

The significance of *p53* codon 72 polymorphism for the development of cervical adenocarcinomas

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Summary Infection with the human papillomavirus is an important co-factor in the development of cervical carcinomas. Accordingly, HPV DNA is recognised in most of these tumours. Polymorphism of the *p53* gene, codon 72, is also considered a risk factor in the development of cervical carcinoma. However, this finding is contradicted by several observers. In the present investigation, 111 cases of adenocarcinoma of the cervix collected through the Swedish Cancer Registry and 188 controls (females with normal cytology at organised gynaecological screening) were analysed with regard to *p53*, codon 72, polymorphism using a PCR- and SSCP-based technique. In the controls, 9% showed pro/pro, 44% pro/arg and 47% arg/arg, whereas in the invasive adenocarcinomas, the corresponding figures were 0%, 29% and 71%, respectively. The difference was statistically significant ($P = 0.001$). HPV DNA was identified in 86 tumours (HPV 18 in 48, HPV 16 in 31 and HPV of unknown type in 7 cases) and 25 tumours were HPV negative. The *p53*, codon 72, genotypes observed in HPV-positive and HPV-negative cervical adenocarcinomas were not statistically different ($P = 0.690$). The results indicate that women homozygous for arg/arg in codon 72 of the *p53* gene are at an increased risk for the development of cervical adenocarcinomas. However, this genetic disposition seems to be unrelated to the HPV infection. © 2001 Cancer Research Campaign <http://www.bjcancer.com>

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Cervical carcinoma is the second most common type of cancer in females worldwide, with over 500 000 new cases diagnosed each year. However, the incidence is declining in developed countries owing to established cytological screening programmes.

In most cancer registries, close to 75% of the tumours are reported to be of the squamous type. Adeno- and adenosquamous carcinomas represent about 10–15% and invasive tumours with unspecified histology constitute the remaining 10–15% (Paloma et al, 1998).

It is now widely accepted that the human papillomavirus (HPV) is the major causal factor in most, if not all cervical cancers. This fact makes cervical cancer probably the most common virally caused cancer (Zur Hausen, 1991).

However, the prevalence of HPV in squamous carcinoma is almost 100% (Walboomers et al, 1999), but in cervical adenocarcinoma, it is only about 70%, according to recent reports (Iwasowa et al, 1996; Paloma Vizcaino et al, 1998).

HPV 16 is the most commonly detected genotype in pre-invasive and invasive squamous carcinoma (50–60%), followed by HPV 18 which is present in about 15% of the tumours (Zehbe and Wilander, 1997a). However, in cervical adenocarcinoma, HPV 18 is the predominating type of virus followed by HPV 16 (Tenti et al, 1996).

Molecular biological studies have revealed that a polymorphism at codon 72 of the human tumour-suppressor gene, *p53*, results in translation to either arginine or proline. The risk of the development of cervical cancer associated with the human papillomavirus is

considered higher for women homozygous for arginine than for those who are heterozygous. (Storey et al, 1998). However, this observation is both confirmed and contradicted by several groups (Hildesheim-A et al, 1998; Josefsson et al, 1998; Rosenthal et al, 1998; Zehbe et al, 1999; Agorastos et al, 2000; Makni et al, 2000). The aim of the present investigation was to examine polymorphism in *p53*, codon 72, in women with invasive cervical adenocarcinomas and to compare the results with those of previous studies performed on patients with pre-invasive and invasive cervical squamous carcinomas. Furthermore, we intended to examine whether the *p53* polymorphism was correlated with an HPV infection in the tumours.

MATERIAL AND METHODS

Tumour material

A collection of cervical adenocarcinomas was used for the study. Tumour blocks were obtained from different Departments of Pathology, with the aid of the Cancer Registry of the National Board of Health and Welfare in Sweden. The material has previously been described in detail (Andersson et al, 2001).

The original collection consisted of formalin-fixed (tumour blocks) from 180 women which were sectioned and stained with haematoxylin-eosin for light microscopic analysis. From the light microscopic examination and the women's medical records, 23 tumours were considered to be of non-cervical origin, 14 tumours were classified as squamous carcinomas and 9 as adenosquamous carcinomas. 131 relevant cases, selected after microscopic analysis and evaluation of the medical records of the women, were further used. However, due to a lack of sufficient material for DNA extraction or a negative β -globin test (see below) an additional

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20 cases had to be excluded. The remaining 111 cases were used for HPV tests and the examination of p53, codon 72, polymorphism.

DNA extraction

DNA was isolated in one 10- μ m thick section from each selected tissue block (Lungu et al, 1992). The sections were treated for 3 hours with 500 μ g ml⁻¹ proteinase K in 150 μ l digestion buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) at 65°C. The enzyme was then inactivated by boiling for 10 min.

HPV-testing

The HPV tests were performed as described earlier (Zehbe et al, 1996; Zehbe and Wilander, 1997). Briefly, the analyses were performed on extracted DNA (Lungu et al, 1992) obtained from sections of the paraffin blocks of which the preceding section had been used for morphological diagnosis. In the first sequence, the availability of tissue DNA was examined by performing a β -globin test (Saiki et al, 1988). Secondly, the extracted DNA was analysed with the SHARP Signal System (Digene Diagnostics, Beltsville, MD) (Manos et al, 1989; Zehbe and Wilander, 1997) using MY09/MY11 primers from the L1 gene of HPV.

Specimens showing HPV of a high-risk type were subjected to HPV typing (HPV of a low-risk type was not observed in our material). HPV DNA from the L1 region was amplified with GP5+/GP6+ primers and the amplicon products were HPV-typed by single-strand conformational polymorphism (SSCP). The method has previously been described in detail (De Roda Husman et al, 1995; Zehbe et al, 1996).

To optimise the HPV PCR analysis, all HPV-negative adenocarcinomas were subjected to a second HPV test using specific primers from the E6 genes of HPV 16 and 18 for the PCR reaction (Alemi et al, 1999) (Table 1). Thereafter, PCR products, indicating infection with HPV 16 or 18, were identified on agarose gels.

p53 polymorphism test

The p53 exon 4 PCR reaction was performed with two 21 bp primers (Table 1), and at an annealing temperature of 62°C resulting in a 155-bp reaction product.

Table 1 Primers used for PCR

Primer pair	Sequence 5'-3'	Position of gene	Length (bp)	Product size (bp)
β -globin/PCO4	CAACTTCATCCACGTTCCACC	54-73	20	268
β -globin/GH20	GAAGAGCCAAGGACAGGTAC	195-176	20	
HPV/MY09	CGTCCMARRGGAWACTGATC ^a	L1 (HPV6)	20	398
HPV/MY11 ^b	GCMCAGGGWCATAAAYAATGG ^a		20	
HPV/GP5+	TTTGTTACTGTGGTAGATACTAC	L1 (HPV16)	23	141
HPV/GP6+	GAAAAATAAACTGTAAATCATATTC	L1 (HPV16)	25	
HPV 16	CTAAAATTAGTGAGTATAGACATTA	E6	25	176
HPV 16	CCTTATATTATGGAATCTTTGC	E6	22	
HPV 18	ATGTTGCCTTAGGTCCATGCA	E6	21	570
HPV 18	ACCGAAAACGGTCGGGACC	E6	19	
p53, codon 72	GACCCAGGTCCAGATGAAGCT	Exon 4	21	155
p53, codon 72	ACCGTAGCTGCCCTGGTAGGT	Exon 4	21	

HPV, Human papillomavirus.

^aDegenerate code, M = A + C; R = A + G; W = A + T; X = G + C.

^bBiotinylated at its 5' end.

For p53 genotyping, single-strand conformation polymorphism (SSCP) was used, employing the Gene Gel Excel 12.5 kit (Pharmacia Biotech, Uppsala, Sweden). The 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 Plus One DNA Silver Staining kit (Figure 1).

DNA preparations obtained from cytobrush specimens in the 188 normal females (25-60 years old), participating in the organised gynaecological screening in the county of Uppsala, were used as controls in the p53, codon 72 genotype study.

Statistical analysis

The χ^2 test for trend was used for the statistical comparison of the proportions (Breslow and Day, 1980).

RESULTS

Of the 131 adenocarcinomas collected for the study, a successful β -globin test and a p53, codon 72 analysis were obtained in 111 tumours. They were of the papillary or glandular type and lacked a squamous component.

HPV analysis

By using the MY09/MY11 primers from the HPV L1 gene for the PCR reaction and the SHARP Signal System, 77/111 (69%) of the tumours were determined HPV positive. With the application of the specific HPV 16 E6 and HPV 18 E6 primers on the remaining HPV negative tumours, an additional 9 cases 86/111 (77%) showed HPV DNA, of which HPV 16 was identified in 4, and HPV 18 in 5 tumours, respectively. In the HPV positive adenocarcinomas, 48/86 (43%) were infected with HPV 18, 31/86 (31%) with HPV 16 and the remaining 7/86 (7%) with other or unknown types of HPV. 25 (23%) cases were HPV negative (Table 2).

p53 analysis

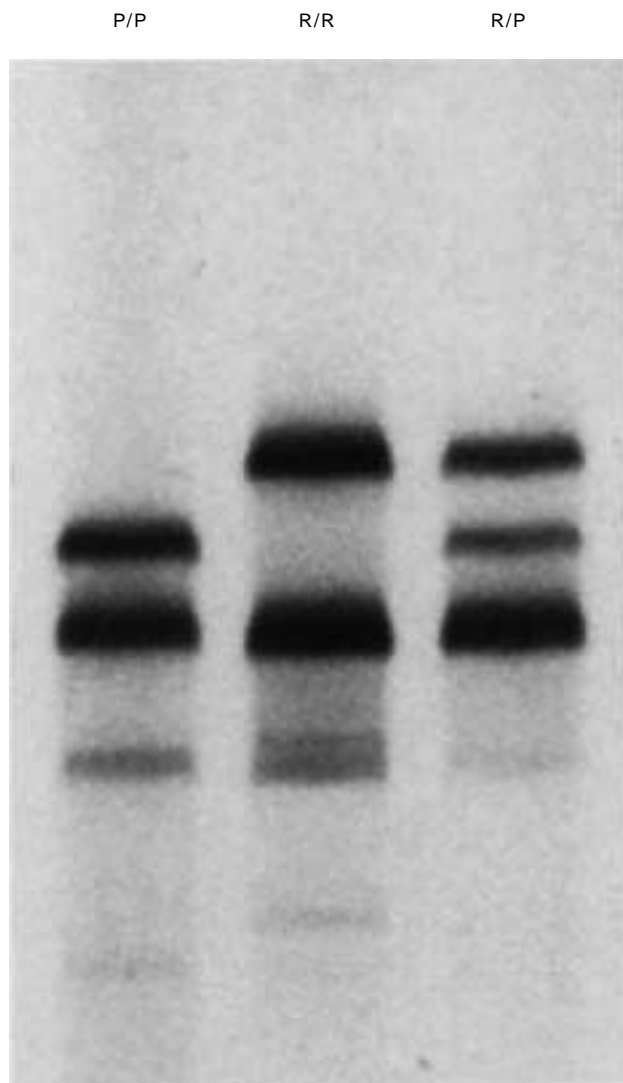
The 3 p53, codon 72, genotypes arg/arg, arg/pro and pro/pro all gave divergent band patterns with the PCR-SSCP analysis as illustrated in Figure 1.

Table 2 Prevalence of various genital types of HPV identified in the cervical adenocarcinomas

Cervical adenocarcinomas	Number of cases	(%)
HPV-18 pos	48	43
HPV-16 pos	31	28
HPV pos, other types	7	6
HPV neg	25	23
Total	111	

Table 3 p53, codon 72, genotypic distribution in normal controls and HPV positive and negative cervical adenocarcinomas

	Pro/Pro (%)	Pro/Arg (%)	Arg/Arg (%)	Total
Normal controls	16 (9)	83 (44)	89 (47)	188
Adenocarcinomas				
HPV-18 pos	0 (0)	15 (31)	33 (69)	48
HPV-16 pos	0 (0)	7 (23)	24 (77)	31
HPV-X pos	0 (0)	2 (29)	5 (71)	7
HPV neg	0	8 (32)	17 (68)	25
Total	0	32 (29)	79 (71)	111

**Figure 1** PCR-SSCP genotyping of the p53 codon 72 polymorphism. The lanes show women homozygotic for pro/pro (lane 1), heterozygotic for arg/pro (lane 3) and homozygotic for arg/arg (lane 2), respectively

In the 188 normal female controls, the distribution of genotypes was arg/arg 47%, arg/pro 44% and pro/pro 9%. The corresponding figures for the cervical adenocarcinomas were arg/arg 71%, arg/pro 29% and pro/pro 0%. The difference between normal controls and women with cervical adenocarcinomas was statistically highly significant ($P < 0.001$). However, the small difference obtained between HPV-positive and HPV-negative tumours was not statistically significant ($P = 0.690$) (Table 3).

DISCUSSION

The prevalence of HPV observed and the distribution of the different types of HPV that we identified in the cervical adenocarcinomas are in general agreement with previous observations of these tumours (Iwasawa et al, 1996; Paloma Vizcaino et al, 1998). These findings emphasise the existence of some pathogenetic difference in comparison to the cervical squamous carcinomas, which are almost always HPV positive and where HPV 16 predominates over HPV 18. Recently, it was shown that women with HPV positive adenocarcinomas are younger than those with HPV negative adenocarcinomas (Tenti et al, 1996; Andersson et al, 2001). Moreover, this observation indicates that carcinogenic factors other than HPV are involved in the development of at least some cervical adenocarcinomas.

Since the original report by Storey et al (Storey et al, 1998) that women with the p53 codon 72 arg/arg genotype are at an increased risk of developing cervical carcinomas, a series of publications has occurred dealing with this enigma. The results of several investigations are concordant with those of Storey et al (Storey et al, 1998; Zehbe et al, 1999), whereas several others are unable to support the finding (Minaguchi et al, 1998; Klaes et al, 1999; Brooks et al, 2000). All these studies are performed on squamous carcinomas or tumours without defined histological typing.

In the present report of pure cervical adenocarcinomas, we found a significant increase ($P = 0.001$), of the arg/arg in comparison with normal controls. Thus, in Swedish women, adenocarcinomas and squamous carcinomas of the cervix seem to manifest identical behaviour, since in a previous investigation in Sweden, women with the arg/arg genotype were correlated to an increased risk of developing squamous carcinoma (Zehbe et al, 1999). The distribution of p53 codon 72 genotypes, which we found in the normal female population, is equivalent to that observed in a previous study of Swedish women (Zehbe et al, 1999). However, the prevalence of the different p53, codon 72, genotypes is unequally distributed in several countries and therefore the importance of the arg/arg genotype in the development of cervical cancer is of variable significance (Tenti et al, 1998). Furthermore, since the p53 polymorphism was not distributed differently among HPV-positive and -negative adenocarcinomas, it is suggested that the increased risk of cervical adenocarcinoma in arg/arg women is not directly related to the HPV infection. Our results further support the opinion that polymorphism in the human genome, at a critical site, can affect the rate of incidence of cancer (Rosenthal et al, 1998; Storey et al, 1998). In addition, single-point mutations in the DNA of oncogenic types of HPV can have a corresponding biological potential. For instance, an amino acid shift in codon 83 of the E6 protein of HPV 16 Leu < Val seems to increase both the persistence

of infection and the malignant properties of the virus in Swedish women (Zehbe et al, 1998; Andersson et al, 2000).

Apparently, the interplay between the human genotype and oncogenic variants of HPV are of importance for the risk of the development of cervical cancer. However, the evaluation of this interplay is complicated by the normal variation of the human genome in different geographic regions and by an uneven distribution of the types and variants of HPV in the world (Koutsky, 1997). The introduction of gynaecological screening, about 30 years ago, has been successful in reducing the rate of incidence of squamous carcinoma of the cervix in the developed countries (Möhlcck et al, 1994; Bergström et al, 1999). However, during the same time period, the rate of incidence of adenocarcinoma of the cervix has increased, especially in younger women (Miller et al, 1993). Evidently, cytological screening has not been able to reduce the incidence of adenocarcinoma. The introduction of HPV testing, an increased knowledge of the importance of polymorphism in the human genome for the development of tumours and continued research on the oncogenic properties of the different types and variants of HPV are considered as instrumental in reducing the incidence of cervical adenocarcinoma.

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