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Original Paper

Abnormal Expression or Mutation of *TP53* and HPV in Vulvar Cancer

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HPV (human papillomavirus) plays an important role in cervical cancer and may also play a role in vulvar cancer. *TP53* mutation is common in a variety of cancers but its role in vulvar cancer is not well established. The aim of this study was to assess the prevalence of HPV infection and *TP53* mutation as well as their correlation in vulvar cancer. Also, HPV detection and abnormal p53 expression were assessed in relation to age, co-existing vulvar intraepithelial neoplasia and vulvar dystrophy. Forty-eight samples of vulvar cancer were studied. DNA was extracted from formalin-fixed paraffin embedded tissue for polymerase chain reaction/Southern blot study with HPV 16 and 18 and L1 primers. Paraffin sections were immunostained (IHS) for p53 protein using three antibodies, p1801, CM1 and DO7. The p53 mutation was also screened using polymerase chain reaction (PCR) single-stranded conformation polymorphism (SSCP) and confirmed by sequencing. Overall, HPV was detected in 48% (23/48), of which 96% (22/23) were HPV 16 or 18. By IHS, p53 overexpression was detected in 46% of cases whilst *TP53* mutations were identified in 21%. In HPV positive and negative tumours, p53 abnormal expression was detected in 39% and 52%, respectively, and *TP53* mutation was found in 22% and 20%, respectively. Mutations were mainly found at codons 273 and 204. Age was not found to be associated with HPV detection. However, the presence of HPV (71%) or absence of abnormal p53 expression (65%) were higher in tumours with VIN3, but were not correlated with dystrophy. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

THE ROLE of HPV (human papillomavirus) in pathogenesis of cancer lies in the degradation of the p53 protein by the E6 protein of HPV 16 or 18 [1]. HPV infection is common in cervical cancer and probably plays a major role in its pathogenesis [2]. Both vulvar and cervical cancer arise from squamous epithelium and are exposed to similar external factors, so it is possible that they share a common pathogen. Since condyloma infection is not uncommon in vulva, HPV infection may also play a role in its pathogenesis similar to that in cervical carcinoma.

Although *TP53* mutation or abnormal expression was found to be uncommon in cervical cancer in our previous study [2], *TP53* is a common tumour suppressor gene in the pathogenesis of a variety of cancers [3] and, therefore, may play a role in vulvar cancer. If HPV and *TP53* do play a role in the pathogenesis of vulvar cancer, *TP53* abnormal expression or mutation is probably more common in HPV negative tumours as an alternative pathway to p53 degradation by HPV. The aim of this study was to assess the prevalence of HPV and abnormal p53 status and their correlation in vulvar cancer. Other factors such as age, vulvar intra-epithelial neoplasia (VIN) and dystrophy were also assessed in relation to HPV and abnormal p53 status.

PATIENTS AND METHODS

Patients with vulvar cancer were identified by going through archival pathology reports kept at the Department of Pathology and clinical files kept at the Department of Obstetrics and Gynaecology, Queen Mary Hospital during the period 1974–1992. Paraffin blocks of 48 patients with

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squamous cell carcinoma of the vulva were retrieved. All slides were reviewed and confirmed to be squamous cell carcinoma of the vulva. Since some of the clinical files were not available, stage and survival rate could not be analysed in this study. However, age and other histological features such as co-existing VIN and dystrophy were assessed and correlated with HPV and abnormal p53 status.

Extraction of DNA from paraffin sections

Ten paraffin 5 μm sections were dewaxed in 1 ml of xylene in an Eppendorf tube twice and washed with 95 and 100% ethanol, respectively. The tissue was then dried in a Speed-vac and resuspended in TE (Tris/EDTA) buffer. Details of tissue digestion and DNA extraction were described previously [2].

HPV detection and typing by polymerase chain reaction and Southern blotting

This procedure has been described previously [4]. Briefly, DNA extracted from paraffin sections was subjected to three polymerase chain reaction (PCR) assays using consensus primers for HPV L1 and specific primers for HPV 16 and 18 E6. PCR products were run on 4% agarose gels and blotted on to Nylon membranes for hybridisation. DNA from Caski and HeLa cell lines were used as positive controls for HPV 16 and 18, respectively. DNA from the C33 cell line and water were used as negative controls. DNA amplification control was performed using a beta-globin gene primer.

Immunohistochemical staining for p53

Paraffin 5 μm sections thickness were cut and mounted on APES (3-amminopyropyl triethoxysilane) coated slides. Details of the immunohistochemical staining (IHS) have been reported previously [4]. Briefly, after deparaffinisation and rehydration, sections were incubated with 0.3% hydrogen peroxide to block endogeneous peroxidase activity, and subsequently preheated in 6 M urea for 5 min with microwave for antigen retrieval. The monoclonal antibodies p1801 (Ab2) (Novocastra, U.K.) and DO7 (Novocastra Lab Ltd, Newcastle upon Tyne, U.K.), raised against both wild and mutant p53, were used at dilutions of 1:60 and 1:50, respectively. The polyclonal antibody, CM1 raised against a full-length recombinant generated human p53 protein (Novocastra), was applied at 1:2400 dilution. Immunostaining was performed using the ABC immunoperoxidase method, Vectastain ABC kit (Vector Laboratories Inc, Burlingame, California, U.S.A.). Positive and negative controls were included in each IHS assay.

Screening of p53 exons 5–8 mutation by PCR/SSCP

Exons 5–8 of the *TP53* gene were amplified by PCR according to published procedures [5, 6]. The primer for exon 5 [5] and primers for exons 6–8 [6] were used as reported in the two previous publications. r-^{32}P-end labelled primers were prepared by incubating 5 pmol of each left and right primer with 10 units of T4 polynucleotide kinase and 2 μl of r-^{32}P-ATP (5000 Ci/nmol, Amersham) in 10 μl of kinase buffer at 37°C for 30 min. Five units of TaqDNA Polymerase (Perkins–Elmer, California, U.S.A.) were added subsequently to the diluted mixture containing 2.5 nM $MgCl_2$ and 78 μM of dNTP. Four microlitres 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 5 min at 94°C, followed by 35 cycles of PCR (40 sec at 94°C for denaturation; 40 sec incubation at 53–60°C for annealing; 90 sec at 72°C for polymerisation and finally at 72°C for 7 min). Formamide dye (45 μl) was added to stop the reaction. Two microlitres of the final product was loaded onto an 8% polyacrylamide gel with 3% glycerol and electrophoresed at 2 Watt at room temperature overnight. The gel was dried and exposed to X-ray film at −70°C for 6–12 h with an intensifying screen.

PCR/SSCP was repeated in the 15 samples including the ten with shifted bands. On seven of the ten samples with shifted bands, PCR/SSCP was repeated on normal tissue dissected around the tumour from the paraffin block. The remaining three samples had inadequate normal tissue for extraction of DNA.

Direct cycle-sequencing of PCR product

DNA bands that displayed a mobility shift in SSCP gel were excised and eluted in 50 μl of sterile water at 37°C overnight. An aliquot of 20 μl eluted DNA was reamplified. PCR products were purified by electrophoreses on a 5% acrylamide gel and extraction of phenol: chloroform. The procedure used was essentially that described previously [2]. DNA sequencing was according to the procedure described by Mok and associates [7]. The Taq Sequencing Kit (US Biochemicals, Cleveland, Ohio, U.S.A.) was used under the conditions described by the manufacturer. The final product was run on a 6% sequencing gel. The gel was dried and exposed to X-ray film.

Statistical analysis

A logistic regression using SAS (SAS Institute, Cary, North Carolina, U.S.A.) was applied to examine the presence or absence of vulvar intraepithelial neoplasia (VIN3) and vulvar dystrophy with the other covariates, for example, age, HPV infection rate, p53 expression and *TP53* mutation. Testing for the difference in age distribution for the groups of HPV infection rate and vulvar cancer was also conducted. Chi-square test was used for analysis of nominal data. All the significant results were given at 5% level of significance. A P value of less than 0.05 was considered as significant.

RESULTS

HPV detection

HPV 16 was detected in 18 samples (38%) and HPV 18 in 7 samples (15%). Among these samples, HPV 16 and 18 were both found in 3 (6%). HPV 16 or 18 was detected in 22 samples (46%). One sample (2%) which was HPV 16 and 18 negative was positive with the HPV consensus primers, but the type of HPV was not sought. Thus, 23 samples were HPV positive (48%) and 25 (52%) were HPV negative (Table 1).

Table 1. *Summary of HPV detection in vulvar cancer*

Detection of HPV	Number (%)
Only HPV 16 positive	15 (31)
Only HPV 18 positive	4 (8)
Both HPV 16 and 18 positive	3 (6)
Either HPV 16 or 18 positive	22 (46)
Both HPV16 and 18 negative, L1 positive	1 (2)
HPV negative	25 (52)

Table 2. p53 *mutations in* 10 *samples of vulvar cancer*

	No.	Codon	Nucleotides	Amino acids
Exon 6	5	204	CGT→CAT	Ala→Thr
Exon 8	6	273	CGT→CAT	Ala→Thr
	1	283	CGC→CAC	Arg→His

Table 3. *Correlations of HPV and p53 status*

	p53 IHS		p53 mutation		
	Negative No. (%)	Positive No. (%)	No No. (%)	Yes No. (%)	Total No. (%)
HPV negative	12 (48)	13 (52)	20 (80)	5 (20)	25 (52)
HPV positive	14 (61)	9 (39)	18 (78)	5 (22)	23 (48)
Total	26 (54)	22 (46)	38 (79)	10 (21)	48 (100)

TP53 *status*

14 (29%) samples stained positively with the antibody Ab2, 18 (38%) with CM1 and 19 samples (40%) with DO7. The overall positivity rate using the three antibodies was 46% (22 samples).

Using SSCP for exons 5–8, shifted bands were detected in 10 samples (21%). Sequencing of the shifted bands confirmed 12 mutations. Three mutations were found in exon 6, five in exon 8 and two mixed mutations in exons 6 and 8 (Table 2). The seven samples from normal tissues around the cancer cells showed normal *TP53* sequencing. All samples showed positive p53 IHS reaction.

Correlation between HPV status and p53 abnormal expression/ mutation

Of the 25 HPV negative samples, 13 (52%) were p53 positive by IHS, whilst in the 23 HPV positive samples, 9 (39%) were p53 positive. The difference was statistical non-significance (Table 3). Five of the 25 (20%) HPV negative samples and 5 of the 23 HPV positive samples (22%) had p53 mutations (Table 3).

Among the 25 HPV negative samples, 16 (64%) had either positive *TP53* IHS or mutation. In the 23 HPV positive samples, 11 (48%) had either positive *p53* IHS or mutation. The difference did not reach statistical significance.

Correlations between HPV, TP53 *status and age, VIN3 and vulvar dystrophy*

The age of the 48 patients ranged from 45 to 88 years with a mean ± standard deviation (S.D.) of 64.8 ± 14.6 years. The median age was 66 years. The mean ± S.D. of 23 patients with no HPV detected in the tumour were 67.7 ± 11.1 years and that of 22 patients with HPV detected were 61.8 ± 17.3 years. There was no statistically significant difference in the mean age between the two groups. Similarly, there was no statistical significant difference in age between samples with or without abnormal p53 expression.

All 48 samples were squamous cell carcinoma of the vulva. VIN3 was present in 17 (35%) samples. HPV was detected in 12 of these 17 (71%) samples which was significantly higher than that detected in samples without co-existing VIN3 (P value = 0.016; Table 4). Abnormal *TP53* expression/mutation was detected in 21 (68%) of 31 samples without VIN3 and 6 (35%) of the 17 samples with VIN3 (P = 0.024; Table 4).

Vulvar dystrophy was present in 19 (40%) samples: 12 had hyperplasia, five lichen sclerosis and two mixed pathology. HPV was detected in 7 (37%) of 19 samples. In samples with no vulvar dystrophy, HPV was detected in 16 samples (55%). Results of backward elimination of the logistic regression suggested that only the factor HPV was significantly related to the presence of VIN3 (P = 0.027). The other variables, for example, age and p53 were not significant (data not shown). The only factor significantly correlated with the vulvar dystrophy was VIN3, which was negatively related to vulvar dystrophy (P value < 0·05).

16 samples had neither VIN3 nor dystrophy and HPV was detected in 7 of these (44%) compared with 16 of 32 (50%) samples with either VIN3 or dystrophy present. Abnormal p53 expression was detected in 10 of 16 (63%) samples without VIN3 or dystrophy, but in 17 of 32 (53%) samples with either lesion.

DISCUSSION

HPV was detected in 48% (23/48) of squamous cell carcinoma of the vulva in our Hong Kong Chinese series, a figure similar to the 31–57% found in other studies [8–11]. The majority (96%) were HPV 16 or 18. A high percentage (83%) of HPV 16 was also detected in another study [12]. Although the percentage of HPV infection was lower in vulvar cancer than cervical cancer, HPV 16 and 18 were the major HPV types, supporting the oncogenic potential of these HPV types probably through degradation of p53 protein. Abnormal *TP53* expression or mutation seemed to occur more often in HPV negative tumours (64%) than HPV positive tumour (48%) although the difference did not reach statistical significance. This lack of correlation of abnormal p53 expression with HPV status was also found in a study by Kagie and associates [12]. However, both HPV and p53 probably play a role in the pathogenesis of vulvar cancer as indicated by the high proportion with oncogenic HPV and abnormal p53 expression.

p53 positivity by IHS was detected in 46% (22/48) of vulvar cancer in this study. Although different antibodies were used, both Kagie and associates [12] and Kohlberger and associates [13] also detected approximately 50% of p53 overexpression by IHS in vulvar cancer. p53 positivity by IHS could be due

Table 4. *Correlations of HPV, abnormal p53 status with VIN3 and dystrophy co-existing with vulvar cancer (chi-square used)*

VIN3	HPV absent No. (%)	HPV present No. (%)	P value	Normal p53 No. (%)	Abnormal p53 No. (%)	P value
Absent (n = 31)	20 (65)	11 (35)	P = 0.016	10 (32)	21 (68)	P = 0.024
Present (n = 17)	5 (29)	12 (71)		11 (65)	6 (35)	
Dystrophy						
Absent (n = 29)	13 (45)	16 (55)	P > 0.05	12 (41)	17 (59)	P > 0.05
Present (n = 19)	12 (63)	7 (37)		8 (42)	11 (58)	

to staining of the stabilised mutant p53 or stabilised wild-type protein via other mechanisms which lead to interruption of the normal degradation pathway of p53 [14]. Wild-type p53 protein may be stabilised by binding to the murine double minute oncoprotein MDM-2 [15] or the growth arrest DNA damage inducible gene GADD-4 [16]. However, there may be other unknown mechanisms which stabilised the wild-type protein. The significance of this abnormal expression in prognosis and survival has been shown in other cancers [17, 18]. Though positive p53 IHS does not necessarily correlate with mutation of *TP53*, abnormal expression of p53 seems to indicate deranged function of this tumour suppressor gene. This may contribute as an alternative pathway in the pathogenesis of vulvar cancer.

TP53 mutation was detected in 21% (10/48) of vulvar cancer in this study using SSCP screening confirmed by direct sequencing. Lee and associates [9] using a similar technique detected *TP53* mutation (23.8%) in his study. However, *TP53* mutation was found in approximately 20% of either HPV negative or positive tumours in our study, whilst Lee and associates [9] found, in 21 vulvar cancer cases, *TP53* mutation in only 8% of HPV positive tumours and in 44% of HPV negative tumours. This discrepancy needs further investigation for clarification.

TP53 mutations were found in exons 6 and 8 in this study. Although mutations have been found in quite a number of codons in exons 5/6, 7, 8/9 in vulvar carcinoma cell lines [19], mutations were found mainly in two codons in our study. The commonest spot was codon 273 with 6 samples showing the same mutation. Codon 273 is also a hot spot for a variety of cancers such as ovarian (11.2%), breast (6.7%), lung (8.9%), colorectal (9.5%) and liver (4.1%) [20]. Another hot spot found in this study was at codon 204 in exon 6. This is not commonly found in other cancers. The mutation was unlikely to be a new polymorphic site since a normal genotype was found in the normal tissue around the cancer cells. All 10 mutations were G→A transitions suggesting a spontaneous mutation. Further study is required to confirm whether the two codons are hot spots for vulvar cancer.

This study did not demonstrate a higher incidence of HPV infection in younger patients with vulvar cancer. Toki and associates [11] showed that the mean age of patients with HPV positive vulvar cancer was 22 years younger than those with HPV negative tumour. Bloss and colleagues [10] found that all his patients who were younger than 45 years had HPV positive vulvar cancer. Since none of the patients in our study were under 45 years, this could be the reason for the lack of correlation of age with HPV status.

The association of HPV and VIN3 suggests that, like cervical cancer, HPV infection predisposes to dysplastic changes in squamous epithelium. However, unlike cervical cancer, progression from HPV infection to VIN3 and cancer has not been well established in vulvar carcinoma. Indeed, only 39% of vulvar cancer with VIN3 was found in this study. However, the high association of HPV in vulvar cancer with VIN3 suggests an association of HPV and VIN3 in some vulvar cancer. In contrast, vulvar dystrophy, though found in 40% of vulvar cancers, did not show a preponderance for HPV infection. p53 abnormal expression was higher in tumours without VIN3, suggesting an alternate non-HPV pathway of pathogenesis where p53 may play a part.

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