p53 Codon 72 Polymorphism Does Not Affect the Risk of Cervical Cancer in Patients from Northern Italy[1]

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Abstract

A case-control study was performed to investigate the risk of cervical cancer associated with *p53* polymorphism at codon 72, encoding either arginine or proline. It has been recently suggested that the arginine isoform increases the susceptibility to invasive cervical cancer; however, data remain controversial. The polymorphism was examined by both allele-specific PCR and RFLP analysis in 101 patients with primary cervical cancer and in 140 healthy women of the same age and from the same geographical area. The distribution of *p53* genotypes in cervical cancer patients and in controls was not significantly different ($P = 0.445$), and homozygosity for arginine at residue 72 was not associated with an increased risk for cervical cancer (odds ratio, 0.81; 95% confidence interval, 0.47–1.42; $P = 0.52$). Similarly, different genotype distribution and increased risk were not observed when patients *versus* controls were analyzed according to human papillomavirus status and cancer histotype. Therefore, no evidence of association between homozygosity for *p53* arginine and cervical cancer was found in our population sample.

Introduction

Certain HPV[3] genotypes are involved in the development of cervical cancer (1). HPV16 and HPV18 are the most frequently detected genotypes in invasive cervical carcinoma. Oncoprotein E6 of tumor-associated HPVs binds to tumor suppressor protein p53, inducing its degradation through the ubiquitin pathway (2, 3). A common *p53* polymorphism at codon 72, encoding either arginine or proline, has been shown to affect E6-mediated degradation of p53 *in vitro* (4). Moreover the arginine form of p53 has been associated with an increased risk of HPV-related cancer in humans by Storey *et al.* (4) and Zehbe *et al.* (5). This observation, however, remains controversial because it has not been confirmed in other studies on different populations (6–10).

To examine whether the arginine polymorphic variant of *p53* could represent a risk factor for cervical carcinoma in the Northern Italy population, we performed a retrospective study using both ASP and RFLP analysis.

Materials and Methods

Cases and Specimens

A total of 241 women, all born in the same geographical area, were investigated for the *p53* polymorphism at codon 72:101 subjects with primary cervical cancer (median age, 49.0 years; range, 32–67) and 140 healthy women of similar age (median age, 47.0 years; range, 30–64). The patients included 64 cases with primary cervical SCCs, and 37 cases with primary cervical AdCs. Eighty-six patients (75 cases with HPV-positive and 11 cases with HPV-negative tumors) were consecutively observed in a single gynecological unit between January 1995 and May 1998. The remaining 15 patients were retrieved from cases with HPV-negative tumors observed between 1990 and 1994. All of the patients underwent surgery, and the treatment consisted in type III hysterectomy of Piver with lymph node dissections. The *p53* polymorphism analysis was performed on formalin-fixed, paraffin-embedded normal lymph nodes. The controls, all of whom were women with normal Pap smear and no previous history of cervical dysplasia, were retrieved from 734 subjects who attended consecutively a family planning service between January 1995 and August 1996. Frozen cervicovaginal lavages were available for the *p53* polymorphism analysis of the controls. The HPV status of the patients and controls, ascertained at observation, was reanalyzed for the present study.

Analysis of p53 Codon 72 Polymorphic Alleles

DNA Extraction. DNA was extracted from formalin-fixed, paraffin-embedded nontumoral tissues (lymph nodes) of patients and from frozen cervicovaginal lavages of controls. One to three sections (depending on sample size) of formalin-fixed, paraffin-embedded tissue were incubated at 58°C overnight in 150 μl of extraction buffer [50 mM KCl, 10 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatine, 0.45% NP40, 0.45% TWEEN 20, and 0.5 mg/ml proteinase K]. Exfoliated cervicovaginal cells suspended in PBS solution were concentrated by low-speed centrifugation and incubated for 1 h at 56°C in 100 μl of extraction buffer. The solutions were heated at 95°C for 15 min to inactivate proteinase K and were subsequently centrifuged. One to 5 μl of digested material were used directly for enzymatic amplification by PCR.

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[3] The abbreviations used are: HPV, human papillomavirus; ASP, allele-specific PCR; SCC, squamous cell carcinoma; AdC, adenocarcinoma; OR, odds ratio; CI, confidence interval.

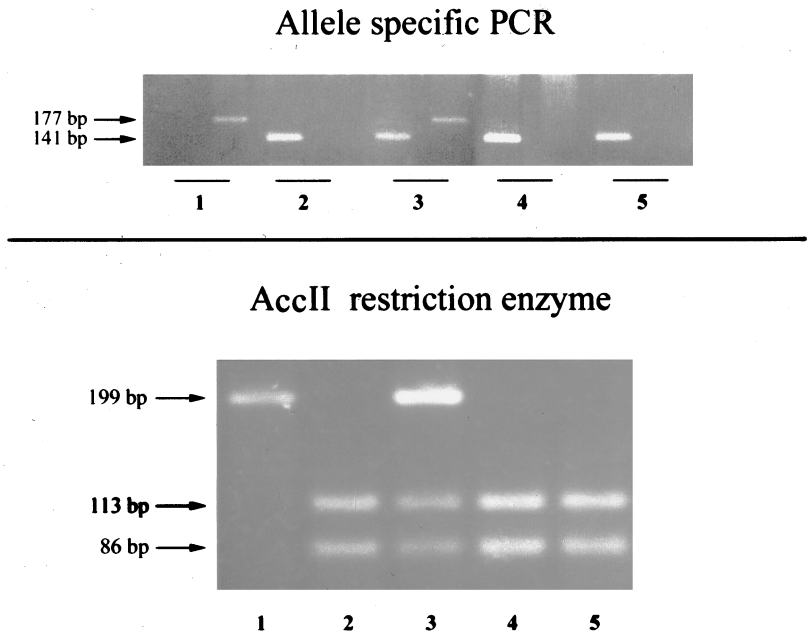

Fig. 1. Analysis of *p53* codon 72 polymorphism in five patients with cervical carcinoma. The same DNA samples were investigated by both ASP and RFLP analysis. The amplification of the *arginine* allele with primer pair $p53^+/Arg^-$ gives a PCR product of 141 bp; the primer pair $Pro^+/p53^-$ amplifies a 177-bp fragment corresponding to the *proline* allele. *Acc*II digestion of the PCR products, obtained with primers described by Ara *et al.* (11), identifies a fragment of 199 bp (proline) or two fragments of 113 bp + 86 bp (arginine). *Case 1, Pro/Pro*; *cases 2, 4,* and *5, Arg/Arg*; and *case 3, Arg/Pro*.

ASP. *p53* arginine and proline sequences were amplified in separate reactions with the primer pairs $p53^+/Arg^-$ (PCR product, 141 bp) and $p53Pro^+/p53^-$ (PCR product, 177 bp) described by Storey *et al.* (4). PCR was performed in a 50-μl volume that contained 30 ng of template DNA, 0.5 μM each primer, 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1 unit of Red Hot Taq polymerase (Advanced Biotechnologies). Cycling parameters were: 95°C for 30 sec, 62°C for 30 sec, and 72°C for 40 sec for 33 cycles with a final elongation at 72°C for 5 min (GeneAmp 9600 Thermal Cycler, Perkin Elmer). Reaction products (10 μl each) were analyzed by electrophoresis on a 3% Metaphor-1% Nusieve gel.

RFLP Analysis. Target sequences were amplified with the primers described by Ara *et al.* (11) in a 25-μl volume containing 30 ng of template DNA, 0.4 μM each primer, 200 μM dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 1.5 units of Taq polymerase (Dynazyme II Finnzymes). Cycling parameters were: 95°C for 2 min, 65°C for 2 min, 72°C for 2 min for 33 cycles with a final elongation at 72°C for 10 min (PTC-200, MJ Research). Twenty μl of the PCR product (199-bp fragment) were incubated overnight with 1 unit of *Acc*II restriction enzyme at 37°C and then were analyzed with electrophoresis on a 4% Nusieve gel. The base substitution causing modification of amino acid residue 72 from proline to arginine generates an *Acc*II cutting site; therefore, the enzymatic digestion of the arginine corresponding sequence yields two DNA fragments of 113 and 86 bp, respectively.

HPV DNA Analysis

All of the cases and controls were reanalyzed for the identification of HPV infection. DNA extraction, primers (consensus MY09/MY11 and type specific for HPV16 and -18), and PCR amplification procedures for the detection of HPV in formalin-fixed, paraffin-embedded cervical cancers and in cervicovaginal lavages were described in previous studies, in which the choice of primers and the strategy of amplification were also discussed (12, 13). In addition, the tumors that were HPV-negative and all of the cervicovaginal lavages were tested by using HPV general primers GP5+/GP6+ (14) not previously utilized.

Statistical Analysis

χ^2 analysis was used to examine differences in the proportions of the three *p53* codon 72 genotypes between cervical cancer patients and controls. Fisher correction was applied when appropriate. OR and 95% CI were used to compare categorical variables.

Results

The analysis of HPV infection from patients and controls confirmed previously obtained results (12, 13). In the cancer group, HPV16 or HPV18 DNA sequences were present in 75 cases (45 SCCs and 30 AdCs); HPV16 was found in 60 tumors (42 SCCs and 18 AdCs), and HPV18 was found in 15 tumors (3 SCCs and 12 AdCs). Twenty-six tumors (19 SCCs and 7 AdCs) were HPV-negative. In the control group, no HPV DNA sequences were detected by using consensus primers MY09/MY11. The amplification of negative tumors and cervicovaginal lavages with general primers GP5+/GP6+ failed to yield any additional HPV-positive cases.

The analysis of *p53* polymorphism was performed by both ASP and RFLP analysis in 230 cases (97 patients and 133 controls) and allowed the detection of the same results in all of the samples (Fig. 1). Eleven cases (4 patients and 7 controls) were classified only on the basis of ASP because PCR products suitable to RFLP analysis could not be obtained. *p53* genotypes and allele frequencies in patients and controls, as well as the distribution of HPV-status and cancer histotypes, are shown in Table 1. Allele frequencies were in Hardy-Weinberg equilibrium in all of the groups studied (Table 1). The distribution of p53 genotypes in cervical cancer patients and in controls was similar ($P = 0.445$) also when cases and healthy subjects were considered according to HPV-status (HPV-positive, $P = 0.285$; HPV-negative, $P = 0.532$) and cancer histotype (SCC, $P =$

Table 1 Genotypes and allele frequencies in 101 patients with cervical cancer and in 140 healthy controls

Samples	Total	Genotypes			*F-proline* allele	HW (*P*)[b]
		A/A[a]	*A/P*	*P/P*		
Controls	140	86	47	7	0.22	0.86
Patients	101	57	35	9	0.26	0.29
HPV^+	75	39	29	7	0.29	0.64
HPV^-	26	18	6	2	0.19	0.19
SCC	64	34	22	8	0.30	0.16
AdC	37	23	13	1	0.20	0.60

[a] F, Frequency; *A/A, Arg/Arg; A/P, Arg/Pro; P/P, Pro/Pro*.
[b] No deviation from Hardy-Weimberg (HW) equilibrium was observed for any group.

0.143; AC, $P = 0.833$). Similarly, homozygosity for arginine at residue 72 was not associated with an increased risk for cervical cancer (OR, 0.81; 95% CI, 0.47–1.42; $P = 0.52$) also for groups with different HPV-status (HPV-positive: OR, 0.68; 95% CI, 0.37–1.25; $P = 0.23$; HPV-negative: OR, 1.41; 95% CI, 0.54–4.02; $P = 0.6$) and cancer histotype (SCC: OR, 0.71; 95% CI, 0.38–1.35; $P = 0.36$; AdC: OR, 1.03; 95% CI, 0.46–2.37; $P = 1.0$).

Discussion

Storey *et al.* (4) showed that the codon 72 arginine variant of *p53* codes for a protein that is more sensitive to HPV16 and HPV18 degradation than the proline variant. These data were further supported by genotype analysis in a group of cervical cancer patients who showed a 7-fold enrichment of the arginine allele over the proline allele as compared to healthy controls (4). Moreover, Thomas *et al.* (15) have recently presented evidence that, *in vitro*, the wild-type p53 arginine variant induces apoptosis with faster kinetics and suppresses transformation more efficiently than the wild-type p53 proline variant. These observations may have implications for the development of cancer in subjects harboring wild-type *p53* sequences and for the responsiveness of tumors to therapy.

The functional differences between the two p53 isoforms described by Storey *et al.* (4) provoked a series of epidemiological studies to verify whether homozygous arginine at codon 72 was a candidate risk factor for cervical HPV-related cancer. In a recent study, Zehbe *et al.* (5) obtained results consistent with those of Storey *et al.* (4); however, the data from the original report were not confirmed by several other authors (6–10).

Our study was based on a large sample so as to give a reliable estimate of *p53* polymorphism and cervical cancer association, and it was designed to minimize sources of bias. Patients and controls were of similar age and were recruited from a homogeneous population born in a restricted geographical area. This population is characterized by a low prevalence of genital HPV infection, which is higher among younger women (13). Most of our controls were older than 35 years and without history of cervical dysplasia or genital warts, accounting for their HPV-negative status. Both cervical SCC and AdC were investigated as well as tumors with different HPV status. Most of the cases were consecutively observed in a single institution, and, among them, the prevalence of HPV-negative tumors was about 13%. Fifteen HPV-negative tumors were retrieved from a previous series; however, we believe that this potential bias should not have hampered the main conclusion of the study. Loss of heterozygosity at the 17p13 chromosomal region, harboring *p53*, has been reported in 15–22% of cervical cancer (16). Therefore, the genotype analysis was performed on normal tissues from patients and not on tumor samples to avoid the risk of overestimating the *p53* homozygosity. Moreover, two different methods were employed to test *p53* genotypes, because allele-specific assays may perform differently according to the source of DNA (formalin-fixed paraffin-embedded tissue or frozen cells).

Our data are comparable to those of most studies as to *p53* allele frequencies. We found no statistically significant differences in the distribution of *p53* genotypes between controls and patients, and homozygosity for arginine at residue 72 was not associated with an increased risk for cervical cancer. Similarly, a different genotype distribution and cancer risk were not observed when patients and controls were analyzed according to HPV status and cancer histotype. Therefore, we can conclude that no evidence of association between homozygosity for *p53* arginine and cervical cancer was present in our population sample.

The discrepancy between our results and those of Storey *et al.* (4) and Zehbe *et al.* (5) might be explained either by the small sample size or by the design of those studies. It should be noted that Joseffson *et al.* (see Ref. 10), by comparing the control group of Zehbe *et al.* (5) with a higher number of cases with cervical cancer, did not demonstrate any significant association.

It is well known that *p53* allele frequencies vary among ethnic groups (17). Because consistent epidemiological relationships between HPV infection and cervical cancer have been reported worldwide, it is crucial that investigations of *p53* polymorphism and risk of cancer should be extended to populations living at different latitudes to verify the reported association. In particular, epidemiological studies in populations living near the equator, who have a higher frequency of the proline variant (17), could help to identify subgroups of subjects at higher risk for cervical cancer.

References

1. Zur Hausen, H. Viruses in human cancers. Science (Washington DC), *254:* 1167–1173, 1991.

2. Scheffner, M., Werness, B. A., Huibregste, J. M., Levine, A. J., and Howley, P. M. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell, *63:* 1129–1136, 1990.

3. Werness, B. A., Levine, A. J., and Howley, P. M. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science (Washington DC), *248:* 76–79, 1990.

4. Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuers, J., Leigh, I. M., Matlashewski, G., and Banks, L. Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature (Lond.), *393:* 229–234, 1998.

5. Zehbe, I., Voglino, G., Wilander, E., Genta, F., Tommasino, M. Codon 72 polymorphism of *p53* and its association with cervical cancer. Lancet, *354:* 218–219, 1999.

6. Rosenthal, A. N., Ryan, A., Al-Jehani, R. M., Storey, A., Harwood, C. A., Jacobs, I. J. *p53* codon 72 polymorphism and risk of cervical cancer in UK. Lancet, *352:* 871–872, 1998.

7. Minaguchi, T., Kanamori, M. M., Matsushima, M., Yoshikawa, H., Taketani, Y., and Nakamura, Y. No evidence of correlation between polymorphism at codon 72 of *p53* and risk of cervical cancer in Japanese patients with human papillomavirus 16/18 infection. Cancer Res., *58:* 4585–4586, 1998.

8. Lanham, S., Cambell, I., Watt, P., and Gornall, R. *p53* polymorphism and risk of cervical cancer. Lancet, *352:* 1631, 1998.

9. Hayes, V. M., Hofstra, R. M. W., Buys, C. H. C. M., Hollerna, H., and van der Zee, A. G. J. Homozygous arginine-72 in wild-type p53 and risk of cervical cancer. Lancet, *352:* 1756, 1998.

10. Joseffson, A. M., Mangnusson, P. K. E., Ylitalo, N., Quarforth-Tubbin, P., Ponteacuten, J., Adami, H. O., and Gyllensten, U. p53 polymorphism and risk of cervical cancer (scientific correspondence). Nature (Lond.), *136:* 530–532, 1998.

11. Ara, S., Lee, P. S. Y., Hansen, M. F., and Saya, H. Codon 72 polymorphism of the *TP53* gene. Nucleic Acids Res., *18:* 4961, 1990.

12. Tenti, P., Romagnoli, S., Silini, E., Zappatore, R., Spinillo, A., Giunta, P., Cappellini, A., Vesentini, N., Zara, C., and Carnevali, L. Human papillomavirus types 16 and 18 infection in infiltrating adenocarcinoma of the cervix: PCR analysis of 138 cases and correlation with histologic type and grade. Am. J. Clin. Pathol., *106:* 52–56, 1996.

13. Tenti, P., Zappatore, R., Migliora, P., Spinillo, A., Maccarini, U., De Beneditis, M., Vesentini, N., Marchitelli, G., Silini, E., and Carnevali, L. Latent human papillomavirus infection in pregnant women at term: a case-control study. J. Infect. Dis., *176:* 277–280, 1997.

14. Van den Brule, A. J. C., Snijders, P. J. F., Gordijn, R. L. J., Bleker, O. P., Maijer, C. J. L. M., and Walboomers, J. M. M. General primer mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cervical scrapes and carcinomas. Int. J. Cancer, *45:* 644–649, 1990.

15. Thomas, M., Kalita, A., Labrecque, S., Pim, D., Banks, L., and Matlashewski, G. Two polymorphic variants of wild-type p53 differ biochemically and biologically. Mol. Cell. Biol., *19:* 1092–1100, 1999.

16. Mullokandov, M. R., Kholodilov, N. G., Atkin, N. B., Burk, R. D., Johnson, A. B., and Klinger, H. P. Genomic alterations in cervical carcinoma: losses of chromosome heterozygosity and human papilloma virus tumor status. Cancer Res., *56:* 197–205, 1996.

17. Beckman, G., Birgander, R., Sjalander, A., Saha, N., Holmberg, P. A., Kivela, A., and Beckman, L. Is p53 polymorphism maintained by natural selection? Hum. Hered., *44:* 266–270, 1994.