a source for normal DNA. Peripheral blood was obtained from 99 healthy individuals serving as the control group and stored at 4°C for further use. All subjects derived from a white Caucasian Greek population living in the genetically homogeneous geographical area of Crete, with no exposure to any known environmental carcinogens. The Ethics Committee of the University of Crete approved this study and written informed consent was obtained from all patients.

2.2. DNA extraction

Blood samples were washed with the Reagent A solution (10 mM Tris–HCl pH 8.0, 320 mM sucrose, 5 mM $MgCl_2 \cdot 6H_2O$ and 1% Triton-X-100), and centrifuged. The residual was washed with the Reagent B solution (400 mM Tris–HCl pH 8.0, 150 mM NaCl, 60 mM EDTA and 1% sodium dodecyl sulphate (SDS)).

Tissue samples were lysed with the Reagent B solution adding 50 μg Proteinase K and incubated for 5 h at 65°C.

All samples were then extracted with phenol/chloroform and chloroform. DNA was precipitated with absolute ethanol, washed with 70% ethanol and resuspended in 100 μl double distilled water.

2.3. Polymerase chain reaction (PCR) amplification of p53 polymorphic sequences

All specimens were examined for the presence of amplifiable DNA using a set of primers for β-globin gene. For the determination of the polymorphism at codon 72 of the *p53* gene two sets of primers were used, one to amplify the Arg allele and the other to amplify the Pro allele (Table 1). One hundred nanograms of the extracted blood DNA were amplified in a PCR reaction containing 1 × buffer (20 mM Tris–HCl pH 8.4 and 50 mM KCl), 1.6 mM $MgCl_2$, 200 μM of each dNTP, 200 nM of each primer and 0.024 U/μl Taq polymerase (Gibco BRL, Life Technologies Inc.) in a final volume of 25 μl. The detection of the two polymorphic variants was done in two different tubes. The amplification was performed as follows: initial denaturation at 94°C for 3 min, amplification for 35 cycles at 94°C for 30 s, 60°C for the Arg allele and 54°C for the Pro allele for 30 s and 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. The PCR product of the Arg allele was 141 bp, while the product of the Pro allele was 177 bp. Heterozygous specimens had both PCR products, whereas homozygous samples exhibited only one of the two products. In each PCR reaction two blank samples were employed as negative controls, to ensure that no contaminants were introduced. The determination of *p53* codon 72 polymorphism in the corresponding blood of the tumor specimens was performed in order to assure that the results were strictly genomic and not due to loss of heterozygosity (LOH). PCR procedures were repeated twice and the results were 100% reproducible.

2.4. PCR amplification for HPV detection

For the detection of HPV genome the general primers GP5 and GP6 were used (Table 1). One hundred nanograms of the extracted DNA of each tumor sample was amplified in a PCR reaction, as described above. The mixture was heated at 94°C for 3 min and the samples were amplified for 40 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 30 s, followed by elongation at 72°C for 5 min. PCR products were expected to be 139–145 bp, depending on HPV type [29].

2.5. Multiplex PCR for the determination of HPV genotypes (HPV typing)

HPV typing of all HPV-positive samples was carried out using multiplex PCR. Specific pairs of primers were used to simultaneously amplify regions of HPV types 11, 16, 18 and 33 in the same reaction tube, giving different lengths of amplified DNA. Each HPV-positive tumor sample was amplified in a PCR reaction containing 1 × buffer, 2.66 mM $MgCl_2$, 0.05% W-1, 533 μM of each dNTP, 166 nM of each primer and 0.04 U/μl Taq polymerase in a final volume of 15 μl. The amplification parameters were: initial heating at 94°C for 2 min and 30 s, 10 cycles of amplification at 94°C for 30 s, 52°C for 40 s and 72°C for 35 s, 30 cycles of amplification at 92°C for 30 s, 47°C for 40 s and 72°C for 45 s and final extension at 72°C for 10 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). Primer

Table 2
Clinical and histopathological data of 50 patients with bladder cancer and results of *p53* codon 72 polymorphism analysis and HPV detection

No.	Arg/Arg	Arg/Pro	Pro/Pro	HPV	Stage	Grade	Sex	Age
1	+				T1	I–II	M	72
2		+			T1	I–II	M	65
3	+			+	Tα	I	M	55
4	+				T2	III	M	59
5	+				T3α	III	F	76
6		+			T1	I–II	M	81
7		+		+	T3α	III	F	72
8		+			T3β	III	M	73
9			+		Tα	I	M	73
10		+			T1	III	M	59
11		+			Tα	I	M	64
12	+				T1	I–II	M	80
13	+				Tα	I	M	57
14		+			T1	I–II	M	68
15	+				T2	III	M	60
16	+			+	Tα	I	M	76
17	+				T1	I–II	M	82
18	+				T1	III	M	74
19	+				Tα	I	M	72
20	+				Tα	I	F	68
21	+			+	Tα	I–II	M	65
22	+				T2	III	M	70
23	+				T1	I–II	M	58
24	+				Tα	I	M	66
25	+			+	Tα	I	M	71
26	+				Tα	I–II	M	70
27	+				Tα	I	F	75
28	+				T1	I–II	M	66
29		+			T1	II	F	62
30		+			T1	I	M	73
31	+			+	T1	II	M	69
32	+				T1	II	M	78
33		+			T1	I–II	M	56
34		+			T1	II–III	M	47
35		+			T1	I–II	F	18
36	+				Tα	I	M	57
37	+				T1	I–II	F	70
38	+				Tα	I–II	M	68
39		+			Tα	II	M	78
40		+			Tα	II	M	76
41		+			T1	I–II	M	67
42	+				Tα	I–II	F	59
43	+				Tα	II	M	63
44	+				T1	II–III	M	73
45			+		T1	II	M	74
46	+				T1	II–III	F	67
47		+			T1	II	M	56
48	+				T1	II	M	60
49		+			T1	II	M	74
50		+			T1	I–II	M	33

sequences and the length of PCR products are shown in Table 1.

2.6. PCR products analysis

The PCR products were analyzed by electrophoresis in a 2% agarose gel, stained with ethidium bromide and photographed on a UV light transilluminator. The photographs were processed using Adobe Photoshop 5.5 for Windows.

2.7. Statistical analysis

Statistical analysis was performed using the Chi-square test with the package SPSS 10.0 for Windows. Statistical significance was regarded at P-value <0.05.

3. Results

Blood specimens from 50 patients with bladder cancer were analyzed for codon 72 polymorphism of the *p53* gene, while the corresponding tissue samples from each patient were tested for the presence of HPV. Mean age at diagnosis was 66.1 ± 11.4 years. The majority of the specimens was obtained from male patients (41 out of 50). The results of these analyses as well as the clinical and histopathological data are shown in Table 2.

The Arg/Arg genotype of the *p53* codon 72 polymorphism was found in 30 patients (60%), the Arg/Pro genotype in 18 (36%) and the Pro/Pro genotype in two (4%) (Figs. 1 and 2). The analysis was performed in blood samples and not in the corresponding tumor samples, in order to exclude the possibility of reduction to homozygosity due to LOH.

In order to determine whether the distribution of the *p53* codon 72 polymorphism in bladder cancer patients is different from that in the general population, we subjected blood specimens from 99 healthy individuals to the same genotyping assay. As *p53* allele frequencies have been shown to vary according to ethnic group, controls and patients were from the same ethnic background. The Arg/Arg genotype was found in 24 individuals (24.2%), the Arg/Pro genotype in 64 (64.7%) and the Pro/Pro genotype in 11 (11.1%). The results of the *p53* genotyping assay in both normal controls and bladder cancer patients are shown in Table 3.

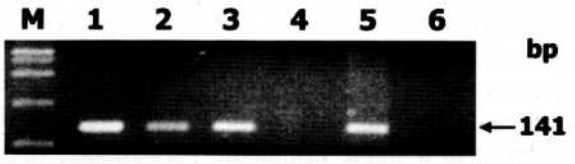

Fig. 1. PCR amplification of the *p53* codon 72 Arg allele (141 bp). Lanes 1, 2, 3 and 5; positive samples. Lanes 4 and 6; negative samples. M; 100 bp molecular weight marker.

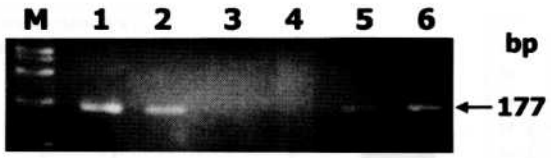

Fig. 2. PCR amplification of the *p53* codon 72 Pro allele (177 bp). Lanes 1, 2, 5 and 6; positive samples. Lanes 3 and 4; negative samples. M; 100 bp molecular weight marker.

Based on these findings, we detected a significant statistical association of the Arg/Arg genotype with bladder cancer ($P < 0.00002$; odds ratio (OR), 4.69; 95% confidence interval (CI), 2.13–10.41). On the contrary, no statistical significance was observed in the Pro/Pro genotype among tumor and normal specimens ($P = 0.15$; OR, 0.33; 95% CI, 0.05–1.70). Further statistical analysis of the PCR findings revealed that the relative frequency of each allele is 0.78 for Arg and 0.22 for Pro in tumor specimens, and 0.565 for Arg and 0.435 for Pro in normal controls. The distribution frequency of the two alleles is considerably different between bladder cancer patients and

Table 3
Distribution of *p53* codon 72 polymorphism in HPV-related and non-related bladder cancer and in normal population

	Arg/Arg	Arg/Pro	Pro/Pro
Bladder cancer			
HPV+	5	1	–
HPV−	25	17	2
Total	30 (60%)	18 (36%)	2 (4%)
Control normal			
HPV+	–	–	–
HPV −	24	64	11
Total	24 (24.2%)	64 (64.7%)	11 (11.1%)

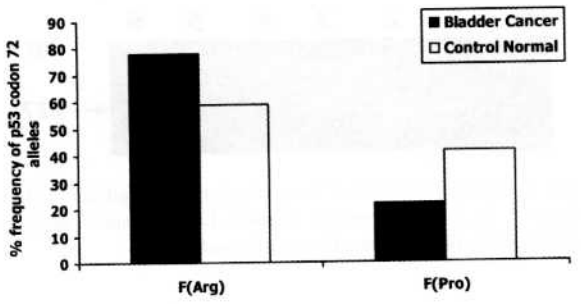

Fig. 3. *p53* codon 72 polymorphism PCR analysis. Shaded columns; frequencies of Arg and Pro alleles in tumor specimens. Unshaded columns; frequencies of the two alleles in normal specimens.

healthy individuals ($P < 0.002$; OR, 2.67; 95% CI, 1.38–5.20) and is presented graphically in Fig. 3.

HPV DNA was detected in six patients (12%). Multiplex PCR revealed that all patients harbored the high-risk HPV-18 type (Fig. 4). Interestingly, five of the six HPV-positive specimens (83.3%) presented the Arg/Arg genotype of the *p53* codon 72 polymorphism, but no statistical association was found between this genotype and HPV ($P = 0.22$; OR, 3.80; 95% CI, 0.37–93.42). Only one HPV-positive sample (16.7%) had the Arg/Pro genotype, while none had the Pro/Pro genotype. No statistical association was found between HPV-positive and HPV-negative tumor samples having the Arg/Pro genotype, nor between HPV-positive and HPV-negative samples with the Pro/Pro genotype.

Finally, the results of the *p53* codon 72 polymorphism analysis were grouped according to patient sex, age, tumor stage and tumor grade. However, no statistical association was found between the *p53* polymorphism and any of these parameters.

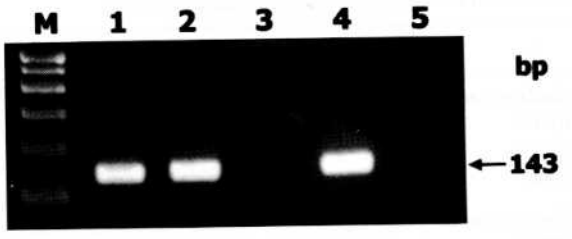

Fig. 4. Detection of HPV-18 by PCR amplification (143 bp). Lanes 1, 2 and 4; positive samples. Lanes 3 and 5; negative samples. M; 100 bp molecular weight marker.

4. Discussion

Since the original publication by Storey et al. in HPV-associated cervical cancer [19], that *p53* homozygotes have a significantly higher risk of developing cancer, numerous studies have been conducted in cervical and other tumors. The results are controversial with several groups confirming the original finding [20,24,30,31], while others have failed to find an association between *p53Arg* and cancer [21–23,32].

In this study, we examined the prevalence of *p53* codon 72 polymorphism in a Greek group of bladder cancer patients and controls. Furthermore, we searched for an association between *p53* alleles and the presence of HPV in the same series of tumor samples. We found a significant statistical association in the distribution of *p53* alleles between bladder cancer patients and healthy individuals. Homozygosity for *p53Arg* was detected in 60% of cancer specimens compared to 24.2% of controls; the Arg/Pro genotype was detected in 36% of the tumors compared to 64.7% of controls, while only 4% of the tumors were homozygous for *p53Pro* allele compared to 11.1% of controls. Our results indicate that individuals with *p53* Arg/Arg genotype have increased risk of developing bladder cancer, which is in agreement with Storey et al. [19], with previous studies by our group in breast [26], skin [27], laryngeal [28] and cervical cancer [33], as well as with another Greek study on cervical cancer [34]. Moreover, considering the statistical difference between genotypes Arg/Arg and Arg/Pro but not between genotypes Arg/Pro and Pro/Pro among tumor and normal samples, it is clear that it is the *p53Arg* homozygous genotype which affects the predisposition. The presence of the *p53Arg* itself in a heterozygous status is not sufficient to develop the tumor phenotype.

Great care was taken to minimize sources of bias and to avoid the overestimation of the results. Patients and controls, derived from a well-defined population, were of similar ages and were recruited from a homogeneous population living in the restricted geographical area of Crete. All cases were consecutively observed in a single institution. Furthermore, since LOH at the *p53* region has been reported in bladder cancer [35], the genotype analysis was performed on blood and not on tumor samples to avoid the risk of

overestimating the *p53* homozygosity [36], suggesting that these tumors arose in individuals who were predisposed by having homozygous *p53Arg* genotype. Finally, we extracted DNA from fresh and not paraffin-embedded samples, ensuring the high quality and integrity of DNA, which can be easily amplified by PCR, since both PCR fragments are relatively short (<200 bp).

The failure of several previous studies to find an association between *p53Arg* and cancer can be attributed to variations in protocols among different laboratories, or to poor selection of the control group [30]. Most of the previous studies were conducted in populations with high Arg frequency (>0.65) and therefore, an increase of the Arg frequency in the tumor patients would possibly not yield a statistically significant result. On the other hand, in populations with higher Pro frequency, such as ours (0.435), the increase in the Arg/Arg genotype is sufficient enough to produce statistically significant results [22].

HPV was detected in 12% of tumor specimens, which is consistent with previous studies performed in bladder cancer [9–11], confirming that HPV is a significant factor in the development of a small percent of the tumors. Typing assay revealed that all tumor samples harbored HPV-18. The high prevalence of this HPV type in the Greek population could be attributed to ethnic variations [37]. Therefore, the mechanism proposed by Storey et al. [19], that HPV E6 oncoprotein binds *p53* and promotes its ubiquitin-mediated degradation [6,7] (especially of *p53Arg* protein), can only be applied to a small percentage of bladder tumors. The lack of a statistical association between HPV infection and the homozygous *p53Arg* genotype in bladder cancer, support the synergistic action of the virus, especially in an environment with exposure to carcinogens, rather than its causative role in the development of the disease.

Recently it was shown that *p53Arg* enhances the ability of certain conformational *p53* mutants to form stable complexes with *p73*, resulting in loss of *p73* DNA-binding capacity, and its ability of serving as a sequence-specific transcriptional activator and an inducer of apoptosis [38]. Furthermore, it was suggested that the Arg allele is preferentially mutated and retained in tumors arising from Arg/Pro germline heterozygotes [39]. Since the mutation rate of *p53* in bladder cancer is 20–60% [14], the above mechanism could explain the higher Arg/Arg genotype in this subgroup of bladder tumors.

The preference for Arg/Arg genotype in human tumors is still unclear. Nevertheless, it is known that codon 72 polymorphism lies within the proline-rich domain of *p53* [12]. This domain is required for the growth suppression activity of *p53* [40] and plays an important role in *p53*-mediated apoptosis [41]. It comprises five PxxP SH3 (SRC-homology-3) binding motifs [40], one of which is lost when the proline at codon 72 is replaced with arginine. Perhaps this substitution affects the biological role of the polyproline domain. The two polymorphic variants of the *p53* protein differ in their ability to bind components of the transcriptional machinery, activate transcription, induce apoptosis and repress the transformation of primary cells, although their DNA-binding affinity is the same [42]. These observations might lead to a new biological mechanism, which could explain the implication of *p53* polymorphism in carcinogenesis.

It is noteworthy that there is significant correlation between the Arg frequency and latitude, implying that the *p53* polymorphism is maintained by natural selection. The increased frequency of the Pro allele in populations closer to Earth's equator, who show little genetic similarity apart from dark skin pigmentation, suggests that codon 72 polymorphism plays an important role in ecological adaptation to ultra-violet radiation, probably through UV-induced *p53*-mediated DNA repair [43].

In conclusion, this is the first study of *p53* codon 72 polymorphism in bladder cancer patients, indicating that *p53Arg* homozygous genotype is associated with the development of the disease. The low incidence of HPV infection suggests that it is not a major oncogenic factor but may have a synergistic action with specific genotypes of *p53*. Further studies are needed in order to elucidate the function of the *p53* codon 72 polymorphism, its evolutional and ecological presence, as well as its role in the development of bladder and other cancer types, as it is indicated to be one of the strongest susceptibility factors in cancer development and progress.

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