Chapter - IV
CHAPTER - IV

APOPTOSIS REGULATORY PROTEINS

INTRODUCTION

Oncogenes are genes whose products are associated with neoplastic transformation whereas proto-oncogenes are normal cellular genes that affect growth and differentiation. They can be converted into oncogenes by carcinogenic factors including viruses (Almog and Rotter, 1998). Cancer may arise not only by activation of growth-promoting oncogenes, but also by inactivation of genes that normally suppress cell proliferation (Cancer suppressor genes, or antioncogenes) (Kerr et al., 1994). HPV infection appears to be an early event in cervical carcinogenesis with additional abnormalities being required for biological transformation (Skomedal et al., 1999; Giannoudis et al., 2000). Moreover, DNA viruses appear to exert some of their proliferative and oncogenic effects on the host cell by the interaction of viral-encoded oncoproteins with critical proteins in the cell cycle regulation (Helland et al., 1993). One of the important aspects of the transforming and immortalizing activities of HPVs is their co-operation with oncogenes. It is also evident from the previous studies that the viral infection by HPV is necessary but not sufficient to induce invasive cervical cancer (Pradip Nair et al., 1999b). Additional factors like, cellular oncogenes such as ras, c-myc, c-erb etc. and apoptosis regulatory proteins such as p53, Rb, bcl-2, bax etc. are shown to be involved in cervical carcinogenesis. It is therefore necessary
to study the impact of HPV on the cell cycle regulators like apoptosis regulatory proteins which may improve the understanding of HPV associated cervical carcinogenesis. The study of molecular markers that may be associated with the process of carcinogenesis and its progression may help in a better understanding of the molecular pathogenesis of the disease.

Involvement of Apoptotic Regulatory Proteins

Apoptosis or programmed cell death (PCD) is a genetically-regulated cellular suicide mechanism that plays a crucial role in the development and defence homeostasis (Aejaz Syeed et al., 2001). Apoptosis is an essential and fundamental process including growth, differentiation, tissue remodelling and immunological development (Ravi et al., 2000).

Cancer is a multifactorial disease where there is a gain of immortality due to defective apoptosis (Ravi et al., 2000). Since only a percentage of infected individuals develop cancer after a long latency period, cervical epithelium must undergo additional genetic changes caused by other factors for malignant transformation to occur, in vivo (Zur Hausen, 1989). Modulation of apoptosis and apoptotic regulatory proteins by high-risk HPV infection has been suggested as an important factor in the development of cervical cancer (Pradip Nair et al., 1999b). The tumour suppressor protein p53 and antiapoptotic protein bcl-2 play a major role in the regulation of cell cycle (Kirsch and Kastan 1998; Popescu et al., 1998). Alterations in these apoptotic regulatory proteins
may have a role in the multistaged development of cervical cancer (Pradip Nair et al., 1999b). Although several hundred genes are known to control growth, molecular genetic studies in cancer have shown that a few of them are consistently involved in the natural history of human cancer.

**Involvement of Tumour Suppressor Protein (p53) in Cervical Cancer**

The control of apoptosis is pivotal to embryonic development and the sustenance of adult life. It is conceivable that in many human primary tumour cells, loss of apoptosis is linked to p53 inactivation (Almog and Rotter, 1998). At the gene level, p53, a tumour suppressor gene, located on 17 p13.1, is mutated in more than 50% of almost all human cancers (Harris and Hollstein, 1993; Harris, 1996). Those who inherit a mutant copy of the p53 gene are at a high-risk of developing malignant tumours (Hollstein et al., 1991). However, p53 gene mutations are not common in cancers of the cervix uteri (Helland et al., 1993). At the protein level, p53 is a 53 kDa nuclear phosphoprotein and inactivation of p53 seemed to be important in the tumourigenesis of the uterine cervix (Helland et al., 1998).

The function of the normal wild type p53 is to prevent the propagation of genetically damaged cells by initiating transcription of several genes that cause cell cycle arrest and DNA repair. However, if DNA damage cannot be repaired, p53 induces apoptosis by increasing transcription of the pro-apoptotic genes and by repressing the expression
of anti-apoptotic genes (Ravi et al., 2000). With loss of p53 function, DNA damage goes unrepaired and cells carrying mutant genes continue to divide and eventually give rise to cancer (Kaelin, 1999). p53 alterations provide a selective advantage for clonal expansion of neoplastic cells (Vogelstein and Kinzler, 1992). At the protein level this p53 can also be functionally inactivated by products of DNA oncogenic viruses (Pradip Nair et al., 1999b). A study has shown that the p53 protein overexpression observed was not associated with the presence of mutation in p53 gene (Helland et al., 1993) supporting the view of inactivation of p53 proteins by HPV.

*In vitro* experiments have shown that the high-risk HPV E6 proteins stimulate the degradation of p53 via the ubiquitin dependent proteolysis system (Scheffner et al., 1990) and seems to occur in cytoplasmic proteosomes (Almog and Rotter, 1998). Several previous studies have reported contradictory results on the expression of p53 protein in HPV-associated cervical cancer (Bosari et al., 1993; Pollanen et al., 1993; Crook et al., 1994; Mittal et al., 1995). They concluded that the p53 aberration is not an early event but may be acquired in the course of cervical cancer and is involved in its progression. Furthermore, p53 function is altered in many tumours that retain a wild type p53 allele often found in cervical cancer and other anogenital cancers (Storey et al., 1998). Although the exact sequence of molecular events has not fully been elucidated, loss of p53 function is believed to play an important role in the pathogenesis of carcinoma of the uterine cervix (Miwa et al.,
1995). However, the role of p53 in cervical cancer progression is of considerable controversy.

**Association of p53 with HPV Infection in Cervical Cancer**

*In vitro* studies have shown that HPV-negative cervical carcinoma cell lines exhibit mutation in p53 gene, whereas no such mutations occur in the HPV-positive cell lines (Crook *et al.*, 1991; Yaginuma and Westphal, 1991; Iwasaka *et al.*, 1993). It is therefore hypothesised that p53 can either be inactivated by mutation or by complex formation with HPV oncoproteins and is an essential step for the development of cervical carcinoma (Helland *et al.*, 1998). However, *In vivo* studies have shown the p53 mutation to be infrequent in cervical cancer, irrespective of the HPV status (Borresen *et al.*, 1992; Choo and Chong, 1993; Helland *et al.*, 1993; Paquette *et al.*, 1993; Milde-Langosch *et al.*, 1995; Miwa *et al.*, 1995; Pradip Nair *et al.*, 1999b). An earlier study also has indicated that the p53 protein over expression observed in their study was not associated with gene mutation (Helland *et al.*, 1993) and which may be explained partly by the presence of high-risk HPV subtypes (Skomedal *et al.*, 1999). Further, *in vitro* experiments have shown that the high-risk HVP E6 proteins stimulate the degradation of p53 via the ubiquitin dependent proteolysis system (Scheffner *et al.*, 1990). The binding of p53 and HPV E6 is thought to be mediated by a host cell transcription factor called E6-associated activator protein (E6-AP) (Huibregtse *et al.*, 1993; Scheffner *et al.*, 1993). It has also been shown that the inactivation of p53 is one of the major predictors of failure to
respond to radiotherapy and chemotherapy in many tumour types (Mcllwraith et al., 1994; Buttitta et al., 1997; Cuttilli et al., 1998) and it could be due to high incidence of p53 inactivation as evidenced in squamous cell carcinoma of head and neck disease (Ganly et al., 2000).

However, the understanding of the molecular mechanism underlying HPV-associated cervical carcinogenesis is still incomplete and studies are required to establish the association between p53 and HPV infection.

Involvement of Anti-apoptotic Protein bcl-2 in Cervical Cancer

The bcl-2 gene family seems to act as a regulator of the apoptotic pathway (Tjalma et al., 1997; Adams and Cory, 1998; Tjalma et al., 1999). The two most important apoptosis regulating proteins of this family are bcl-2, a member of the anti-apoptotic family and bax, a member of the pro-apoptotic family (Oltvai et al., 1993). Together they probably act as a rheostat for the cell death program (Oltvai et al., 1993; Adams and Cory, 1998). Bax is a dominant inhibitor of bcl-2 and has ~ 21% homology to the bcl-2 protein. It can form heterodimers with bcl-2 and abrogate its ability to suppress apoptosis (Oltvai et al., 1993). The bcl-2 can rescue cells from apoptosis. Although bcl-2 was extensively studied in hematopoietic tissues, its increased expression has also been described in several epithelial tumours, such as colonic neoplasms, associated with a better clinical prognosis (Bosari et al., 1995).
In breast cancer, a strong correlation was observed between expression of bcl-2 and estrogen receptor-positive and epidermal growth factor receptor-negative tumours (Dimitrakakis et al., 2000). Similarly, in the prostate, another hormone-regulated organ, high concentrations of bcl-2 protein were found in androgen-independent carcinomas (Colombel et al., 1993). Furthermore, an inverse relationship may exist between p53 and bcl-2 expression in breast (Haldar et al., 1994) and ovarian cancer which in turn implies that bcl-2 may be regulated by an estrogen receptor signalling pathway (Leek et al., 1994). Unlike breast cancer, bcl-2 expression in neuroblastoma has been associated with poorly differentiated, high-grade histological types and with amplification of the N-myc oncogene (Castle et al., 1993). The above data suggest that the bcl-2 and p53 pathway leading to programmed cell death may not be regulated in the same way among different cancers. The regulation of cell life and death in vivo, however is complex and the relative contributions of various genes may be tissue specific.

**Association between bcl-2 and HPV Infection**

An *in vitro* study has demonstrated increased bcl-2 expression in the absence of HPV infection and in the presence of inactive p53 (Liang et al., 1995). In contrast to lymphomas, little or no evidence for gross alterations in bcl-2 gene structure has been obtained for other types of cancer, suggesting that alternative mechanisms for dysregulation of bcl-2 gene expression may exist in human malignancies (Miyashita et al., 1994). Little is known about the relationship between bcl-2 and HPV
infection in cervical cancer. Few studies conducted on the association of bcl-2 with HPV infection also have reported contradictory results. No correlation was found between HPV positivity and bcl-2 expression in earlier studies conducted by Brychtova (Crawford et al., 1998; Brychtova et al., 2000). But another in vitro study (Radhakrishna Pillai et al., 1996) and an in vivo study (Pradip Nair et al., 1999b) have found strong association between the presence of HPV E6 protein and bcl-2 expression. However, the significance of bcl-2 in HPV associated cervical cancer still remains to be determined. Studies are therefore needed to understand the complete molecular mechanism underlying HPV associated cervical carcinogenesis.

WORKING HYPOTHESIS

Although HPV plays an important role in the pathogenesis of cervical cancer, it alone is not sufficient for the induction of cervical cancer. Additional factors contribute to the multistage process leading to cervical cancer (Giannoudis et al., 2000). Alteration in oncogenes and/or tumour suppressor genes appear to be required in parallel to infection (Herrington, 1995). Modulation of apoptosis and apoptotic regulatory proteins by high-risk HPV infection may be an important factor in the development of cervical cancer (Pradip Nair et al., 1999b). Obviously, the regulation of apoptosis in neoplastic tissue seems to be a complex process that is probably differently regulated depending on tumour type (Staunton and Gaffney, 1995). Studies are therefore necessary to elucidate the involvement of apoptotic regulatory proteins in HPV
associated cervical carcinogenesis. Several genes are known to regulate apoptosis and of which, the tumour suppressor p53 and anti-apoptotic gene bcl-2 play a major role in HPV induced cervical cancer. A hypothesis evolved that p53 can either be inactivated by mutation or complex formation with HPV oncoproteins (Ho et al., 1998c). It is also accepted that the neutralization of p53 function by any form of inactivation, is a common, and possibly a requisite step in human cancer (Kaelin, 1999). Based on this view, we have made an attempt to analyse the p53 expression in relation to histopathologic progression and HPV status of cervical cancer.

The over expression of bcl-2 protein can block apoptosis (Reed, 1994). Increased bcl-2 expression was observed in cervical carcinoma cell lines containing mutated or HPV E6 inactivated p53 (Liang et al., 1995). However its part in the development or progression of epithelial malignancies in *in vivo* is not yet understood. The significant role of these apoptotic regulatory proteins in the progression of cervical cancer from dysplasia into invasive cancer has not been established (Pradip Nair et al., 1999b; Clarke and Chetty, 2001). However, when p53 function is abrogated as in the case of HPV-infected cervical carcinoma cells or in cervical carcinoma cells containing mutated p53, the repression of bcl-2 gene by p53 is removed, allowing its over expression. The present study has been evolved based on this hypothesis, to analyse the tissue type specific expression of bcl-2 and its role in cervical cancer progression. It is also important to study the association between bcl-2 and HPV infection in cervical cancer. The present study was therefore focussed to
assess the expression of p53 and bcl-2 proteins in different stages of HPV associated cervical lesions. We believe that these findings may help in the early diagnosis and pave the way for immunotherapeutic approach.

**STUDY DESIGN**

This study was carried out in one hundred and twenty five cervical specimens, consisting of normal cervical epithelium (n=20), lesion with histologic features of mild dysplasia (n=20), lesion with histologic features of severe dysplasia (n=25) and invasive cervical carcinomas (n=60), in which the HPV status have been analysed already (Chapter III). Histopathologic grading of specimens were carried out by Haematoxylin Eosin staining and classified based on the Bethesda system of classification. Both p53 and bcl-2 immunoreactivity were analysed in the same biopsy samples. The data obtained were subjected to correlation analysis with cancer progression and HPV status.

The mean age at the time of the diagnosis was 38 years (range 23-60 years) for patients with dysplasias and 54 years (range 32 to 67 years) for patients with invasive cancer. Normal samples were obtained from women with an average age of 45 years (21 to 60 years) who had no pathological findings in uterine cervix in a pathologist's examination.
METHODOLOGY

Study Sample

Cervical tissues were collected and processed as given in Chapter III. Paraffin tissue sections of 4μm thick, were placed on poly-L-lysine coated glass slides. One section from all samples was stained with hematoxylin-eosin for routine histopathologic and conventional light microscopic analysis (Appendix) and duplicate serial sections were used for immunocytochemistry for p53 and bcl-2 expression.

Immunocytochemistry for p53 and bcl-2

Paraffin was removed from tissue sections by incubating at 60°C for 1 hr and then washed three times in xylene for complete dewaxing. The sections were gradually rehydrated using alcohol and distilled water. Slides were then incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. The sections were incubated in citric acid buffer and pressure cooked for antigen retrieval. Sections were then incubated overnight with the respective primary monoclonal antibodies such as, p53 (Anti p53, Clone DO-7, DAKO, DAKO A/S, Denmark, dilution 1:50, kindly gifted by Prof.M.Radhakrishna Pillai, Division of Lab Medicine, TVM). And bcl-2 (anti bcl-2, DAKO A/S, Denmark, dilution 1:50, kindly gifted by Prof.M.Radhakrishna Pillai, Division of Lab Medicine, TVM). The reaction products were visualized using a streptavidin-biotin-immunoperoxidase complex (DAKO) as secondary antibody complex with DAB. All sections were then counter stained with haematoxylin (detailed protocol is given in Appendix).
Sections of normal lymph node tissue and breast cancer sample with p53 gene mutation were used as positive controls for bcl-2 and p53 immunoreactivity. Sections without primary antibody treatment were used as negative control.

Assessment of Immunoreactivity

Immunoreactivity of p53 and bcl-2 was graded based on the intensity of the brown DAB colour development and the cell layer showing positivity. In invasive cancer, immunoreactivity was recorded on the basis of the percentage of basaloid cells which had positive immunoreactivity because the epithelial morphology was lost. To analyse the immunoreactivity of p53 (nuclear), total of 100 cells were evaluated in all sections and the percentage of positive cells was calculated. Expression of p53 was considered "Significant" when characteristic nuclear immunoreactivity was present in at least 10% of the tumour cells. Similarly, bcl-2 (cytoplasmic) was considered "Significant" when cytoplasmic immunoreactivity was evident in at least 10% of tumour cells. An immunoreactivity index was created by classifying the protein expression into four categories as given in Table 6. Normal samples showed insignificant immunoreactivity for p53 as well as bcl-2 and taken as negative immunoreactivity index.

Data Analysis

Correlation analysis was carried out between the immunoreactivity and histopathologic grades for both p53 and bcl-2 using Spearman's
Table 6: Immunoreactivity Assessment for p53 and bcl-2 Proteins in Cervical Cancer

<table>
<thead>
<tr>
<th>Grades</th>
<th>Immunoreactivity (%)</th>
<th>Expression index</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0-10</td>
<td>Insignificant (negative)</td>
<td>(-)</td>
</tr>
<tr>
<td>2.</td>
<td>11-25</td>
<td>Mild</td>
<td>(+)</td>
</tr>
<tr>
<td>3.</td>
<td>26-50</td>
<td>Moderate</td>
<td>(++)</td>
</tr>
<tr>
<td>4.</td>
<td>above-50</td>
<td>Intense</td>
<td>(+++)</td>
</tr>
</tbody>
</table>
correlation test. This is to find out the association of apoptotic regulatory proteins with tumour progression. The association between HPV status and the respective apoptotic regulatory proteins (p53 & bcl-2) was also studied by Univariate statistical correlation analysis.

RESULTS

p53 expression in Relation to Histopathologic Grades of Cervical Cancer

A distinct nuclear immunoreactivity for p53 was judged as positive. The expression of p53 protein was represented in 4 groups. This was carried out by counting 100 cells in one area for both positive and negative cells and the difference was taken as expression index. The groups have values 0-10 (negative for p53), values 11-25 (mild expression), values 26-50 (moderate expression) and values above 50 (intense expression).

The histopathologic grades were assigned into 4 groups as mentioned earlier and the p53 expression was correlated with these histopathologic stages in the same 125 study population. The different intensities of p53 expression in relation to the histopathologic stages are shown in Plate 3. The significant expression of p53 protein increases as the tumour progresses from dysplasia to invasive cancer and insignificant in normal sample.

The data for p53 immunoreactivity among the 4 histopathologic stages are given in Table 7. We observed that all the 20 normal samples analysed were negative for p53 expression (100%). In mild dysplasia
Plate 3. Nuclear p53 immunoreactivity in different histopathologic grades of cervical lesions

a. Normal cervical epithelium showing negative immunoreactivity for p53 (40X)
b. Negative control showing negative Immunoreactivity for p53 (40 X)

c. p53 immunoreactivity in dysplastic lesions of cervical epithelium (20 X)
d. p53 immunoreactivity in invasive cervical cancer epithelium (40 X)
Table 7: Association between p53 and Histopathologic Stages

<table>
<thead>
<tr>
<th>Study population (n=125)</th>
<th>p53 negative (values 0-10)</th>
<th>Mild expression (values 11-25)</th>
<th>Moderate expression (values 26-50)</th>
<th>Intense expression (values above 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=20)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild dysplasia (n=20)</td>
<td>14</td>
<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Severe dysplasia (n=25)</td>
<td>0</td>
<td>7</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Invasive cancer (n=60)</td>
<td>0</td>
<td>1</td>
<td>33</td>
<td>26</td>
</tr>
</tbody>
</table>
cases, 6 were positive for p53 (30%) and 14 were negatively stained for p53 (70%). But in severe dysplasia and invasive cancer there were no p53 negative cases and in invasive cancer 26 (43.3%) cases were intensively stained for p53 out of the 60 cases analysed.

There was a highly significant correlation between p53 expression and increasing histopathologic stages of cervical cancer \( (r=0.88842; p=0.00001) \) (Fig.9). In addition, p53 accumulation was highly associated with advanced invasive cancer with intense expression.

**Association between HPV Infection and p53 Protein Expression**

The immunoreactivity of p53 was analysed in the same 125 study population in which HPV subtypes were analysed and statistically correlated with the HPV status. Table 8 explains the data analysis.

Among the total 42 HPV negative cases 26 were also negative for p53 expression (61.9%). The remaining samples showed mild to intense positive expression for p53. All the low-risk HPV6 infected cases were negative for p53 and only one low-risk HPV 11 positive case showed moderate p53 expression. Whereas, out of 67 HPV 16 positive cases analysed, only 5 were negative for p53 (7.4%) and remaining 62 cases showed mild to intense expression, 62/67 (92.5%). In the case of HPV 18 positive cases none of them showed negative expression for p53 and all the 12 cases showed mild to intense p53 expression. These results indicate the higher degree of overexpression of p53 in HPV positive cases when compared to HPV negative cases and also, we observed intense expression in cases infected with high-risk type HPV 16/18. This indicates the close association between the HPV infection and p53 accumulation in cervical carcinogenesis.
Figure 9  Correlation between p53 Expression and Histopathologic Progression in Cervical Cancer

1.00 - Normal
2.00 – Mild dysplasia
3.00 – Severe dysplasia
4.00 – Invasive cancer
* p53 immunoreactivity is in mean expression grouped in a range
Table 8: Association between HPV Status and p53 Expression

<table>
<thead>
<tr>
<th>Study population (n=125)</th>
<th>p53 negative (values 0-10)</th>
<th>Mild expression (values 11-25)</th>
<th>Moderate expression (values 26-50)</th>
<th>Intense expression (values above 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV negative (n=42)</td>
<td>26</td>
<td>5</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>HPV 6 positive (n=3)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV 11 positive (n=1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>HPV 16 positive (n=67)</td>
<td>5</td>
<td>6</td>
<td>35</td>
<td>21</td>
</tr>
<tr>
<td>HPV 18 positive (n=12)</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>
Statistical analysis of this data revealed a highly significant correlation between HPV status and p53 accumulation in cervical cancer tissues \( (r = 0.62015, p = 0.000001) \) (Fig.10).

**Bcl-2 Expression in Relation to Histopathologic Grades of Cervical Cancer**

The immunoreactivity for bcl-2 was analysed in the same 125 study population and the results were correlated with the four histopathologic stages to find out whether there is any relation between bcl-2 expression and cancer progression. Bcl-2 immunoreactivity was found throughout the cytoplasm with low concentration in the perinuclear zone in tumour cells. The intensity of immunostaining and the distribution of positive cells were heterogenous. Plate 4 shows the increased expression of bcl-2 as the cancer progresses histopathologically.

The 20 normal subjects analysed showed negative expression for bcl-2. Among the 20 mild dysplasia cases 13 were negative for bcl-2 (65%) and 7 cases showed mild and moderate expression. In severe dysplasia and invasive cancer none of them showed negative expression. Out of 60 invasive cancer 2 cases showed mild expression 43 cases showed moderate expression (71.7%). Also, 15 cases stained intensively for bcl-2 among the 60 invasive cases (25%). Table 9 elaborates the data regarding this.

From the statistical analysis we suggest that there is a highly significant correlation between bcl-2 expression and histopathologic
Fig. 10 Association of HPV infection with p53 expression in cervical cancer
Plate 4. Cytoplasmic bcl-2 immunoreactivity in different histopathologic grades of cervical lesions

a. Normal cervical epithelium showing negative bcl-2 immunoreactivity (40 X)

b. bcl-2 immunoreactivity in dysplastic lesion of cervical epithelium (20 X)

c. bcl-2 immunoreactivity in carcinoma in situ epithelium of cervix (40 X)

d. bcl-2 immunoreactivity in invasive cervical cancer epithelium (40 X)
Table 9: Association between bcl-2 Expression and Histopathologic Stages

<table>
<thead>
<tr>
<th>Study population (n=125)</th>
<th>bcl-2 negative (values 0-10)</th>
<th>Mild expression (values 11-25)</th>
<th>Moderate expression (values 26-50)</th>
<th>Intense expression (values above 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=20)</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mild dysplasia (n=20)</td>
<td>13</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Severe dysplasia (n=25)</td>
<td>0</td>
<td>3</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Invasive cancer (n=60)</td>
<td>0</td>
<td>2</td>
<td>43</td>
<td>15</td>
</tr>
</tbody>
</table>
progression \((r=0.86929; p=0.00001)\) (Fig.11). Increased expression of bcl-2 was observed with different intensity as the cancer progresses.

**Association between HPV Infection and bcl-2 Expression**

Distinctive cytoplasmic immunoreactivity for bcl-2 was taken as positive. Like p53, bcl-2 expression was also assigned into four groups such as negative, mild, moderate and intense expression and correlated with HPV status. The data are given in Table 10 and show that among the 42 HPV negative cases 26 were negative for bcl-2 (61.9%) and the remaining were positive for bcl-2 expression. All the 3 HPV 6 infected cases showed negative reaction for bcl-2 and only one HPV 11 analysed showed moderate expression for bcl-2. Among the 67 HPV 16 positive cases only 4 were negative for bcl-2 and 94% (63/67) cases showed mild to intense bcl-2 expression. In HPV 18 positive cases none of them showed negative expression. All of them showed mild to intense positive reaction for bcl-2.

Statistical analysis revealed highly significant correlation between HPV status and bcl-2 expression \((r=0.68130; p=0.00001)\) (Fig.12). This result is more or less similar to the correlation between p53 and HPV status. This suggests the co-overexpression of p53 and bcl-2 in HPV associated cervical cancer.
Figure 11  Correlation between bcl-2 Expression and Histopathologic Progression in Cervical Cancer

1.00 - Normal  
2.00 – Mild dysplasia  
3.00 – Severe dysplasia  
4.00 – Invasive cancer  
* bcl-2 immunoreactivity (mean expression)  
Values < 10 - bcl-2 negative  
Values 11-25 – mild expression  
Values 26-50 – moderate expression  
Values > 50 – intense expression
Table 10: Association between HPV Status and bcl-2 Expression

<table>
<thead>
<tr>
<th>Study population (n=125)</th>
<th>bcl-2 negative (values 0-10)</th>
<th>Mild expression (values 11-25)</th>
<th>Moderate expression (values 26-50)</th>
<th>Intense expression (values above 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV negative (n=42)</td>
<td>26</td>
<td>8</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>HPV6 positive (n=3)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPV11 positive (n=1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>HPV16 positive (n=67)</td>
<td>4</td>
<td>2</td>
<td>48</td>
<td>13</td>
</tr>
<tr>
<td>HPV18 positive (n=12)</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 12 Association of HPV Infection with bcl-2 Expression in Cervical Cancer
DISCUSSION

p53 Expression in Relation to Histopathologic Progression of Cervical Cancer

In cancer biology, it is becoming increasingly apparent that many cancer cells circumvent the normal apoptotic mechanism to prevent their self destruction, by harbouring mutations or genetic damage (Cotter and Samali, 1997). Numerous genetic factors have been shown to modulate apoptosis and the interactions among these factors are complex and still incompletely defined (Guchelaar et al., 1997). The p53 gene is one of the most frequently mutated genes in human cancers and is the key factor in cell cycle checkpoint regulation (Sidransky and Hollstein, 1996). The wild type p53 is known to induce apoptosis when over expressed (Kagawa et al., 1997). The p53 protein activates the death gene bax and downregulates the survival genes like bcl-2 (Miyashita et al., 1994).

Several studies reported that p53 over expression is more frequent in squamous cervical carcinomas than in adenocarcinomas (Bosari et al., 1993; Holm et al., 1993). Accumulation of p53 protein is often considered to indicate the mutant form that is unable to regulate the cell cycle and apoptosis (Sidransky and Hollstein, 1996). However, the association between p53 expression and cervical cancer progression is not well understood because of the accumulation of contradictory reports. A recent report has shown that the p53 expression in cervical cancer progression is a late event with increased expression in advanced CIN lesion and invasive cervical cancer (Dimitrakakis et al., 2000). Recently, another study also has observed overexpression of p53 protein only in invasive
cancer and they could not find p53 immunoreactivity in CIN lesions, suggesting the p53 overexpression as a late event in tumourigenesis (Clarke and Chetty, 2001). Some other studies have shown the increased expression of p53 in early stages of cervical cancer also (Vassallo, 2000).

The present study showed a much higher percentage of cases with p53 expression in the study population. The results show an obvious significant correlation between p53 expression and increasing histopathologic grades of cervical cancer (r=0.88842; P=0.00001) Table 7 and Fig (9). We have also observed mild to moderate expression of p53 in few mild dysplasia cases (30%) in addition to the overexpression of p53 in severe dysplasia (100%) and invasive cancer (100%). Our report is therefore in agreement with the report of Pradip Nair et al. (1996) which showed the significant correlation between p53 expression and tumour progression.

**Involvement of p53 in HPV Infection**

The p53 gene promotes growth arrest and cell death by apoptosis in response to genotoxic insults, and has been termed as the "guardian of the genome". Impaired function of this p53 protein by viral genomes such as HPV and SV40 may lead to direct stabilization of the wild-type protein (Boabang et al., 2001). Cells lacking wild type p53 or expressing inactivated and stabilised p53 protein or mutant p53 fail to arrest the cell cycle in response to DNA damage. Unlike most of the solid tumours, the mutation in p53 gene is found to be infrequent in cervical cancer (Koshida et al., 1997, Popescu et al., 1998; Pradip Nair et al., 1999b),
which suggests the involvement of some other mechanism for the overexpression of p53 in cervical cancer. It is evident from the studies on cell lines that the inactivation of p53 through interaction and complex formation with the HPV E6 oncoproteins is a characteristic feature of cervical cancer (Ku et al., 1997; Thomas et al., 1999). It has been suggested that the binding of these proteins in vivo may alter cellular regulatory functions such as proliferation and transformation (Herrington and McGee, 1992). In the present study we made an attempt to evaluate the association between the apoptosis regulatory protein, p53 and HPV status in cervical cancer.

In the present study, an obvious association was observed between p53 expression and HPV status in the study population. The result has shown higher p53 expression in HPV positive cases when compared to HPV negative cases. Patients infected with low-risk HPV subtypes 6/11 failed to show p53 immunoreactivity (except 1 case with HPV 11 infection). A relatively higher level of p53 immunoreactivity was observed in HPV16 infected cases. Interestingly, all the HPV18 infected cases showed immunoreactivity for p53. In accordance with the reports of Paquette et al. (1993) and Pradip Nair et al. (1999b), our results also suggest that the p53 protein inactivation by complex formation with high-risk HPV 16/18 subtypes may be responsible for the overexpression of p53 observed in cervical cancer.

Crook et al. (1994) have suggested from their study that some degree of modulation of p53 function is necessary in the normal viral life
cycle and also demonstrated a correlation between the efficiency of this activity and oncogenic potential of the virus. It has also been demonstrated that p53 protein complexes with the HPV 16/18 E6 protein in the cytoplasm (Liang et al., 1993). This complex is thought to target p53 for rapid degradation via ubiquitination (Picksley and Lane, 1994). Further, HPV E6 protein can also interfere with the normal function of p53 by its ability to abrogate both transcriptional activation and transcriptional repression function of the p53 gene (Lechner et al., 1992; Mietz et al., 1992). The loss of function of the p53 check point regulation due to its interaction with high-risk HPV E6 may thus impair the apoptotic response to virally infected cells.

However, certain other studies could not find any significant correlation between p53 accumulation and HPV status (Kurvinen et al., 1996; Vassallo et al., 2000). Further, Kurvinen et al., (1996) have reported very little or absence of p53 expression in high grade lesions with high-risk HPV 16/18 infection when compared to low-risk lesions with HPV 6/11 infection, which is contradictory to our results.

bcl-2 in Relation to Histopathology of Cervical Cancer

The proto-oncogene bcl-2 has been proven to be a central player in mammalian cell death pathways (Miyashita et al., 1994). The bcl-2 gene becomes transcriptionally deregulated in the majority of Non-Hodgkins’s B-cell lymphomas where it is involved in t(14;18) chromosomal translocation (Tsujimoto et al., 1985). High levels of bcl-2 protein and/or aberrant expression of bcl-2, however have been described in a wide
variety of human cancers in the absence of gross structural alterations in the bcl-2 gene, including many leukemias, neuroblastomas and carcinomas of the lung, prostate, colon and nasopharynx (Reed et al., 1991; Mc Donnell et al., 1992; Campos et al., 1993; Lu et al., 1993; Pezzella et al., 1993). An investigation on bcl-2 transcription and protein expression in cultured cervical carcinoma cell lines has shown that all the cell line had increased bcl-2 expression but there was no chromosomal translocation or rearrangement of the bcl-2 gene. Each cell lines of that study also had (or) expressed inactive p53 either by mutation or by complex formation with HPV oncoproteins (Liang et al., 1995).

Only a limited number of studies are available on bcl-2 protein expression in cervical cancer progression and the results of most of them are contradictory to each other. Currently available studies on bcl-2 have concentrated on the prognostic value of bcl-2 in cervical cancer. The understanding of the involvement of bcl-2 protein in cervical cancer progression is the fundamental aspect for diagnostic and prognostic, approaches. Few studies made on bcl-2 expression have shown a relatively increased expression of bcl-2 in the early stages of cervical lesions (Crawford et al., 1998; Dimitrakakis et al., 2000), breast cancer (Binder et al., 1995) and gastric cancer (Koshida et al., 1997). However, bcl-2 expression was also observed in advanced stages of cervical lesions like severe dysplasia (Kurvinen et al., 1996; Radhakrishna Pillai et al., 1996), invasive cancer (Brychtova et al., 2000) and in both HSILs and invasive cancer (Pradip Nair et al., 1999b).
In the present study we have also analysed the bcl-2 expression in cervical cancer progression and our results are more consistent with the studies showing, increased expression of bcl-2 in severe dysplasia and invasive cancer (Brychtova et al., 2000). Our study also has shown the gradual increase in bcl-2 expression from mild dysplasia to invasive cancer with a statistically significant correlation ($r=0.86929; p=0.00001$), which suggest the strong association between bcl-2 expression and cervical cancer progression (Table 9 & Fig 11). Thus our study supported the hypothesis that the modulation of apoptosis and apoptotic regulatory proteins are the prerequisites for carcinogenesis, which was suggested earlier by Pradip Nair et al., (1999b) and Brychtova et al. (2000).

**Association between bcl-2 and HPV Infection**

Most of the tumours including breast (Haldar et al., 1994; Binder et al., 1995), gastric (Koshida et al., 1997), colorectal (Popescu et al., 1998) and Ovarian (Diebold et al., 1996) shows mutation in p53 and downregulation of bcl-2. Wild type p53 has the ability to downregulate bcl-2, *in vivo* (Ravi et al., 2000). In the case of cervical cancer, p53 gene mutation is found to be less frequent (Pradip Nair et al., 1999b), suggesting the existence of wild type p53 protein in cervical cancer. But the published data as well as our present study has shown overexpression of both p53 and bcl-2 protein. This suggests the influence of some other major factor in the apoptosis regulatory mechanism in cervical cancer. Increased bcl-2 protein expression has been reported in cervical carcinoma cell lines containing mutated or E6-inactivated p53
(Liang et al., 1995). Another study also has demonstrated a strong association between the presence of HPV E6 protein and bcl-2, and suggested that the detection of which may be helpful for identification of women at increased risk for developing cervical cancer (Radhakrishna Pillai et al., 1996).

In the present study, a significant association between bcl-2 expression and HPV infection \( (r=0.68130; \ p=0.00001) \) was observed on correlation analysis. The correlation observed between bcl-2 protein and HPV status was nearly similar to the correlation observed for p53 expression, with HPV status. This suggests that the aberrant expression of both p53 and bcl-2 are interlinked with high-risk HPV infection in cervical cancer. All the HPV 6 infected cases showed negative immunoreactivity for bcl-2 and among the 67 HPV 16 infected cases, except 4 cases (2.68%) all others were positive for bcl-2. All the HPV 18 infected cases showed bcl-2 immunoreactivity. Thus our study showed higher percentage of bcl-2 immunoreactivity in high-risk HPV 16/18 infected cases. Our result also supports the view of Radhakrishna Pillai, reported earlier (Radhakrishna Pillai et al., 1996).

Evidences are also emerging that viruses interact with the cell death pathway at the check points defined by the bcl-2 family proteins for their site of intervention. It is also known that DNA viruses parasitize the host cellular machinery to drive their own replication (Oltvai and Korsmeyer, 1994). This suggests a strong effect of HPV on
bcl-2 at the gene level. But it is not clear whether the upregulation of bcl-2 is a direct effect of HPV infection or not (Liang et al., 1995).

However, when p53 function is abrogated, as in the case of HPV infection, cervical carcinoma cells or in cervical carcinoma cells containing mutated p53, the repression of bcl-2 gene by p53 is removed, allowing its expression.