



p53 codon 72 polymorphism is linked to the development and not the progression of benign and malignant laryngeal tumours

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Abstract

The *p53* codon 72 polymorphism, resulting in either an arginine or a proline residue has been proposed to affect the susceptibility of *p53* protein to human papilloma virus (HPV) E6-mediated degradation in vitro. However, there are controversial results from several clinical studies in various human tumours. The purpose of our study was to investigate the significance of this *p53* genotype with respect to the risk of neoplasia development in Greek patients with benign and malignant laryngeal tumours. Furthermore, we searched for an association between *p53* alleles and the presence of HPV in the same series of samples. We found a significant statistical association in the distribution of *p53* genotypes between laryngeal lesions and normal samples ($P < 0.001$). Allelic analysis of the patients with both benign and malignant tumours revealed a striking over-representation of the homozygous *p53Arg* allele compared to normal population ($P < 0.0003$). HPV was detected in only 3 laryngeal samples (1 benign and 2 malignant tumours). This is the first study correlating the *p53* codon 72 polymorphism in laryngeal tumours. Our results provide evidence that this *p53* polymorphism may be implicated at the early stages of the disease and concerns predisposition to premalignant laryngeal lesions rather than to progression from benign tumour toward malignancy. Moreover, we demonstrate that the *p53Arg* homozygous genotype affects the predisposition for laryngeal tumours while the heterozygous status does not. The low incidence of HPV infection suggests that it is not a major oncogenic factor in the development of laryngeal tumours but may have synergistic action with specific genotypes of *p53* gene. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *p53* codon 72; HPV; Laryngeal malignant tumours; Laryngeal benign tumours

1. Introduction

Laryngeal cancer constitutes up to almost 2% of all human cancers and 90–95% are squamous cell carcinomas. Epidemiological studies suggest a considerable geographical variation of the disease, as well as an association with the smoking and drinking history of the patients [1]. In general the predicted mortality is 30% (thus a survival rate of approximately 70%) [2]. These observations point to the existence of particular mutagens causing various genetic alterations [3,4], and strongly involved in laryngeal tumorigenesis. Therefore, it is of great importance to identify factors that increase the risk of laryngeal tumours.

Human papilloma virus (HPV) infection has been considered as a potential oncogenic factor for the

development of laryngeal cancer. Several of the 60 types of HPVs have been detected in laryngeal tumours [5,6] although the prevalence of HPV is estimated to range between 3–85% [7–9]. HPV-16/18 encodes E6 protein, which binds to cellular tumour suppressor protein *p53* and directs degradation through the ubiquitin pathway [10,11]. Therefore, it is widely assumed that *p53* is functionally inactivated by the viral E6 protein in HPV-associated cancer cells and that infection with high risk HPV types leads to the same phenotype as a loss of *p53* function due to *p53* gene mutation.

The *p53* gene is frequently mutated in laryngeal tumours either through mutations or via altered expression of the gene product [12–14]. A polymorphism in the wild-type *p53* gene at codon 72 of exon 4 has been described, resulting in either a proline (CCC, *p53Pro*) or an arginine (CGC, *p53Arg*) residue [15]. Storey et al. reported a significant overexpression of the *Arg/Arg p53* genotype in cervical cancer patients relative to the normal population [16]. Women homozygous

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for *p53Arg* were about seven times more susceptible to HPV-associated tumorigenesis than heterozygotes. In addition, the data were supported by results from an in vitro assay demonstrating that the *p53Arg* form of the protein was more susceptible to degradation by the HPV E6 protein than the *p53Pro* variant. Similar studies on cervical cancer have shown controversial results [17–25]. Studies on *p53* codon 72 polymorphism and HPV have also been carried out in other human cancers such as esophageal [26], head and neck [27], lung [28,29], breast [30] and skin [31]. However, the contribution of *p53* polymorphism has apparently not been documented. The existence of a similar risk factor for a given *p53* genotype in HPV-related laryngeal tumours would facilitate screening of HPV-related individuals.

In the present study, we investigated the genotypic frequency of *p53* codon 72 polymorphism in correlation with the presence of HPV in both benign and malignant laryngeal tumours compared to matched healthy controls. Our data provide evidence for the first time that the *p53Arg* homozygosity present a potential risk factor in laryngeal tumorigenesis and the *p53Arg* allele, in the absence of *p53Pro* allele confers a susceptibility for the development rather than the progression of laryngeal tumours.

2. Materials and methods

2.1. Tumour specimens and DNA extraction

Thirty-seven cytological specimens of the larynx were obtained from the General Hospital Nikaias, Pireus [3]. Seventeen samples were benign neoplasias while 20 specimens were squamous cell carcinomas of the larynx. The biologic material was obtained through direct laryngoscopy, by a brush. Part of the material was smeared on slides and fixed by alcohol for Papanicolaou stain and conventional cytologic diagnosis. The remaining part was rinsed in normal saline and was stored at -80°C for DNA extraction. A matched normal DNA control from blood obtained from each patient was analyzed. Furthermore, 40 healthy individuals without evidence of cancer served as the control group. Specimens obtained from all 37 patients and 40 controls were distributed in blind fashion for *p53* codon 72 polymorphism analysis. Informed consent was obtained from all patients who participated in this study. DNA was extracted as previously described [32] and stored at 4°C until PCR amplification.

2.2. PCR amplification for HPV detection

All specimens were examined for the presence of amplifiable DNA using a set of primers for β -globin gene. For the detection of the HPV genome, the general

primers GP5 and GP6 [33] were used. The extracted DNA of each sample was amplified in a volume of 50 μl containing 200 μM of each dNTP, 0.5 μM of GP5 and GP6 primers, 1.5 mM MgCl_2 and 1.25 U Taq polymerase (Gibco BRL) in its reaction buffer. 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 40 cycles of amplification at 94°C for 50 s, 52°C for 45 s and 72°C for 45 s (HPV), followed by elongation at 72°C for 5 min. PCR procedures were repeated twice, the products were analyzed on a 10% polyacrylamide gel and silver stained.

2.3. Multiplex PCR

HPV typing of all HPV positive samples was carried out using multiplex PCR. We used a specific pair of primers [34] to amplify virus types HPV 11, 16, 18 and 33 in a single tube reaction. In each tube, sequences of various virus types were amplified simultaneously, giving different lengths of amplified DNA. By electrophoresing the PCR product of each multiplex reaction we could observe the bands corresponding to each HPV type. The multiplex PCR for HPV types 11, 16, 18, 33 included amplification at 94°C for 1 min, 55°C for 50 s and 72°C for 50 s. 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 10% polyacrylamide gel and silver stained. The PCR products for HPV 11 was expected to be 236 bp, 315 bp for HPV 16, 143 bp for HPV 18 and 171 bp for HPV 33.

2.4. PCR amplification of the *p53* codon 72 polymorphism

The polymorphic region of *p53* gene was PCR-amplified from the genomic DNA of laryngeal cytological specimens (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 [16]. In every 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, 60°C for 40 s and 72°C for 30 s (*p53*+/*Arg*-), at 94°C for 40 s, 54°C for 40 s and 72°C for 30 s (*p53Pro*+/*p53*-). Elongation was at 72°C for 5 min. PCR products were analyzed on a 10% polyacrylamide gel and silver stained. Heterozygous samples showed fragments for both pairs of primers while homozygous specimens exhibited DNA bands only in one of the two

sets of primers. To examine the accuracy of our polymorphism analysis in the laryngeal tumour patients, we performed the same genotyping assay not only in tumour specimens but also in the corresponding DNA from blood of the same patients and the genotype was identical in both.

2.5. Loss of heterozygosity (LOH) analysis

The DNA samples of laryngeal lesions were examined for allelic imbalance using three highly polymorphic microsatellite markers, D17S515, TP53 and D17S678 (Research Genetics, Inc., USA) located at the *p53* region. PCR analysis and LOH evaluation were performed as previously described [3].

2.6. Contamination assay

To assess the possibility of contamination of biopsy material with adjacent normal cells, we mixed 200 ng of total DNA from samples known to be homozygous for either *p53Pro* or *p53Arg* at ratios 95:5 and 5:95. PCR was carried out as described above and it was shown that the minor DNA component was amplified significantly less than the major component.

2.7. Statistical analyses

The statistical analyses was performed with the package SPSS. Statistical significance was set at P -value < 0.05 .

3. Results

3.1. HPV analysis in laryngeal lesions

All specimens of laryngeal benign and malignant tumours were analyzed by PCR for the presence of HPV genome. The integrity of DNA was confirmed by the amplification of *β-globin* as a reference gene (data not shown). Using general primers we identified three out of 37 specimens infected with HPV (8%). Two were malignant and one was a benign tumour. Furthermore, we performed HPV typing using specific primers for HPV 11, 16, 18, 33, since these HPV types are of high risk and the commonest in Greek patients. Multiplex PCR revealed the presence of HPV 18 in all three HPV-related samples (Fig. 1).

3.2. *p53* codon 72 polymorphism

We studied a total of 77 individuals: 37 laryngeal benign and malignant lesions and 40 non-cancer controls. To analyze the *p53* codon 72 polymorphism, we used a PCR-based assay that specifically detects

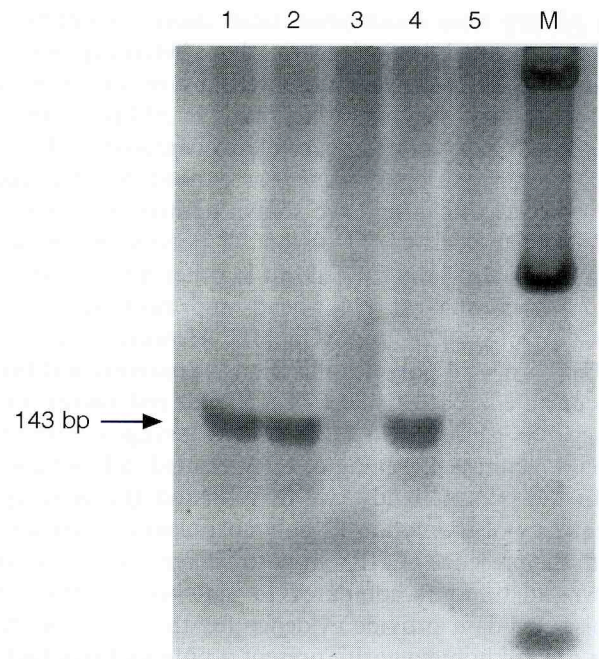


Fig. 1. Detection of human papilloma virus 18 type by PCR amplification (143 bp). Lanes 1, 2 and 4, positive samples; lanes 3 and 5, negative samples; M, molecular weight marker.

either the *p53Pro* or the *p53Arg* allele. The primer pair *p53+/Arg-* yields a PCR product of 141 bp of the arginine allele (Fig. 2) whereas the *p53+/Pro-* primer pair gives a DNA fragment of 177 bp of the proline allele (Fig. 3).

At the end of genetic analysis the code was broken, and we compared the distributions of *p53* genotypes as well as the frequencies of *p53Arg* and *p53Pro* alleles among benign laryngeal lesions, malignant tumours and the control group. As *p53* allele frequencies have been shown to vary according to ethnic group, we analyzed controls and patients from the same ethnic background. The results of the *p53* codon 72 polymorphism in benign and malignant laryngeal tumours as well as in non-cancerous samples are summarized in Table 1. The overall distribution of the *p53* genotypes between laryngeal lesions (both benign and malignant tumours) and control samples was statistically significant ($P < 0.001$). Statistically significant correlation was found between the distribution of *p53* genotypes among healthy and malignant samples ($P = 0.016$) and among healthy and benign laryngeal specimens ($P < 0.001$). On the contrary no statistical association was observed between the distribution of *p53* genotypes among benign and malignant samples ($P = 0.197$). We also searched for statistical correlation between the genotype *Arg/Arg* versus *Arg/Pro* plus *Pro/Pro* genotypes and observed that there was an association among normal and benign samples ($P < 0.004$), among normal and malignant samples ($P = 0.01$) and among normal and both benign and malignant samples ($P < 0.0002$), whereas the same comparison

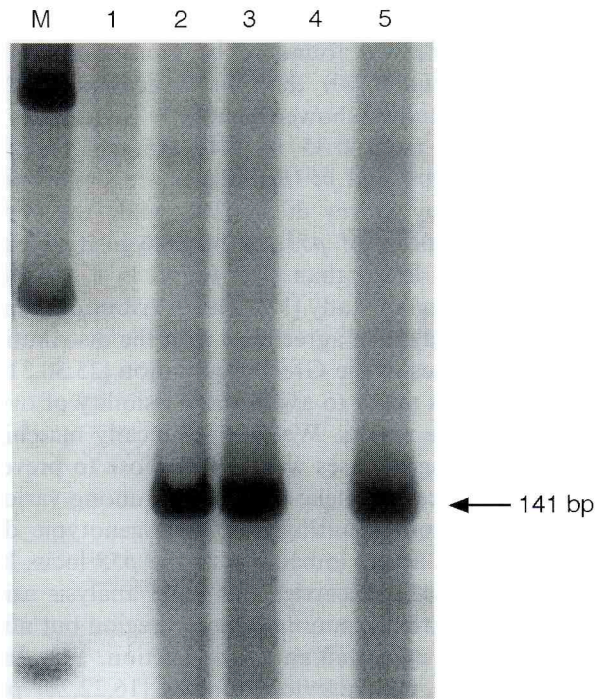


Fig. 2. *p53Arg* allele amplification employing PCR (141 bp). Products were electrophoresed through a 10% polyacrylamide gel and silver stained. Lanes 2 and 3, positive samples; lanes 1 and 4, negative samples; M, molecular weight marker.

between benign and malignant specimens did not reveal any statistically significant association ($P=0.209$).

We found a marked difference in the frequency of *p53Pro* and *p53Arg* alleles between laryngeal specimens and healthy controls. *p53Arg* allele was detected more frequently in laryngeal lesions, compared to controls ($P<0.0003$). The comparison in *p53* allele frequencies between benign and malignant tumours individually to the frequency of control samples revealed statistical significance in both cases, $P<0.0004$ and $P<0.016$, respectively. On the contrary, no statistically significant association was found between the distribution of *Arg/Pro* alleles in laryngeal benign and malignant specimens.

Since LOH on *p53* region has been reported in laryngeal tumours [35] the frequency of our laryngeal patients homozygous for *p53Pro* or *Arg* may be over-estimated. We performed microsatellite analysis to determine the LOH status of the tumour DNA that were homozygous for *p53Pro* or *p53Arg* using three microsatellite markers, D17S515, TP53 and D17S678. None of the specimens tested (all homozygous for *p53Arg*) exhibited LOH indicating that this is not an important mechanism for over-representation of *p53Arg* genotypes in laryngeal lesions.

Biopsies of laryngeal cytological specimens could possibly be contaminated with adjacent normal cells. To assess contamination of biopsy material, samples known to be homozygous for *p53Pro* or *p53Arg* were mixed at

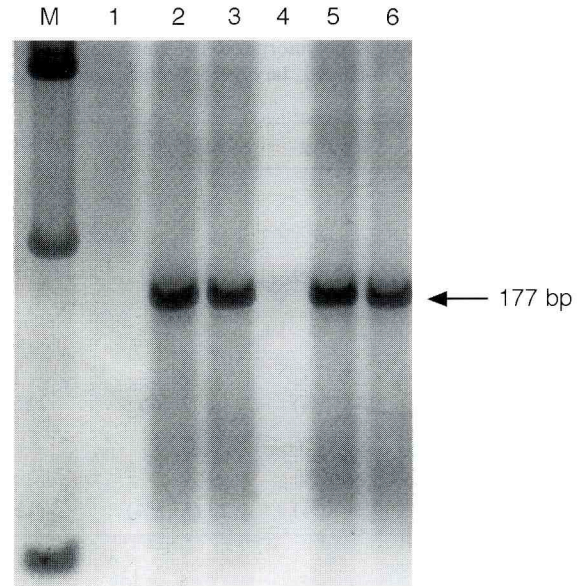


Fig. 3. *p53Pro* allele amplification employing PCR (177 bp). Products were electrophoresed through a 10% polyacrylamide gel and silver stained. Lanes 2, 3, 5 and 6, positive samples; lanes 1 and 4, negative samples; M, molecular weight marker.

ratios 95:5 and 5:95. In each case, the minor DNA *p53* allele was amplified much less than the major *p53* allele, indicating that even a low degree of contamination cannot affect the assessment of genotypes (data not shown). Finally, the accuracy of our polymorphism analysis in the laryngeal tumour patients was proved by performing the same genotyping assay not only in tumour specimens but also in the corresponding DNA from blood of the same patients. The genotype in both cytological material and blood samples was identical.

4. Discussion

The *p53* codon 72 polymorphism has been proposed as a risk factor mainly for the development of HPV related human cancers. In combination with data from in vitro assays indicating that the E6 protein of high risk HPV types can cause p53 degradation rather than those from low risk, it has been suggested that individuals carrying the arginine allele may be more susceptible to the development of invasive cervical carcinomas [16]. However, there is a considerable number of studies which failed to uncover any significant over-representation of the *p53Arg* genotype in tumour samples [17,19,20,22,36].

Our study was designed to investigate the importance of this *p53* genotype with respect to the risk of neoplasia development in Greek patients with benign and malignant laryngeal tumours. Furthermore, we searched for an association between *p53* alleles and the presence of HPV in the same series of samples. We found a

Table 1
Distribution of *p53* codon 72 polymorphism in human papilloma virus (HPV) -related and non-related laryngeal benign and malignant tumours

	Arg/Arg	Arg/Pro	Pro/Pro
<i>Laryngeal benign</i>			
HPV ⁺	1	–	–
HPV [–]	13	3	–
Total	14 (82%)	3 (18%)	–
<i>Laryngeal malignant</i>			
HPV ⁺	2	–	–
HPV [–]	11	6	1
Total	13 (65%)	6 (30%)	1 (5%)
<i>Control normal</i>			
HPV ⁺	–	–	–
HPV [–]	12 (30%)	23 (57.5%)	5 (12.5%)

significant statistical association in the distribution of *p53* genotypes between laryngeal lesions and normal samples. Homozygosity for *p53Arg* was detected in 73% of laryngeal lesions, compared to 30% of controls; the *Arg/Pro* genotype was found in 24% of the tumours compared to 57.5% of controls while only 3% of the tumour specimens were homozygous for *p53Pro* allele versus 12.5% of healthy controls. The *p53Arg* genotype was observed more frequently in benign and tumour samples even when they were compared individually to the normal group. On the contrary, no statistical significant association was found between the distribution of *Arg* and *Pro* alleles among laryngeal benign and malignant tumours. Similar correlations were observed when the frequencies of *p53Arg* and *p53Pro* alleles were compared to the corresponding frequencies in normal samples. The *p53Arg* allele was detected significantly more frequently in both benign and malignant tumours than in normal specimens. Our results provide evidence that the *p53* codon 72 polymorphism may be implicated at the early stages of the disease and concerns predisposition to premalignant laryngeal lesions rather than the progression from benign tumour toward malignancy. Moreover, considering the statistical difference between genotypes *Arg/Arg* and *Arg/Pro* but not between *Arg/Pro* and *Pro/Pro* genotypes among tumour and control samples, it is clear that it is the *p53Arg* homozygous genotype which affects the predisposition. The presence of the *p53Arg* allele itself in a heterozygous status is not sufficient to develop the tumour phenotype.

The genotype distribution of *p53* codon 72 polymorphism is significantly different among ethnic groups. Beckman et al., reported that there was a significant decrease in the frequency of *Pro* allele with increasing latitude, ranging from 0.63 in Africa Blacks to 0.17 in Swedish Saamis [37]. Weston et al., also reported that

the frequency of the *Pro* allele varied by ethnicity [38]. The *p53Pro* allele was found to be more common in African-Americans (0.50) than in Caucasians (0.29). Two Japanese studies showed genotype frequencies of *p53Pro* ranging from 0.35 to 0.40 [15,39]. The frequencies of *p53Arg* and *p53Pro* alleles are similar with those reported by Storey et al. [16], while the corresponding frequencies of *p53Arg* homozygosity in our control group is lower than that found in a Japanese [22] and a Norwegian study [17]. The distribution of *p53* alleles in our study is in agreement with the distribution of previous studies in the Greek population [25,30,31].

Great care was taken to avoid the possibility of overestimation of the results. We used ethnically matching controls to laryngeal cases with the purpose to prevent possible differences in allelic frequencies among various ethnic groups from resulting in biased genotypic distributions. Since allelic imbalance at the *p53* locus has been demonstrated, we carried out LOH analysis using microsatellite markers spanning the *p53* region but none of the specimens exhibited an allelic deletion. This finding is consistent with previous studies [16,22,26] presenting a low rate of LOH at this locus. Furthermore, in order to confirm the results of the *p53* codon 72 polymorphism we performed the same analysis in the corresponding normal DNA of the patients as well, obtaining identical genotypes while an assay was carried out to assess the possibility of tumour contamination by normal cells. Finally, the DNA of the samples examined was intact since it was extracted from fresh tissue and not from formalin-fixed tissue, excluding the possibility of poor DNA quality and the presence of PCR inhibitors.

HPV was detected in only three samples, one benign and two malignant tumours. HPV typing revealed type HPV-18. This finding is in agreement with previous studies in the Greek population [25,40,41] and has been attributed to ethnic variations of HPV types and the geographical location of Greece. Interestingly, all HPV positive samples were homozygous for *p53Arg* allele. The role of HPV in laryngeal carcinogenesis has not been demonstrated yet but the present findings support the idea that HPV is not a major oncogenic factor in the development of laryngeal tumours. However, the presence of viral proteins may have synergistic action with specific genotypes of *p53* gene, such as *p53Arg*, suggesting a model by which E6 oncoprotein eliminates the *p53* protein and leads to HPV-associated laryngeal tumours.

This is the first study in laryngeal tumours, indicating the difference in the frequency of the two *p53* alleles in both benign and malignant tumours and normal control samples and suggesting that the *p53Arg* allele, in the absence of *p53Pro* allele confers susceptibility to laryngeal tumours and may be implicated at early stages during oncogenesis. Further investigation is required to determine the influence of this *p53* polymorphism on

laryngeal carcinogenesis and its potential utility for the identification of patients at either low or high risk.

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