10.1053.paor.1999.0161 available online at http://www.idealibrary.com on IDEAL

ARTICLE

Decreased Programmed Cell Death in the Uterine Cervix Associated with High Risk Human Papillomavirus Infection

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The relationship between apoptosis, apoptosis regulatory proteins, cell proliferation and human papillomavirus infection during various phases of tumor progression in the uterine cervix was studied. Apoptosis was defined by morphological criteria and the TUNEL assay. Expression of p53, bcl-2, bax, cyclin D1, Ki 67 and E6 protein was evaluated by immunocytochemistry. Presence of mutant p53 was detected using a mutant specific ELISA. Type of HPV infection was determined by PCR using type specific primers. Apoptosis showed significant negative correlation with increasing histological abnormality (p=0.0005). Higher tumor cell proliferation was associated with increasing histological abnormality (p=0.001 for Ki 67 and cyclin D1). There was significant correlation between histological grade and immunoreactivity of p53 (p=0.0001) and bcl-2 (p=0.0002). However, mutant p53 was expressed by only 12 of the 230 samples. Expression of bax and the bax/bcl-2 ratio showed an inverse correlation to histological grade (p=0.0003 and 0.0001, respectively). There was also an inverse correlation between extent of apoptosis and immunoreactivity of p53 (p=0.0001) and bcl-2 (p=0.0001). A significant positive correlation between expression of the bax protein and apoptosis was evident (p=0.0001). HPV infection significantly correlated to the extent of histological abnormality (p=0.0001). High risk HPV-E6 protein also showed this significant correlation (p=0.0002). There was an inverse correlation between apoptosis and HPV infection (p=0.0002). High risk HPV infection was associated with decreased apoptosis and also increased human cell proliferation. Lowest levels of bax/bcl-2 ratio was also associated with HPV 16 and 18 infection (p=0.0001). Modulation of apoptosis and apoptotic regulatory proteins by high risk HPV infection may be an important factor in the development of cervical cancer. (Pathology Oncology Research Vol 5, No 2, 95–103, 1999)

Key words: HPV, apoptosis, cervical cancer

Introduction

Cancer of the uterine cervix remains one of the most common female malignancies in India. It accounts for about 26% of all female cancers and about 90,000 women are expected to develop the disease annually.[6] There is substantiai evidence to suggest a role for human papillomavirus (HPV) infection in the development of cervical cancer.[1] Recent studies also suggest that deregulation of programmed cell death (PCD or apoptosis) contributes to the development of malignancy. Apoptosis is a distinct form of cell death that can result from activation of a genetically regulated cell suicide program or from cell injury induced by various stimuli.[32] Apoptosis may therefore be of significance, both to tumor progression owing to its role in total cell turnover and in tumor response to therapy due to the apoptotic response of cells to irradiation and chemotherapy.[4,8] Although the molecular mechanisms underlying the development of cervical cancer including the role of HPV have been elucidated during the last few years,[26] the role of apoptosis is still unclear. The possibility of alterations in programmed cell death during cervical carcinogenesis stems from the description of changes in tumor associated genes involved in the regulation of apoptosis. We had previously reported alterations in expression of p53,[11] as well as the modulation of

Received: Nov 20, 1998; *accepted:* March 5, 1999

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 1219-4956/99/020095+09 $ 12.00/0

apoptotic regulatory bcl-2 and p53 proteins by the high risk HPV E6 transforming protein.[22] p53 produced in response to DNA damage acts to restrain cellular proliferation by binding to specific regions of DNA and regulates the expression of other genes responsible for cell cycle arrest, DNA repair and initiation of apoptosis.[19] The p53 gene induces the production of other pro-apoptosis proteins such as bax and represses apoptosis inhibitors such as the bcl-2.[32] Inactivation or mutation in p53 gene may therefore result in overexpression of bcl-2 and down regulation of bax contributing to continuous cell accumulation. This is the background and rationale for the present study to examine the effect of HPV infection on the expression of apoptosis regulatory proteins p53, bax and bcl-2, as well as on the expression of cell proliferation markers as cyclin D1 and Ki 67. This study is extended to various phases of tumor progression in the cervical epithelium, ranging from normal epithelium through low and high grades of squamous intraepithelial lesions and invasive cervical cancer.

Materials and Methods

Patient selection

Tissue samples for the study were obtained from patients attending the gynaecological clinics of the SAT Hospital and the Regional Cancer Centre, Thiruvananthapuram. The study was approved by the Research Advisory Committee of the Regional Cancer Centre. A total of 230 cervical tissue samples were analyzed and 40 cases of apparently normal cervical epithelium, 37 low grade squamous intraepithelial lesions (SILs), 43 high grade SILs, 36 well differentiated squamous cell carcinoma (WDSCC), 31 moderately differentiated (MDSCC) and 43 poorly differentiated carcinoma (PDSCC) were also included. Tissue samples were obtained as punch biopsies. Apparently normal cervical epithelium were obtained from patients undergoing hysterectomy for various non-malignant reasons. Paraffin sections, 4 µm thick, were placed on poly-L-lysine coated glass slides. One section from all samples was stained with hematoxylin-eosin for routine histopathology and duplicate serial sections used for immunocytochemistry, apoptosis evaluation and determination of HPV infection.

TUNEL technique for determination of apoptosis

Estimation of apoptosis in the tissue was determined using the TUNEL assay, employing an in situ cell detection kit (Boehringer Mannheim, Germany) as explained in detail by us earlier.[11,22] Briefly, sections were dewaxed in xylene, hydrated through decreasing grades of ethanol, and washed in distilled water. The nuclei in tissue sections were stripped from proteins with 20 µg/ml of proteinase K (Sigma, St Louis) followed by quenching of endogenous peroxidase with 0.3% H_20_2 in methanol. The sections were covered with 50 µl of a label mix containing the modified nucleotide and the enzyme, in a humidified chamber for 60 minutes at room temperature. For signal conversion analysis, 50 µl of converter peroxidase reagent was used. The substrate reaction was developed using diaminobenzedine (DAB) and counterstained with haematoxylin. To confirm reaction specificity of the TUNEL procedure, a negative control was done omitting the Tdt enzyme. Rat involuting breast tissue was used as positive control.

Evaluation of TUNEL results

Since the enzymatic reaction may also label diffuse areas of necrosis, only those labelled cells that showed additional characteristics of apoptosis, i.e., isolated localization within an intact cell complex without an inflammatory reaction were regarded as positive. Grading of the TUNEL reaction was done as explained by us earlier.[21,24] Briefly, to evaluate differences in various rates of TUNEL reactivity in each sample, 3000 cells were counted at random under high power and an apoptotic index (AI) was expressed as

AI = (number of TUNEL reactive nuclei / total number of cells counted) x 100

Results were graded into 4 classes.[21,24] Class 1 included samples with an AI 0-1.0% (insignificant apoptosis), Class 2 had 1.1–2.0% (mild apoptosis), Class 3 had 2.1–3.0% (moderate apoptosis) while Class 4 had >3.1% (intense apoptosis)

lmmunocytochemistry for p53, bax, E6, cyclin D1, Ki 67 and bcl-2 expression

Immunocytochemistry was performed as described earlier.[9,23] All sections were processed for antigen retrieval using Target Unmasking Fluid (TUF, Kreatech, Amsterdam). Sections were then incubated overnight with the respective primary antibody p53 (Ab-6), Bax (Ab-1), Cyclin D1 (Ab-3), Ki 67/MIB-1 (Ab-1) and HPV16/18 E6 (Calbiochem, Oncogene Science, Cambridge, MA) and anti bcl-2 (Boehhringer Mannheim). Positive control for p53 protein a breast cancer sample with p53 gene mutation, for bcl-2 the tonsil tissue showing negative reaction in germinal centres, and for E6 smears from the HeLa cell line were used. Negative controls omitted the primary antibody. Reaction were visualised using a streptavidin-biotin-immunoperoxidase system (DAKO) with DAB. All sections were then counterstained with haematoxylin.

Evaluation of p53, bax, bcl-2, cyclin D1, Ki 67 and E6 expression

To analyze the expression of p53, bcl-2, bax, cyclin D1 and Ki 67 protein expression, a total of 1000 cells were evaluated in all sections. Expression of p53, cyclin D1 and Ki 67 was considered "significant" when characteristic nuclear immunoreactivity was present in at least 10% of the tumor cells. Similarity, bcl-2 and bax expression was considered significant if cytoplasmic immunoreactivity was evident in at least 10% of tumor cells. In addition to this, an expression index was created as earlier.[9,11,32] This was done by classifying the protein expression into four categories: grade 1 samples where less than 10% of cells showed positivity (insignificant), grade 2 with 11-30% expression (mild expression), grade 3 with 31-50% expression (moderate expression), and grade 4 with greater than 51% expression (intense expression). For E6 expression, only presence or absence of immunoreactivity was considered.

Detection of mutant p53 protein

Analysis of the mutant p53 protein assay was done using a p53 mutant selective ELISA kit (Oncogene Research Products, Cambridge, MA) as described by us in detail elsewhere.[21,24] Specificity of this ELISA kit and cut off absorbency as 0.125 OD for ELISA positive mutant p53 protein has also been described.[24]

Determination of HPV infection

HPV infection in cervical tissues was analyzed by PCR as described in detail elsewhere.[28,33] Briefly, tissues fixed in 10% neutral buffered formalin and embedded in paraffin were used to detect HPV DNA. Three to four sections (10 µ thick) were cut, placed in an eppendorf tube and dewaxed in two changes of xylene. After two washes in 95% ethyl alcohol, the tissue was allowed to dry, suspended in 180 µl sterile water and 20 µl of proteinase K (10 mg/ml) and incubated for 15 hours at 55°C. The proteinase K was inactivated by heating (95°C for 10 min) and the supernatant was used for PCR. Custom designed primers specific for HPV 6, 11, 16 and 18 were used (Genosys, Cambridgeshire, UK). The sequence details of the primers have been described elsewhere.[3] PCR reactions were performed in 40 µl volumes containing 10 mM Tris.HCl, pH 8.3; 50 mM KCI; 1.5 mM $MgCl_2$; 200 µM dNTPs; 1.5 unit Taq polymerase (Perkin Elmer, USA), 4 µM primers and 1 µl sample. The samples were subjected to 30 cycles of DNA amplification in a DNA thermal cycler (Perkin-Elmer Gene Amp PCR system 2400). A cycle represents primer extension for 2 min at 72°C, denaturation for 1 min at 95°C and reannealing for 1 min at 55°C followed by primer extension. To control the PCR reactions, one positive control (consisting of one fragment of the HPV type in question) and one negative control (DNA from a guinea pig muscle) was amplified. The reactions also contained a negative control (buffer solution without sample tissue). After amplification, the samples were incubated at 70°C for 15 minutes. An aliquot of the reaction mixture was then electrophoresed on an 8% polyacrylamide gel and visualized by ethidium bromide staining. The specificity of the amplified DNA was then confirmed by Southern Blot, using type specific probes.

Data analysis

The expression of p53, bcl-2, bax, cyclin D1, Ki 67, and E6, extent of TUNEL reactive nuclei and HPV infection in different tissue samples were statistically analyzed by Kruskal Wallis one way ANOVA, Mann Whitney and the Speannan correlation tests. Analysis showing a confidence interval above 95% ($p<0.05$) was considered significant. Odds ratio and confidence intervals (CI) were calculated using the Fisher's exact test. For this all tissue samples were grouped into cases (all high grade SILs and Invasive Cancer) and controls (normai and low grade SILs).

Results

Extent of apoptosis

Apoptotic cells detected by TUNEL were visualized as cells with dark brown bodies in the nucleus *(Figure 1).* The majority of the 40 apparently normal cervical tissue showed substantial levels of TUNEL reactive nuclei. There was a progressive decrease in the intensity of apoptosis as histological abnormality increased. The pattern of apoptosis was similar in well differentiated and moderately differentiated carcinomas with the majority of samples

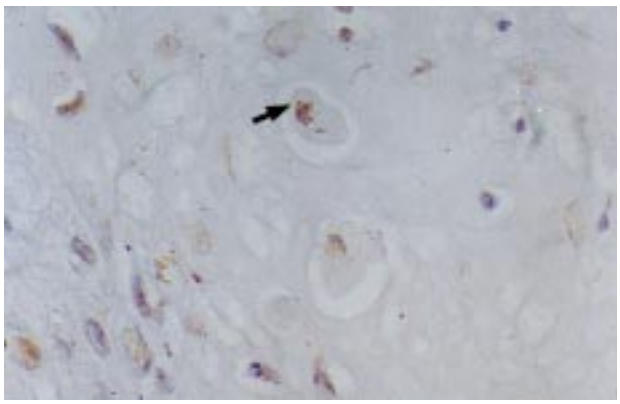

Figure 1. *TUNEL immunoreactivity in poorly differentiated squamous cell carcinoma with apoptotic cells with marked chromatin condensation (arrow) (x40).*

Table 1. **Apoptosis in cervical tissue**

	Apoptotic indices			
	Insignificant (0–1)	*Mild (1.1–2)*	*Moderate (2.1–3)*	*Intense (>3.1)*
Normal (n=40)	6 (15%)	8 (20%)	5 (12.5%)	21 (52.5%)
Low grade SIL (n=37)	5 (13.5%)	17 (45.9%)	4 (10.8%)	11 (29.7%)
High grade SIL (n=43)	15 (34.8%)	18 (41.8%)	8 (18.6%)	2 (4.65%)
WDSCC (n=36)	13 (36.1 %)	18 (50%)	– –	5 (13.8%)
MDSCC (n=31)	11 (35.5%)	15 (48.4%)	3 (9.7%)	2 (6.4%)
PDSCC (n=43)	10 (23.2%)	15 (34.9%)	12 (27.9%)	6 (13.9%)

Figures in paranthesis refer to percentage values

exhibiting Class 1 or 2 TUNEL reactions. However, an interesting observation was a relatively high apoptotic index in 18 of 43 (42%) poorly differentiated tumors *(Table 1)*.

Immunoreactivity of p53

p53 protein expression was apparent from the clear nuclear immunoreactivity in tumor cells *(Figure 2)*. Expression of the tumor suppressor p53 protein was differentially distributed among the various histological grades of lesions *(Table 2)*. None of 40 normal cervical tissue samples were immunoreactive for p53. Of the 37 low grade SILs, 8 showed mild immunoreactivity and 1 moderate immunoreactivity. In high grade SILs, 19/43 samples showed mild p53 expression while the other 24 samples showed moderate immunoreactivity. The expression of p53 was similar in all three histological grades of invasive carcinoma with moderate to intense immunoreactivity in almost all samples.

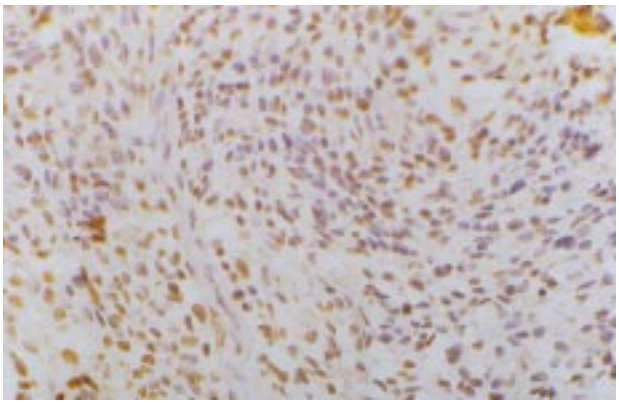

Figure 2. *Intense nuclear immunoreactivity of p53 in poorly differentiated squamous cell carcinoma of the uterine cervix (x20).*

Expression of Bcl-2 and Bax proteins

Expression of the anti-apoptotic bcl-2 protein was predominantly cytoplasmic and sometimes in the perinuclear areas *(Figure 3)*. Bcl-2 was not expressed in any of the normal cervical tissue samples. The majority of low grade SILs also showed insignificant expression of bcl-2. In high grade SILs and invasive carcinomas bcl-2 expression was similar with all lesions expressing the protein. Most of the normal cervical tissue samples and low grade SILs expressed bax *(Figure 4)*. In high grade SILs bax expression was mostly mild while the protein expression was similar in well differentiated and moderately differentiated tumors with the majority of lesions either not expressing the protein or showing mild expression *(Table 2)*. Howev-

Table 2. **Intensity of p53, bcl–2, bax, cyclin D1 and ki 67 expression in cervical tissue**

	Normal (n=40)	*Low grade SIL (n=37)*	*High grade SIL (n=43)*	*WDSCC (n=36)*	*MDSCC (n=31)*	*PDSCC (n=43)*
p53						
Negative	40	28	0	0	0	0
Mild	0	8	19	1	0	0
Moderate	0	1	24	24	2	22
Intense	0	0	0	11	29	21
Bcl–2						
Negative	40	27	0	0	0	0
Mild	0	9	4	7	1	1
Moderate	0	1	37	19	27	34
Intense	0	0	2	10	3	8
Bax						
Negative	0	0	13	17	18	11
Mild	19	23	29	17	11	19
Moderate	21	14	1	2	2	13
Intense	0	0	0	0	0	0
Cyclin D1						
Negative	0	0	0	0	0	0
Mild	26	27	0	0	0	0
Moderate	14	9	19	1	1	3
Intense	0	1	24	35	30	40
Ki 67						
Negative	0	0	0	0	0	0
Mild	34	26	0	0	0	0
Moderate	6	11	11	0	0	0
Intense	0	0	32	36	31	43

er, in poorly differentiated tumors, 13/43 cases (30%) showed moderate bax expression while 19 (44%) showed mild immunoreactivity. The bax to bcl-2 ratio was similar in normai cervical tissue and low grade SlLs while it was reduced in high grade SILs and invasive carcinomas.

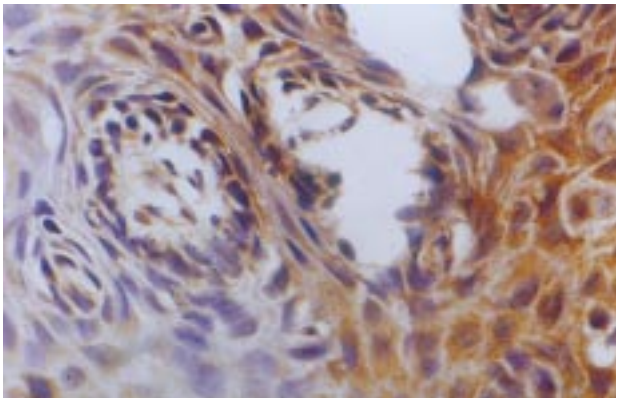

Figure 3. *Intense cytoplasmic immunoreactivity of bcl -2 in well differentiated squamous cell carcinoma of the uterine cervix (x40)*

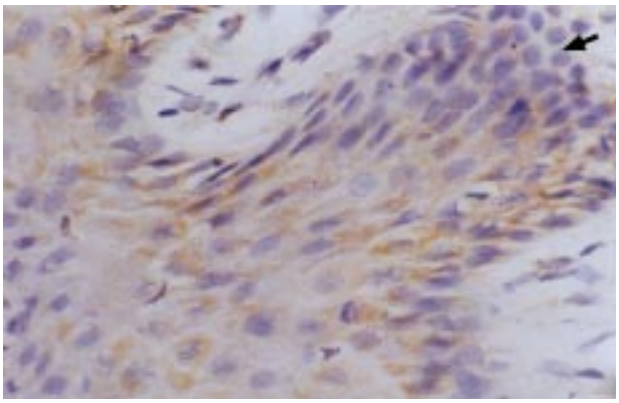

Figure 4. *Mild cytoplasmic immunoreactivity of bax in the spinal cells in normal cervical epithelium. Basal cells show no immunoreactivity (arrow) (x40).*

Tumor proliferative compartment

The total proliferative status of tissue samples was assessed by the expression of Ki 67 and Cyclin D1 *(Figs 5, 6).* The results obtained by both methods were similar although the number of cyclin D1 positive cells were slightly lower than for Ki 67 *(Table 2).* There was an apparent increase in the proliferative compartment as the lesions progressed through increasing histological abnormality.

Human papillomavirus infection

PCR amplification of HPV–DNA showed most of the cervical tissue and low grade SILs to be negative for the HPV types analyzed. Two normal tissues and seven low grade SILs showed presence of HPV16. The distribution of HPV subtypes were similar in the high grade SILs and invasive cancer with the majority of samples positive for HPV 16. One sample was positive for both HPV 16 as well as HPV 18 *(Table 3).* Expression of the high risk HPV 16/18 transforming protein was also similar to the HPV infection pattem in various histological types. Neither of the two normai cervical tissue samples positive for HPV 16 expressed E6. Of the eight low grade SILs which showed presence of HPV 16/18, three expressed E6. These results were more significant in high grade SILs and invasive cancer, where the majority of HPV 16 or HPV 18 infected samples expressed E6. Expression of E6 is shown in *Figure 7.*

Correlation between apoptosis, apoptotic regulatory proteins and histology

There was significant correlation between the extent of apoptosis, expression of p53, bcl-2 and bax with histological grade. The extent of apoptosis showed a significant negative correlation with increasing histological abnormality (r=–0.244, p=0.0005). Fischer's exact test analysis revealed that the odds ratio of a tissue sample having insignificant or

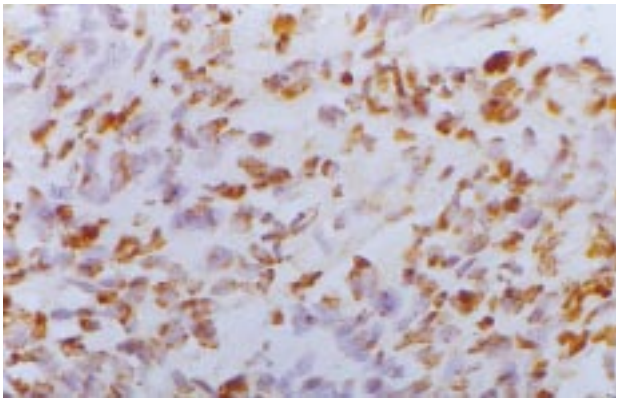

Figure 5. *Intense nuclear immunoreactivity of cyclinD1 in poorly differentiated squamous cell carcinoma (x40)*

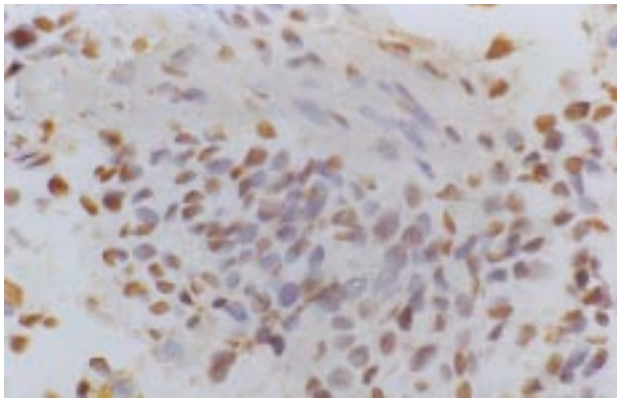

Figure 6. *Intense nuclear immunoreactivity of Ki 67 in poorly differentiated squamous cell carcinoma of the uterine cervix (x40).*

Table 3. **HPV infection in cervical tissue**

	No HPV[*]	*HPV 6*	*HPV 11*	*HPV 16*	*HPV 18*
Normal (n= 40)	35	3	–	2	–
Low grade SIL (n=37)	24	3	2	7	1
High grade SIL (n=43)	14	–	2	22	5
WDSCC (n=36)	7	–	–	23	6
MDSCC (n=31)	5	–	–	24	2
PDSCC (n=43)	13	–	–	29	1

[*]Contains no evidence for the presence of the 4 HPV types analyzed

low apoptotic index being a case was 0.2901 (p<0.0001, 95% CI 0.1626 to 0.5716). There was also significant correlation between histological grade of the lesion and immunoreactivity of p53 (r=0.866, p=0.0001) and bcl-2 (r=0.779, p= 0.0002). The expression of bax and the bax to bcl-2 ratio however showed an inverse correlation to histological grade (r=–0.339, p=0.0003 for bax and r=–0.781, p= 0.0001 for bax/bcl-2 ratio). The extent of TUNEL reactive cells also showed correlation to the expression of p53, bcl-2 and bax. There was an inverse correlation between extent of apoptosis and immunoreactivity of p53 (r= –0.305, p=0.0001) and bcl-2 (r=–0.402, p=0.0001). However, the apoptotic index had a significant positive correlation to the expression of bax protein (r=0.508, p=0.0001) and as shown in *Figure 8* with the bax/bcl-2 ratio (r=0.423, p=0.0001).

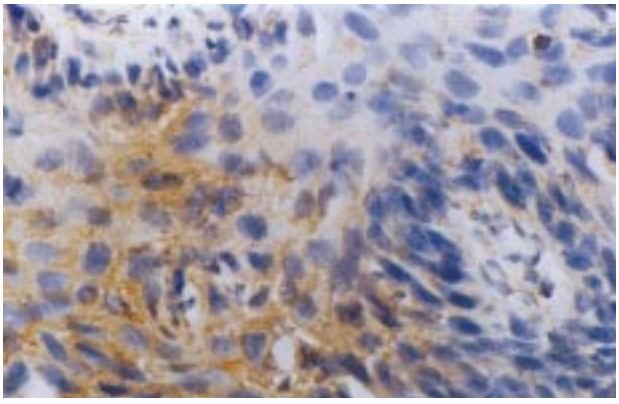

Figure 7. *HPV E6 protein expression in well differentiated squamous cell carcinoma of the uterine cervix (x40).*

Correlation between apoptosis, HPV infection and histology

Presence of HPV infection significantly correlated to the extent of histological abnormality (r=0.467, p=0.0001). Expression of the high risk HPV E6 protein also showed this significant correlation (r=0.644, p=0.0002). Relationship between HPV 16/18 infection and E6 protein expression is shown in *Figure 9*. Fischer's exact test revealed that the odd's ratio of a lesion expressing E6 being a case was 67.382 (p<0.0001), 95% CI: 20.118 to 225.69. The odds ratio of an HPV 16/18 infected sample being a case was0.0546, 95% CI: 0.0256 to 0.1162. There was also an inverse correlation between extent of apoptosis and HPV infection (r=-0.467, p=0.0002). HPV 16 infected cases showed lower levels of apoptosis. This was also the case with high risk E6 expression where an inverse correlation was also evident (r=-0.362, p=0.0001). The odds ratio for a sample positive for HPV 16/18 and showing insignificant or low apoptosis was 6.776 (p<0.0001, 95% CI: 3.647 to 12.59).

Correlation between apoptosis, HPV infection and tumor proliferative fraction

There was significant correlation between the increasing tumor cell proliferation and increasing histological abnormality (r=0.815, p=0.0001 for Ki 67 and r=0.777, p=0.0001 for cyclin D1). Increasing tumor cell proliferation was also associated with decreasing apoptosis. Hence, a negative correlation was evident between apoptotic index and Ki 67 (r=-0.398, p= 0.001) and cyclin D1 (r=-0.390, p=0.001). HPV infection also resulted in increased tumor cell proliferation with the maximum associated with HPV 16 and 18 (r=0.560, p=0.000 for Ki 67 and r=0.664, p=0.0001 for cyclin D1).

HPV modulation of apoptosis regulatory proteins

HPV infection resulted in modulation of the expression of apoptosis regulatory proteins. Expression of bcl-2 and accumulation of p53 was significantly higher in HPV 16 infected lesions (r=0.644, p=0.0001 for bcl-2 and r=0.518, p=0.0001 for p53). However, levels of bax protein expression decreased with HPV 16 expression (r=–0.561, p=0.0001). Accordingly, lowest levels of bax : bcl-2 ratios were associated with HPV 16 and 18 infection (r=–0.640, p=0.0001). These results thus are in agreement with the data showing the modulation of apoptosis by HPV infection.

Discussion

In cancer biology, it is becoming increasingly apparent that many cancer cells circumvent the normal apoptotic mechanisms to prevent their self destruction, which would

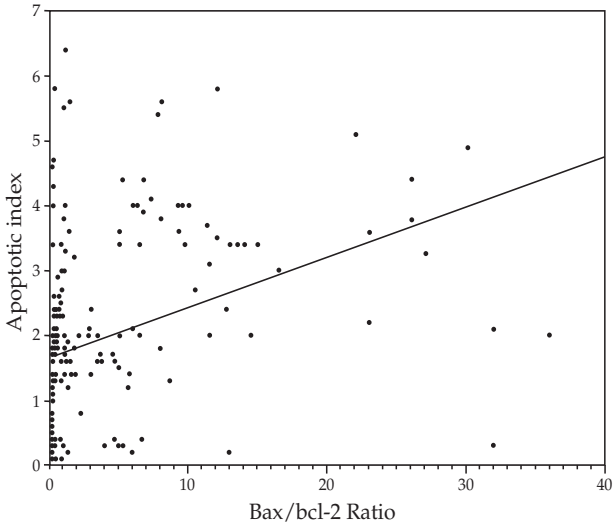

Figure 8. *Scatter plot illustrating the relationship between the bax/bcl-2 ratio and the Tunel index.*

have been indicated because of the mutations or genetic damage they may harbour.[2] Indeed, disarming apoptosis and other surveillance mechanisms may be of fundamental significance in allowing the development of the malignant and metastatic phenotype. The present study, using an *in situ* technique that identifies characteristic chromatin cleavage occurring during apoptosis, shows significant decrease as the cervical epithelium becomes increasingly neoplastic: Thus our results agree with the earlier observation of others,[3,27] showing reduced apoptosis in invasive cervical tumors as compared to CIN lesions. In contrast, Isacson et al report apoptosis to increase with histological abnormality.[5] A similar finding has also been reported by Shoji et al,[29] who also found a significant correlation with histological grading in CIN and tumor cell invasion into the stroma. We have observed that in breast cancer apoptosis correlated significantly with tumor size, tumor grade, and presence of lymph node metastasis.[21] However, as in the present study, no such correlation could be found during tumor progression in the oral cavity.[24]

Numerous genetic factors have been shown to modulate apoptosis and interactions among these factors are complex and still incompletely defined.[4] The p53 gene is one of the most frequently mutated genes in human cancers and is the archetypal cell cycle checkpoint regulator.[30] The wild type p53 is known to induce apoptosis when overexpressed.[7] The protein activates the death gene bax and down regulates the survival genes like bcl-2.[17] Thus at the G1 check point in the cell cycle, the cell has two alternatives: either to repair the DNA damage in the cell and then resume replication or to induce the death genes and trigger apoptosis.

The present study shows an obvious association between the accumulation of p53 and both HPV 16/18 infection and expression of the E6 protein. It has been demonstrated that p53 protein complexes with the HPV 16/18 E6 protein in the cytoplasm.[14] This complex is thought to target p53 for rapid degradation via ubiquitination.[20] In the present study concurrent accumulation of p53 and E6 expression was significant in the high grade lesions. E6 can interfere with the normal function of p53 by its ability to abrogate both transcriptional activation and transcriptional repression function of the gene.[13,16] In this respect E6 shows similarity to certain mutant forms of p53 which also fail to function as transcriptional regulators themselves and may also inactivate the wild type p53 after oligomerization. Recent data[15] also shows that the E6 protein can interact with cellular MCM proteins, believed to have a key role in regulation of DNA replication. E6-MCM interaction may therefore also influence tumor proliferation and apoptosis in the presence of an inactive p53. There is data to suggest that the HPV E6 protein may also function in inhibiting apoptosis in certain cells.[20,26] p53 has been shown to be required for apoptosis induced by adenovirus EIA, ionizing radiation, and etoposide.[34] The loss of func-

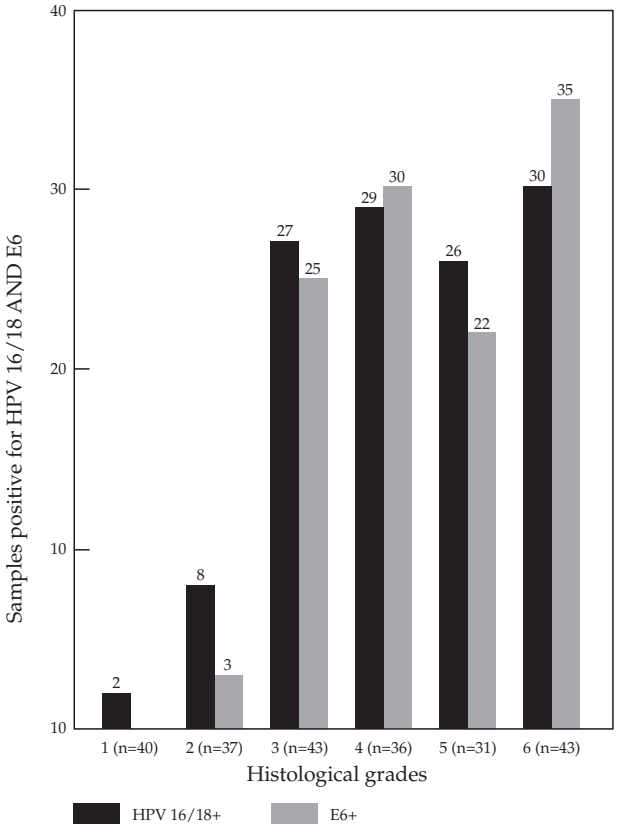

Figure 9. *Bar diagram showing the relationship between HPV 16/18 infection and E6 protein expression in various histological grades of the uterine cervix. 1 = Normal. 2 = Low Grade SIL. 3 = High grade SIL. 4 = Well differentiated squamous cell carcinoma. 5 = Moderately differentiated squamous cell carcinoma. 6 = Poorly differentiated squamous cell carcinoma.*

tion of the p53 check point regulator due to its interaction with high risk HPV E6 may thus impair the apoptotic response to virally infected cells.

Since p53 is a negative regulator of proliferation, this also could explain the higher rates of tumor proliferation as reflected by increased cyclin D1 and Ki 67 expression. The antibody used in the present study for the detection of p53 can detect both mutant and the wild type p53 protein. Wild p53 protein has a short half life (6–20 minutes) but mutant forms have a half life up to 6 hours. Thus detection of p53 by immunocytochemistry is often considered to reflect the mutant form.[25] However this may not be true in all cases since, immunocytochemical analysis of p53 protein may vary according to the antibody used, an issue we have stressed earlier.[11] Moreover in cervical cancer, published data suggests p53 gene mutation to be infrequent.[10] We verified this fact by analysing for mutant specific p53 and found only 12 positive samples of the 230 samples studied. The function of normal p53 protein can be attenuated by the E6 of high risk HPVs (or possibly by other endogenous proteins such as mdm-2), resulting in a functionally inactive form. The strong positive corrlation observed between p53 detected by immunocytochemistry and extent of histological abnormality, could also be on account of the higher synthesis of the p53 protein as a result of excessive DNA damage, accumulation of the E6-p53 complex or due to some factor prolonging the half life of the p53 protein, allowing its detection by immunocytochemistry.

The bcl-2 family of proteins have been shown to be important in the regulation of apoptosis.[19,27] The 21 kD bax protein shares extensive sequence homology with the bcl-2 protein and is capable of forming homodimers or heterodimers with the latter. Increased cellular levels of bax has been found to trigger apoptosis by repressing bcl-2.[23] Expression of bax is known to be upregulated by p53.[18] Inactivated or mutant p53 therefore presumably fails to repress bcl-2 function owing to the low levels of cellular bax protein. We have recently shown that high levels of bcl-2 blocks chemotherapy induced apoptosis.[31] Our current results show a definite correlation between the bax/bcl-2 ratio, p53 accumulation and apoptotic index. There was also a significant positive correlation between bax expression with apoptosis as well as a negative correlation between bcl-2 and apoptosis. This pattern therefore indicates that the bax to bcl-2 ratio is critical in regulating apoptosis.

This study therefore presents data suggesting that modulation of apoptosis and apoptotic regulatory proteins by high risk HPV infection may be an important factor in the development of cervical cancer. The progression of precancerous processes of the uterine cervix to invasive cancer continues to remain an intensely studied problem in gynaecological pathology. Cellular and molecular characteristics of the pathobiology of cervical cancer and its precancerous lesions needs to be identified and elucidated in the context of diagnostic pathology. Such an unified approach may provide the basis for the identification of high risk premalignant lesions, may provide potential targets for intervention and in addition surrogate end point biomarkers for chemopreventive approaches.

Acknowledgement

This study was funded by a research grant from the Department of Science and Technology, Govt of India (No-SP/SO/B-56/95). Pradip Nair is supported by a Research Fellowship from the Council of Scientific and Industrial Research, Govt of India. The authors are most grateful to Professor Balraman Nair of Doctor's Diagnostic Research Centre, Thiruvananthapuram for providing the histopathology reports and the gynecologists of the S.A.T Hospital, Thiruvananthapuram for providing cervical biopsy samples.

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