

Abnormal expression and mutation of p53 in cervical cancer—a study at protein, RNA and DNA levels

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Objectives: The objectives of this study are to document the status of p53 expression and mutation in cervical cancer at protein, RNA and DNA levels and to relate this to the presence of HPV.

Materials and methods: Biopsy specimens from one hundred and three squamous cell carcinoma of the cervix and histologically normal ectocervix were analysed. Fresh tissues were extracted for protein, RNA and DNA and flash frozen tissue cryostat sectioned for immunohistochemical staining. HPV DNA status was determined by PCR using L1 consensus primers and typed for HPV 16 and 18 with E6 specific primers. p53 expression was determined at the protein level by Western blotting on protein extracts and at RNA level by Northern blotting.

Results: There was no p53 overexpression or mutation detectable in the protein extracts. Three of 65 (4.6%) of the carcinomas were positive for p53 by immunostaining with the polyclonal antibody CM1. Overexpression at the RNA level was detected in 2 of 32 (6.3%) carcinomas. p53 mutation was screened for by PCR/SSCP (single strand conformation polymorphism) followed by sequencing to define the site of mutation. Two of the cervical cancers (2.0%) showed mutation in p53 in exons 7 or 8. The mutation rate in HPV positive tumours was 1.2% (1/81) and in HPV negative tumours was 5.2% (1/19).

Conclusion: p53 overexpression or mutation does not seem to play a significant role in cervical carcinomas.

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Keywords: p53; RNA; mutation; cervical cancer; HPV

Introduction

There is a strong association between infection with certain types of genital human papillomaviruses (HPVs) and the subsequent development of cervical cancer.¹ The evidence supporting this association has been comprehensively reviewed recently² and HPV types 16 and 18 are considered to be human carcinogens. In the majority of cervical carcinomas viral DNA sequences are integrated into the host genome³ resulting in deregulation of the viral oncogenes E6 and E7. The E6 oncoprotein of the high risk types has been shown in *in vitro* studies to bind to the p53 protein and degrade it via the ubiquitin pathway.⁴ These and other observations suggest that p53 inactivation in HPV containing cervical cancers is effected via E6 binding and implies that p53 mutation is unlikely to play a role in malignant progression in the cervix. Previous studies indicated that p53 mutation in cervical cancer was confined to HPV negative tumours^{5,6} but subsequent studies were unable to confirm this.⁷⁻⁹

In a previous study¹⁰ on a predominantly Chinese population we were unable to correlate abnormal p53 protein expression as determined by immunochemistry with the absence of HPV DNA. Immunohistochemical staining can only detect overexpression of p53 protein, and abnormalities of p53 expression may be at the DNA, RNA or protein level. To document the status of p53 expression in cervical cancers more completely we have extended our study to examine p53 mutation, RNA and protein

expression in 100 cervical carcinomas and the relationship of this to HPV presence.

Materials and methods

One hundred biopsy specimens of squamous cell carcinoma of the cervix were collected in the Department of Obstetrics and Gynaecology, University of Hong Kong over the period January 1990 to March 1994. Ninety one patients were Chinese and the remainder were predominantly Filipino. Specimens were taken from 50 histologically and cytologically normal cervixes from hysterectomies performed for benign uterine or ovarian disease. All tissues were stored in liquid nitrogen until analysis.

Abnormal p53 protein expression was studied using Western blotting on protein extracts as well as by immunohistochemical staining of frozen sections of tissue samples. Overexpression of RNA was studied using Northern blotting followed by hybridisation with labelled p53 probe. DNAs extracted from tissue samples were subjected to polymerase chain reaction (PCR)/Southern blot analysis to detect HPV sequences. P53 mutation was screened for using PCR/single strand conformation polymorphism (SSCP) followed by DNA sequencing to define the site of mutation. Since the amount of tissue obtained by biopsy was limited, not all samples were extracted for protein, RNA and DNA or sectioned. The priority of tissue extraction was

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DNA (n = 100) for HPV and PCR/SSCP studies first, then frozen sections (n = 65) for immunohistochemical staining (IHS), then protein extraction (n = 64) for Western blot and lastly RNA extraction (n = 32) for Northern blot.

HPV detection and typing

The procedure used was essentially that described previously.¹⁰ Briefly, DNA was extracted and subjected to three PCR assays using consensus primers for HPV L1 and specific primers for HPV 16 and HPV 18 E6. PCR products were run on 4% agarose gels and blotted onto nylon membranes. DNA from Caski and Hela was used as positive controls for HPV 16 and 18 respectively and DNA derived from the C33 cell line and water were negative controls.

Protein extraction and Western blotting

The tissue sample was crushed in the frozen state mechanically, transferred to 1.5 ml of buffer and stirred on ice for 30 minutes. After centrifugation at 10 000 g at 4°C for 30 minutes, the supernatant was collected and the protein concentration determined. 40 µg of protein extract (after boiling) was loaded on a 10% acrylamide gel which was then run at 60 mA with a cooling device. The membrane was rinsed and put in 5% non-fat milk powder in TBST (Tris buffered saline and Tween 20) blocking buffer overnight at 4°C. The monoclonal antibody p1801 (p53 Ab2) (Oncogene Science Inc) at 1:2000 dilution in the blocking buffer, was added to the membrane and left for one hour at room temperature. The membrane was washed with blocking buffer three times, 15 minutes each wash. Horse radish peroxidase labelled second antibody (sheep anti-mouse immunoglobulin, Amersham) at 1:5000 dilution in blocking buffer, was added and left for one hour. Washing was repeated twice with blocking buffer and twice with TBS (Tris buffered saline), 15 minutes each. ECL (enhanced chemical luminescence, Amersham) reagents were added and the membrane exposed to Hyperfilm-ECL for 1, 5, 10 and 20 minutes after which films were developed. Protein extracted from C33 cells which contain a mutant p53^S was used as a positive control and protein extract from Caski cells which contain wild type (wt) p53 was used as a negative control.

Immunohistochemical staining

Frozen sections of 6 µm thickness were cut and mounted on APES (3-aminopropyl triethoxysilane) coated slides. Details of the immunohistochemical staining (IHS) and the results of 55 samples have been reported previously.¹⁰ The polyclonal antibody, CM1 raised against a full-length recombinant generated human p53 protein (Novocastra Lab Ltd, UK), was used at a 1:2400 dilution. Immunostaining was performed using the ABC immunoperoxidase method (Vectastain ABC kit, Vector Laboratories, USA). Positive and negative controls were included in each IHS assay.

RNA Northern blot

Total RNA was extracted using the guanidium/phenol method.¹¹ RNA (20 µg) was run on a 1% formaldehyde agarose gel and blotted by alkali transfer to a cellulose membrane. Hybridisation using ³²P labelled p53 probes was performed. After stripping the membrane of p53 probe, re-hybridisation using a labelled 18S RNA probe was performed to quantitate the amount of RNA loading. Measurement of radioactivity was performed using a densitometer (Bio-Rad Model 620 video densitometer) corrected by the 18S loading factor. RNA extracted from C33 cells was used as positive control and that from Caski cells was used as a negative control. Samples with radioactivity two times stronger than that of C33 were considered as having overexpression of p53.

Screening of p53 mutation by PCR/SSCP

Exons 2 to 11 of the p53 gene were amplified by PCR according to published procedures for exons 5 to 8¹² and for exons 2–4, 9 to 11.¹³ Five pmol/µl each of the left and right PCR primers flanking the exons of the p53 gene was radiolabelled with 10 units of T4 polynucleotide kinase and 2 µl [gamma-³²P] ATP (5000 Ci/mmol, Amersham) in 10 µl of reaction mixture for 30 min at 37°C. Five units of TaqDNA Polymerase (Perkin-Elmer) were added subsequently to the diluted mixture containing 2.5 mM MgCl₂ and 78 µM of dNTP. Four µl of this mixture were then mixed with 100 ng of genomic DNA in a total volume of 5 µl. The PCR was carried out in a DNA Thermal Cycler (Perkin-Elmer) under the following conditions: The template DNA was first denatured by incubation for five minutes at 95°C, followed by 40 cycles of PCR (30 s at 94°C for denaturation; one minute incubation at 60°C for annealing; 30 s at 72°C for polymerization and finally at 72°C for seven minutes). Formamide dye (45 µl) was added to stop the reaction. Two µl of the final product was loaded in an 8% polyacrylamide gel with 3% glycerol and electrophoresed at 2 Watt at room temperature overnight except for exon 4 at 30 Watt at 4°C for 5 hours. The gel was dried on Whatmann paper with a vacuum gel dryer and exposed to x-ray film at -70°C for 6–12 hours with an intensifying screen.

Direct cycle-sequencing of PCR product

Based on the results of the SSCP analysis, DNA bands that displayed a mobility shift in the SSCP were cut out. The DNA was extracted with 50 µl of water at 37°C overnight. Twenty µl of the eluted DNA were taken out and reamplified in a buffer containing 100 pmol of each primers, 4 mM MgCl₂, 200 µM of each dNTP and 5U of Taq-polymerase in a total volume of 100 µl. The PCR products were purified by running on a 5% acrylamide gel, extracted with phenol/chloroform and precipitated with two volumes of absolute alcohol. Purified DNA was resuspended in 20 µl of water and 2 µl was used for DNA sequencing according to the procedure as described by Mok *et al.*¹⁴ Either the 5' or 3'

Summary of abnormal IHS (immunohistochemical staining with CM1), RNA (p53 RNA Northern blotting and hybridisation study) and p53 DNA sequencing showing mutation and HPV PCR study in squamous cell carcinoma of the cervix

HPV 16 or 18 primer	HPV 16 or 18 or consensus primers	IHS	RNA	Mutation
-	-	na*	na	Exon 7
+	+	-	++	-
+	+	-	++	polymorphism
+	+	+	na	polymorphism
-	-	+	-	polymorphism
-	+	-	na	Exon 8
+	+	+	na	-

*na—insufficient material.

primer of the corresponding exon of the p53 gene was labelled with the [γ - 32 P]ATP by polynucleotide kinase. The Taq Cycle Sequencing kit (USB) was used under condition as described by the manufacturer. PCR was carried out as described above. The final product was run in a 6% sequencing gel. The gel was dried and exposed to x-ray film.

Results

HPV 16, 18 and consensus primers PCR and Southern blot

Only 1 (2%) of the 50 DNA samples extracted from normal cervical tissue showed amplified E6 sequences of HPV 16. No amplified sequence was detected in normal cervical tissue using HPV 18 E6 specific and HPV L1

consensus primers. Sixty two (62.0%) of 100 DNA samples extracted from cervical cancer showed amplified E6 sequences of HPV 16 and 40 (40.0%) showed amplified E6 sequences of HPV 18. HPV 16 or 18 was found in 74 (74.0%) cervical cancers. Both HPV 16 and HPV 18 were found in 28 (28.0%) cervical cancers. Using the HPV L1 consensus primers on the 26 HPV 16 and 18 negative samples, 19 samples showed no amplification. Thus, 19.0% of cervical cancer samples had no HPV DNA sequence detected using the above primers.

Western blot and immunoreaction

None of the 30 normal cervical protein extract showed positive immunoreaction with p53 Ab2 (p1801). Only three cervical cancer protein extracts showed a faint band on the ECL-hyperfilm. Strong immunoreaction was evidenced with the C33 protein extract. Thus, no specimen was considered as having overexpression.

Immunohistochemical staining

None of the 50 normal cervical sections showed positive staining with CM1. Only three of the 65 (4.6%) samples showed positive staining with CM1; none was found to have mutation by the PCR/SSCP analysis but two showed polymorphism in exon 4 or 8 (table).

RNA Northern blot

None of the RNA extracted from 10 normal cervical tissue samples hybridised with the p53 probe. Two tumour samples showed twice the intensity of signal compared with that of C33 after hybridisation with the p53 probe on the Northern blotting. Thus, two of the 32 (6.3%) tumour samples showed overexpression of p53 RNA and both were HPV positive. None of them showed positive IHS with the CM1 antibody or mutation in PCR-SSCP analysis but one showed polymorphism in exon 4 (table).

Screening of p53 mutation by PCR/SSCP and direct cycle-sequencing of PCR product

No shifted band was detected in 30 DNA samples from the normal cervical tissues. Shifted bands were only identified once in exons 7 and 8. Direct sequencing of the shifted bands confirmed mutations in codon 248 (CGG-CAG, arginine-glutamine) and codon 282 (CGC-CAC, arginine-histidine) (figs 1, 2). The sample with mutation in exon 7 was HPV negative and no IHS was performed because of insufficient sample. The sample with mutation in exon 8 was HPV 16 and 18 negative but positive with the L1 consensus primers. CM1 IHS was negative in this sample. Insufficient material were available for RNA analysis in the two samples (table). The mutation rate was 2.0%.

Figure 1 Missense mutation in exon 7, codon 248, CGG-CAG. N = normal control, T = cancer sample.

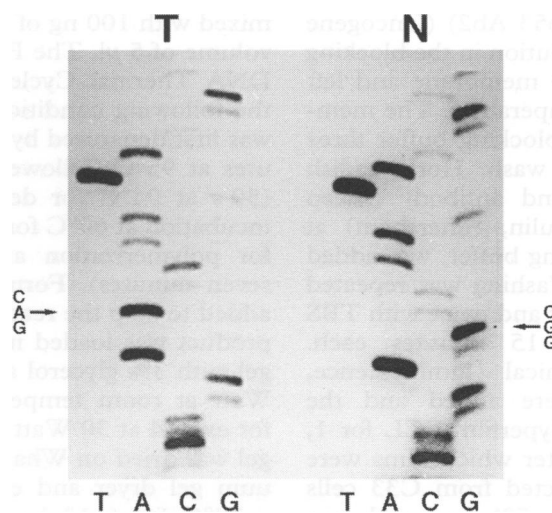
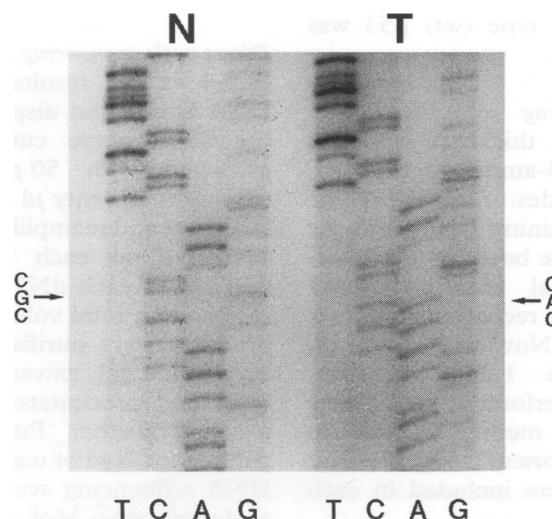


Figure 2 Missense mutation in exon 8, codon 283, CGC-CAC. N = normal control, T = cancer sample.



Discussion

In the present study HPV 16 or 18 E6 DNA sequences were detected in 74% of squamous cell carcinomas of the uterine cervix which

were analysed ($n = 100$), a frequency of detection in cervical cancer comparable to the majority of other studies.² HPV DNA was not detected using L1 consensus primers in 19.0% of cases ($n = 19$). Although it is claimed that these primers should detect at least 30 HPV types including all those known to infect the female genital tract,¹⁵ nonetheless the possibility that minor HPV types or HPV DNA fragments which did not encode the L1 sequences were present in the negative samples cannot be excluded. On the other hand, HPV E6 was detected in one of the normal cervical sample and L1 was not detected in the same sample. This could be due to the loss of the episomal form of the virus. Only 2% of normal cervixes examined in this study were positive for HPV DNA by PCR assay, an incidence of infection in this population comparable with that found in a previous study¹⁶ where 5% of cervical scrapes from pregnant women were HPV DNA positive by the Virapap method. However, the incidence of HPV infection in our population, as determined by the detection of HPV DNA sequences, does appear to be lower than that reported in the Caucasian population. Thus in studies using Virapap for HPV detection in cervical scrapes, an incidence of infection of 12.6% was found in a high risk group attending a local social hygiene clinic¹⁷ and 5% in normal pregnant women.¹⁶ However, the incidence of HPV DNA in cervical carcinoma in the present study is similar to that reported in Caucasians.

Since the E6 protein of the high risk HPVs binds to and degrades p53 via the ubiquitin pathway *in vitro*⁴ it has been assumed that this is the mechanism by which p53 is inactivated *in vivo* in HPV-containing tumours. This has been supported by studies in which p53 mutation has been detected by sequencing in HPV-negative tumours but wild type (wt) protein was present in HPV positive tumours.^{5,6} Mutant p53 protein has a longer half life and enhanced stability compared with the wt protein and can be detected by immunohistochemical staining on histological sections using appropriate antibodies. However, in the present study positively stained cells did not contain mutant p53 but contained wt protein. Mutation is not the only mechanism by which p53 protein stabilisation can be achieved and positive staining is not necessarily an indicator of mutant protein.^{18,19} The wt protein may be stabilised by binding to the murine double minute oncoprotein MDM-2²⁰ or the growth arrest DNA damage inducible gene GADD-4²¹ induced after DNA damage. The anti p53 antibody used in most immunostaining studies, and in the present study, is the polyclonal CM1 which recognises epitopes on both wt and mutant protein.²² Thus positive staining could be a consequence of either overexpression of wt or the presence of mutant p53 protein. It remains to be demonstrated that mechanisms other than or in addition to mutation of p53 do play a role in carcinogenesis *in vivo* and it is of interest that in two studies^{8,9} amplification of the MDM-2 gene in cervical carcinoma could not be demonstrated. In the

present study positive staining could be detected in only 4.6% (65) of the cervical cancer biopsies studied and no overexpression at the protein level could be shown by Western blotting.

However, Northern blotting experiments suggest that overexpression of p53 at the RNA level did occur in 2 (6.3%) cervical cancer biopsy specimens. Both of these were negative by IH staining and had a wt p53 genome and both were HPV 16 positive. These data are difficult to interpret but it is possible that in these cells the translated protein is rapidly degraded as a consequence of HPV E6 binding.

A number of studies have used PCR/SSCP to screen for p53 mutations in cervical cancer biopsy specimens and have consistently shown a low (3–6%) frequency of mutation.^{7,8,23} These studies suffer from a relatively small sample size and the fact that only exons 4–9 were screened. However, other workers using an alternative analysis for detection of mutations, GC-clamped PCR, and larger samples also found a low (2–6%) mutation frequency.^{24,25} Even in a series examining only HPV-negative cancers, in only 2 of 21 cases (9.5%) could mutation be detected.²⁶

Crook *et al.*,⁶ sequenced the entire p53 genome in the 3 HPV negative tumours in their series and showed mutation in all three tumours, whereas Miwa *et al.*⁹ in a study of 47 primary cervical cancers who also screened the complete genome, exons 2–11, could identify only two tumours (4.3%) with mutation. In the present study the entire p53 genome exons 2–11 were screened but in only two tumours (2%) was mutation detected. These consisted of a missense mutation on exon 8 in a sample positive for HPV by consensus PCR but not typed and a missense mutation on exon 7 in a HPV-negative sample. Thus in this study the mutation rate in HPV positive cervical cancer is 1/81 (1.2%) and 1/19 (5.2%) in HPV-negative cancer, indicating that p53 mutation is uncommon in cervical cancer whether HPV negative or positive.

To summarise we have shown in this study of 100 cervical cancer biopsy specimens from patients mainly of Chinese origin an incidence of HPV DNA (predominantly HPV 16 or 18) of 81.0%. In this tumour series over expression or mutation of p53 at the DNA, RNA or protein levels is very uncommon and not related apparently to the presence or absence of HPV DNA sequences.

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