p53 Codon 72 Polymorphism and Various Human Papillomavirus 16 E6 Genotypes Are Risk Factors for Cervical Cancer Development[1]

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ABSTRACT

Risk factors other than human papillomavirus (HPV) infection *per se* for cervical cancer development have been investigated recently. It was suggested that HPV 16 E6 variants and the p53 codon 72 arginine polymorphism could be progression markers. Indeed, it has been demonstrated that specific E6 variants and p53 arginine were both enriched in cancer. However, especially with regard to the latter, divergent results have been reported. Our aim was thus to investigate whether p53 arginine is important for cervical carcinogenesis by scaling up samples of the two European cohorts, the initial results of which were reported previously. In addition, we have assessed the occurrence of p53 codon 72 arginine, in combination with specific HPV 16 *E6* genotypes. We found p53 arginine to be increased in cancer of both cohorts, consistent with our previous concept. Although specific *E6* genotypes increased gradually with the severity of the lesion, p53 arginine was enriched in cancer only. Moreover, the frequency of the arginine allele was similar in groups with different *E6* genotypes. It is concluded that p53 arginine is a risk factor for cervical cancer but probably acts independently of E6 variants.

INTRODUCTION

High-risk HPV[3] types are essential cofactors in cervical carcinogenesis (1). HPV 16 is the most common type, being present in ~50% of cervical cancers worldwide. The majority of high-risk HPV infections regress spontaneously, and only a fraction develops into a preinvasive or invasive lesion, indicating that additional factors are also involved in determining the fate of HPV 16 lesions. It has been proposed recently that two wild-type p53 forms, proline or arginine at the amino acid residue 72, might differently contribute to the development of ICC (2). Storey *et al.* (2) have shown that p53 arginine is more efficiently inactivated by the E6 oncoprotein of human papillomavirus than p53 proline. In the same study, the researchers characterized the p53 polymorphism in cervical cancer specimens from British women and showed that the arginine allele is 7-fold more represented than the proline allele. Our initial investigation of two different European cohorts as well as a Greek and a Brazilian study support these findings (3–5), although several other groups did not observe a significant association of p53 arginine with ICC (6–13).

Intratype HPV 16 variations may represent another potential risk factor for cervical cancer development. Several studies have shown that the HPV 16 genome is polymorphic when compared with the originally published sequence, the so-called prototype (14). The distribution of the different HPV polymorphisms is related to geograph-

ical regions (15). Genotypes of HPV 16 have been characterized as belonging to European, Asian, American-Asian, or African populations on the basis of their nucleotide sequences in the E6, L2, and L1 coding sequences and in the long control region (15). The requirement for a variant in the coding region has been defined as at least one nucleotide exchange leading to an amino acid change (16). The frequently detected European HPV 16 E6 350G variation, harboring an amino acid change at position 83 (leucine to valine) is associated with persistence in British women (17) and can be detected more frequently in cancer than the prototype in Swedish women (16). However, the potential oncogenicity of the L83V variant appears to be population related. Indeed, in Italy, this variant is not enriched in ICC in comparison with precursor lesions (18). Thus, this phenomenon may be explained by the fact that genetic differences between populations contribute to determine the risk of particular HPV 16 variations in disease progression.

The aim of this study was to investigate whether the p53 arginine allele confers a risk factor for cervical carcinogenesis by scaling up the sample size in the two European cohorts, the results of which were reported previously (3). In addition, we correlated the results of the p53 polymorphisms with previously obtained results of HPV 16 E6 genotypes (16).[4] Our data show that p53 arginine and specific *E6* genotypes each constitute a risk factor in cervical carcinogenesis in both cohorts. The frequency of p53 arginine increased abruptly from HCIN to ICC, whereas the presence of specific *E6* genotypes gradually increased or decreased from LCIN to ICC. In addition, the percentage of p53 arginine in the cases of cancer remained the same, irrespective of the E6 genotype. It is concluded that p53 arginine confers a higher risk for cervical cancer independently of E6 variants.

MATERIALS AND METHODS

Clinical Specimens. We have undertaken a cross-sectional study of two independent European cohorts. The selection criteria for both cohorts were HPV 16-positive cervical lesions ranging from LCIN to ICC. The Swedish study material consisted of 170 HPV 16-positive Swedish women diagnosed as having LCIN ($n = 34$), HCIN ($n = 64$) or ICC of squamous cell origin ($n = 72$). The cases were collected between 1990 and 1999 at the Department of Genetics and Pathology, University Hospital, Uppsala, Sweden. Controls consisted of randomly selected 188 HPV-negative and cytologically normal Swedish women from the Uppsala County. The cervical specimens from Italy were collected at the Servizio di Anatomia Patologicala, Ospedale Sant'Anna, Turin, Italy between 1996 and 1998 using the same criteria as described above. The Italian study material consisted of 94 HPV 16-positive women diagnosed as having LCIN ($n = 21$), HCIN ($n = 26$), and ICC ($n = 47$) and 40 ethnically matched controls, *i.e.*, newborn female babies from Turin.

PCR. The p53 exon 4-specific PCR was performed with a hot start modification using the *Taq*Start antibody PT1576-1 (Clontech, Palo Alto, CA) with primers resulting in a 155-bp product, 5′-GACCCAGGTCCAGAT-GAAGCT-3′ and 5′-ACCGTAGCTGCCCTGGTAGGT-3′ (19). Forty amplification cycles were run in the GeneAmp PCR System 2400 with a 94°C denaturation step (1 min), a 62°C annealing step (1 min), and a 72°C extension

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[3] The abbreviations used are: HPV, human papillomavirus; ICC, invasive cervical cancer; HCIN, high-grade cervical intraepithelial neoplasia; LCIN, low-grade CIN; SSCP, single-strand conformational polymorphism; LOH, loss of heterozygosity; OR, odds ratio.

[4] I. Zehbe, G. Voglino, E. Wilander, and M. Tommasino, unpublished data.

step (2 min), including an initial denaturation step of 3 min and a final extension step of 7 min. The PCR mix contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 50 μM of each deoxynucleotide triphosphate, 0.5 μM of each primer, and 0.25 unit of Ampli*Taq* polymerase (PE; Applied Biosystems, Weiterstadt, Germany). PCR products were checked by ethidium bromide agarose gel electrophoresis. The HPV 16 E6-specific PCR and sequencing reaction were performed as described earlier (16).

Typing of p53 Codon 72 Polymorphisms with SSCP. To circumvent the problems involved in performing an allele-specific assay in formalin-fixed tissue, we used SSCP for healthy controls and cases. The benefit of SSCP is that in contrast to allele-specific PCR, it requires only one PCR amplification per sample. Mostly, tumor tissue had to be used for cases because no normal tissue or peripheral blood was available. For SSCP, the GeneGel Excel 12.5 kit was used as recommended by the supplier (Pharmacia Biotech, Uppsala, Sweden). PCR products were run on a GenePhor Electrophoresis Unit at 400 V, 25 mA, 12°C for 100 min. After separation, the 12.5% polyacrylamide gel was silver-stained in a Hoefer Automated Gel Stainer, together with the PlusOne DNA Silver Staining kit.

Microdissection. To exclude the possibility of LOH of p53, a laser microdissection technique was applied to 15 cases of ICC as described earlier (20) but with some modifications. Three paraffin sections of 10 μm were placed on GPET-membranes (Millipore, Eschborn, Germany), stretched by a ring, dried, and stained with H&E, and the selected areas were isolated by liquid laser. DNA extraction for PCR was subsequently performed according to protocols published previously (21). The laser dissection microscope (BTG Biotechnik, Munich, Germany) was equipped with a liquid laser (rhodamine 6G, 590 nm), a TV-camera, and a computer-assisted digitizer system.

Statistical Analysis. To determine the magnitude of differences of p53 polymorphisms with respect to healthy controls (reference) and lesion grade, ORs were calculated. Proportions of polymorphisms in controls and cases were compared, where appropriate, by the Fisher's exact test. The Cochran-Armitage test was used to assess a linear trend of rate of HPV 16 *E6* genotypes with the severity of the lesion. A result of $P < 0.05$ was judged significant, and a result of up to 0.1 was judged to be a borderline significance.

RESULTS

p53 Codon 72 Polymorphism Is Enriched in Invasive Cervical Tumors in Swedish and Italian Women. The *p53* genotyping was performed by SSCP (Fig. 1). The distribution of *p53* genotypes was similar in the healthy controls and both groups of precursor lesions, whereas the percentage of p53 arginine increased in ICC both in Swedish and in Italian women (Table 1). The calculation of the Hardy-Weinberg equilibrium was performed for both cohorts of controls. $P > 0.1$ was obtained by the Fisher's exact test when comparing the observed with the expected relative frequencies in the control groups and thus fits the Hardy-Weinberg equilibrium. The enrichment of the arginine allele in women with invasive tumors as compared with healthy controls was statistically significant in both groups ($P = 0.02$ for each group). However, the OR in the Italian group was 3 and in the Swedish group 2. Because of the larger sample size, the analysis of the Swedish cohort had a high statistical power and was
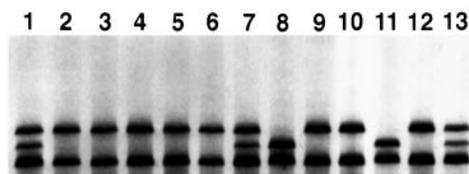

Fig. 1. Determination of p53 codon 72 polymorphisms by SSCP. After denaturing the PCR products, the conformational change patterns are distinct for the p53 proline and arginine homozygote as well as for the heterozygote. An example of 10 cases is illustrated in the figure. *Lanes 2–6, 9,* and *10,* p53 arginine homozygote; *Lane 8,* p53 proline homozygote; *Lanes 1* and *7,* p53 heterozygote. The pattern of the specific p53 polymorphism was determined by using cDNA comprising the 72 arginine or proline alone and in combination (*Lane 11,* p53 proline homozygote; *Lane 12,* p53 arginine homozygote; and *Lane 13,* p53 heterozygote).

Table 1 *Distribution of p53 codon 72 polymorphisms in Swedish and Italian women with HPV 16-positive cervical neoplasia and healthy controls*

	PP[a] (%)	AP (%)	AA (%)	OR (95% CI)[b]
Swedish women				
Controls	16/188 (9.0)	83/188 (44.0)	89/188 (47.0)	1.0 (reference)
LCIN	1/30 (3.3)	14/30 (46.7)	15/30 (50.0)	1.1 (0.5–2.4)
HCIN	7/64 (10.9)	24/64 (37.5)	33/64 (51.6)	1.2 (0.7–2.1)
ICC	4/72 (5.6)	22/72 (30.6)	46/72 (63.9)	2.0 (1.1–3.4)[c]
Italian women				
Controls	1/40 (2.5)	18/40 (45.0)	21/40 (52.5)	1.0 (reference)
LCIN	2/11 (18.0)	3/11 (27.3)	6/11 (54.5)	1.1 (0.3–4.1)
HCIN	1/26 (3.8)	12/26 (46.2)	13/26 (50.0)	0.9 (0.3–2.4)
ICC	3/43 (7.0)	7/43 (16.3)	33/43 (76.7)	3.0 (1.1–7.6)[c]

[a] PP/AA, proline/arginine homozygotes; AP, heterozygotes.
[b] ORs for each diagnostic category in relation to the healthy controls are given with confidence intervals (CI) in parentheses.
[c] For both groups, $P = 0.02$ was obtained by Fisher's exact test when comparing controls with cases of ICC.

Table 2 *Preinvasive and invasive cervical lesions of Swedish and Italian women*

Prevalence of HPV 16 E6 prototype and HPV 16 E6 L83V of Swedish women

	LCIN[a]	HCIN[b]	ICC[c]
Prototype	21/34 (61.8)	27/61 (44.3)	15/64 (23.4)
L83V	10/34 (29.4)	24/61 (39.3)	36/64 (56.3)
Other variants[d]	3/34 (8.8)	10/61 (16.4)	13/64 (20.3)[e]

Prevalence of HPV 16 E6 L83V and other HPV 16 E6 variants of Italian women

	LCIN[a]	HCIN[b]	ICC[c]
Prototype	2/21 (9.5)	6/23 (26.1)	6/47 (12.8)
L83V	17/21 (81.0)	17/23 (73.9)	26/47 (55.3)
Other variants[d]	2/21 (9.5)	0	15/47 (31.9)[e]

[a] For 4 cases of LCIN in the Swedish cohort and for 10 cases of LCIN in the Italian cohort, no p53 data were available.
[b] For 3 cases of HCIN in the Swedish and in the Italian cohort, no E6 data were available.
[c] For 8 cases of ICC in the Swedish cohort, no E6 data were available, and for 4 cases of ICC in the Italian cohort, no p53 data were available.
[d] Other variants denote: African-like and American-Asian multivariants (15, 18), Q3P, R10G, K11T, H24Q, D25N, N85S, L72G, Q3E/L83V, R10G/L83V, I27R/L83V, R55K/L83V, Y60F/L83V, and R10G/D25N/L83V in the Swedish cohort, as well as African-like multivariants (15, 18), H24Y, R48W, R115K, and R10G/L83V in the Italian cohort.
[e] The Cochran-Armitage test for a linear trend resulted in $P = 0.0001$ for the Swedish cohort when comparing cases of prototype with cases of L83V/other variants and $P = 0.002$ for the Italian cohort when comparing cases of L83V with cases of other variants.

therefore able to detect a statistically significant result at an OR of 2.0 in contrast with the Italian cohort, where an OR of 3.0 was required for the same *P*. Only borderline significance ($P = 0.1$) was obtained between ICC and CIN (L/HCIN combined) in the Swedish cohort, although the percentage is very close in healthy controls and in CIN. In contrast, when comparing cases of ICC with healthy controls or cases of CIN in the Italian group, a statistically significant result ($P = 0.02$) was obtained. Next, we analyzed microdissected normal tissue and tumor biopsy material in 15 cases of ICC from the Swedish group to exclude LOH, which may give biased allele frequencies of p53 codon 72 genotypes. The *p53* genotype of the normal tissue was then compared with the *p53* genotype of the tumor tissue and found to be identical in the same patient of all cases tested. These results confirm the notion that p53 codon 72 arginine homozygosity is a risk factor for developing an invasive cervical lesion and that the increase of the arginine allele in invasive tumors is not caused by LOH.

p53 Codon 72 Arginine and HPV 16 E6 Variants Are Independent Risk Factors for Cervical Cancer Development. Subsequent analysis was designed to determine a possible relationship between p53 codon 72 polymorphisms and HPV 16 *E6* genotypes. The distribution frequency of the various HPV 16 *E6* genotypes either decreased or increased with the severity of the lesion in both study groups (Table 2). In the Swedish cohort, the E6 prototype decreased,

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Table 3 *Correlations with p53 codon 72 polymorphisms in invasive cervical lesions of Swedish and Italian women*

Correlation of HPV 16 E6 prototype and HPV 16 E6 variants of Swedish women[a]		
	PP/AP[b] (%)	AA (%)
ICC/Prototype	6/15 (40.0)	9/15 (60.0)
ICC/E6 variants	19/49 (38.8)	30/49 (61.2)

Correlation of HPV 16 E6 L83V and HPV 16 E6 other variants of Italian women		
	PP/AP[b] (%)	AA (%)
ICC/L83V	5/26 (19.2)	21/26 (80.8)
ICC/Other variants[c]	4/11 (36.4)	7/11 (63.6)

[a] For 8 cases of ICC in the Swedish cohort, no E6 data were available. The p53 genotypes detected in these cases were AA ($n = 7$) and AP ($n = 1$).

[b] PP/AA, proline/arginine homozygotes; AP, heterozygotes.

[c] Other variants denote African-like and American-Asian multivariants (15, 18), H24Y and R10G/L83V.

and all types of variants increased with the severity of the lesion ($P = 0.0001$). A certain E6 variation at nucleotide 350, leading to an amino acid change from a leucine to a valine at residue 83 (denoted L83V), was the most common variant. In the Italian cohort, L83V was the most frequent variant in LCIN, and its prevalence decreased with the severity of the lesion. Instead, other E6 variants, especially multivariants of the African and American-Asian genotype, increased with the severity of the lesion ($P = 0.002$). We reason from the data described above that the p53 codon 72 arginine and specific *E6* genotypes constitute independent factors increasing the risk of cervical cancer in both cohorts (Tables 1 and 2). In both study groups, the frequency of p53 arginine increased abruptly from HCIN to ICC, whereas specific E6 genotypes increased or decreased gradually from LCIN to ICC. For the simultaneous analysis of p53 and E6 genotypes, the tumor samples were classified as negatively or positively associated *E6* genotypes. Therefore, the Swedish cohort was divided into two groups including all cases of E6 prototype or all cases of E6 variants, whereas in the Italian cohort, the two groups contained all cases of E6 L83V or all cases of other E6 variants. The prototype did not show a linear trend in the Italian group (Table 2), and therefore, these samples were not considered for this analysis. In the Swedish cohort, the percentage of the cancer cases with arginine was similar, irrespective of the *E6* genotype (Table 3). In the Italian cohort, an enrichment of the arginine allele was noted when grouped with the negatively associated E6 L83V compared with other E6 variants, which are positively associated with progression. This difference was, however, statistically not significant (Table 3). Together, these results suggest that both risk factors are independent of each other.

DISCUSSION

This study provides evidence that p53 codon 72 arginine homozygosity constitutes a risk factor for the development of invasive cervical carcinoma. Specific HPV 16 *E6* genotypes have also been shown to be risk factors in this respect (16, 18). Because p53 arginine is only enriched in ICC, whereas the LCINs and HCINs have a similar proportion to the healthy controls, we conclude that p53 polymorphism plays a role only in the transformation of a HCIN into an invasive lesion. In contrast, specific *E6* genotypes gradually increase or decrease with the severity of the lesion. This may indicate that they are involved in determining the persistence of viral infection. The same frequency of p53 arginine was observed when grouping cases of cancer with specific *E6* genotypes in the Swedish cohort. The reason why the difference detected in the Italian cohort was not statistically significant might be the small sample size. Together, these data suggest that p53 arginine acts independently of E6 variations in cervical carcinogenesis.

The percentage of p53 codon 72 arginine homozygosity in Swedish women with ICC decreased from 73.3 to 63.9% and was thus less pronounced in the present study than in our previous investigation (3). However, the frequency of the arginine allele remained the same in our Italian study group (78%) also after scaling up the number of cases. In addition, a similarly high distribution (75%) of the arginine allele has been observed in HPV 16-positive adenocarcinomas of Swedish women.[5]

The discrepancy in the previous results regarding the enrichment of p53 arginine in cervical cancer has been under debate in the scientific community, and thus far, no consensus has been reached. Considering the functional *in vitro* data, which have shown that the arginine genotype is a better substrate for E6 than the proline genotype (2), it is not surprising to find an association of the arginine allele with cancer. In the meantime, further evidence, consistent with the concept that p53 arginine is a risk factor for cervical carcinogenesis, was reported in a Greek study and in a Brazilian study (4, 5). In addition, a Dutch study has shown that p53 arginine, together with the HPV 16 E6 prototype, conferred a higher risk for cancer development (22). The analysis of each factor separately failed to reveal such a risk. Interestingly, the percentages of p53 arginine in the HPV-negative controls and in the cases of cancer of the Dutch cohort were similar to the ones we have obtained in our Swedish cohort (Ref. 22; Table 2). Nevertheless, no statistical significance was observed in the Dutch study. This might be because of the fact that the size of the control group was smaller than the group of carcinoma cases. In the above-mentioned Brazilian study, it was demonstrated that interlaboratory differences may lead to underestimation or loss of the ability to detect an association between p53 arginine and cervical cancer (5).

In conclusion, several independent studies have now confirmed that p53 arginine represents a potential risk for cervical cancer development. Moreover, the functional data, which have not been challenged thus far, favor such an association. In addition, it has clearly been demonstrated that biases in study design could explain the divergence of the results (5).

ACKNOWLEDGMENTS

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