Chapter - VI
CHAPTER VI

INTERRELATIONSHIP BETWEEN THE APOPTOSIS REGULATORY PROTEINS AND PROLIFERATION MARKER IN CERVICAL CANCER

INTRODUCTION

Over the last 20 years, considerable information has been gathered on regulation of cell growth and proliferation leading to the identification of proto-oncogenes and tumour suppressor genes. But still the interrelated process involved in carcinogenesis is not completely understood because of the conflicting reports in various malignancies. In gastric carcinoma, it has been shown that apoptosis is closely associated with cell proliferation, bcl-2 and bax protein expression (Oltavi et al., 1993). But in ovarian carcinoma, apoptosis, bcl-2 expression and p53 accumulation were not correlated with each other (Diebold et al., 1996). The regulation of cell life and death, in vivo, however is complex and the relative contributions of various genes may be tissue specific and in cervical carcinogenesis also, the interrelated mechanisms are not yet elucidated.

The development and progression of cervical cancer is likely to be associated with alterations in apoptosis, disturbances in immune surveillance, increased cell growth and/or loss of growth suppression (uncontrolled proliferation) (Tjalma, 1999b). Apoptosis is a complex
network of biochemical pathways with fine regulatory mechanisms controlling death events in a cell (Ravi et al., 2000). However, escape from apoptosis alone may not be associated with tumour growth and progression because, tumour cell accumulation may occur if excessive proliferation offsets cellular loss by apoptosis and the central control of proliferation is through cell cycle control (Darnton, 1998).

Both the tumour-suppressor gene p53 and apoptosis control gene bcl-2 have been shown to be associated with carcinogenesis (Dimitrakakis et al., 2000). In carcinogenesis, proto-oncogenes can be activated to produce oncogenic activity and tumour suppressor genes can be lost or inactivated to release control of cellular proliferation (Darnton, 1998).

The loss of p53 function can increase both the pool of proliferating cells and the probability of their neoplastic transformation (Harris and Hollstein, 1993) bcl-2 expression also has been demonstrated in many tumours (Kokawa et al., 1999). The importance of bcl-2 in cervical cancer progression and the interaction with other genes involved in carcinogenesis are, however, not yet understood. It has also been suggested that a better understanding of their role may probably provide the basis for more rational cancer therapies in the future (Tjalma et al., 2001). The present study was also focused to analyse the interaction between the apoptosis regulatory proteins p53, bcl-2 and the proliferation marker PCNA, by correlation analysis.
Interaction between p53 and bcl-2 Proteins in Cervical Carcinogenesis

Though much has been known recently about the mechanisms by which p53 suppresses cell cycle progression (Soddu and Sacchi, 1997; Darnton, 1998), little is known about how p53 induces apoptosis (Sidransky and Hollstein, 1996). The p53 protein resides primarily in the nucleus, binds to specific DNA sequences and functions at least in part as a transcriptional regulator of certain genes responsible for cell cycle arrest, DNA repair and initiation of apoptosis (Vogelstein and Kinzel, 1992; Sidransky and Hollstein, 1996).

p53 can induce apoptotic cell death through an unknown mechanism (Miyashita et al., 1994), which can however be blocked by elevations in the levels of bcl-2 protein (Wang et al., 1993), suggesting that p53 and bcl-2 may participate in a common pathway for regulation of cell life and death. The high level expression of both p53 and bcl-2 proteins in HPV associated cervical carcinomas was reported by many studies (Pradip et al., 1999b; Dimitrakakis et al., 2000; Tjalma et al., 2001) and also our present study, prompted us to explore the interrelationship between these apoptotic regulatory proteins. There appears to be an inverse relationship between p53 and bcl-2 expression in breast cancer (Haldar et al., 1994), ovarian cancer (Henriksen et al., 1995), lymphoma (Nguyen et al., 1996) and colorectal cancer (Popescu et al., 1998). This suggests an interaction between these two factors in the regulation of apoptosis in various malignancies. The correlation
between p53 and bcl-2 expression in cervical cancer is however not clear because of conflicting reports.

A study of the association between these apoptotic regulatory proteins may be a prerequisite for the complete understanding of the molecular pathogenesis underlying cervical cancer development. It has also been suggested that coincident expression of c-myc, proto-oncogene, and bcl-2 effects the subcellular localization of p53 during the cell cycle, which may result in functional inactivation of normal p53. The overexpression of bcl-2 protein can block apoptosis and prolong cell survival and it may play an important role in the process of carcinogenesis (Garcia et al., 1992; Reed, 1994).

**Association between Apoptosis Regulatory Proteins p53, bcl-2 and Proliferation Marker PCNA**

It is widely accepted that an aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies (Levine, 1992; Haldar et al., 1994; Lane and Benchimol, 1994; Miyashita et al., 1994; Diebold et al., 1996; Pradip et al., 1999b). p53 has been suggested to be a cell cycle protein because of its involvement in the control of cell proliferation and localizes in the nucleus (Vogelstein and Kinzel, 1992; Oka et al., 2000) and its exact biochemical function is not known (Dimitrakakis et al., 2000; Tjalma et al., 2001). It has been postulated that p53 could have both a role in regulating transcription of genes that suppress cell proliferation (Fields and Jang, 1990) and a biological function as a G1 checkpoint control
allowing the repair of DNA (Lane, 1992). p53 is also regarded as a multifunctional protein, with the ability to adopt two alternative conformations. One of these, the wild type phenotype, has a suppressor function for cell proliferation, whereas its mutant form has a promoting effect on proliferation (Milner, 1991). The regulation of G1/S boundary is a critical check point in the cell cycle and is potentially inhibited by the p53-induced p21 inhibitor (Darnton, 1998).

It has also been suggested that the p53-mediated cell cycle inhibitor p21 may interfere with DNA synthesis directly by binding to PCNA, (Flores-Rozas et al., 1994; Waga et al., 1994; Soddu and Sacchi, 1997), an essential factor in DNA replication. p53 is also shown to induce another factor, GADD 45 (growth arrest DNA damage) protein, which can also bind to PCNA and inhibit DNA synthesis (Sidransky and Hollstein, 1996; Soddu and Sacchi, 1997; Darnton, 1998). However the association between p53 and PCNA in cervical cancer is not clear. Further studies on the functions of p53 in normal, dysplastic and neoplastic tissues would facilitate our understanding of its role in the regulation or deregulation of cell proliferation during carcinogenesis and tumour progression. In order to understand the tumour progression in cervical carcinoma, it is necessary to analyse the association between p53 immunoreactivity and cell proliferation status.

Overexpression of bcl-2 specifically prevents cells from initiating apoptosis in response to a number of stimuli, whereas it has little or no ability to promote cell cycle progression or cell proliferation (Crawford
et al., 1998). But still, in the present study, we have analysed the association between bcl-2 and PCNA expression in cervical cancer in a hope that the HPV-related cancer may have interlinkage between bcl-2 protein and cell proliferation.

WORKING HYPOTHESIS

According to the hypothesis of Nowell (1976) cancer develops through the stepwise accumulation of genetic events that lead to genetic instability and the progressive loss of growth regulation. This suggests the role of a highly interlinked mechanism underlying molecular pathology of cancer development. As far as the cancer of cervix is concerned, the HPV infection causes an even more complicated process by interacting with host cell gene regulation system. This viral infection may lead to cellular transformation through inactivation of p53 activity and/or upregulation of bcl-2 expression in cervical cancer (Liang et al., 1995). p53 acts as an extra defense mechanism by selectively destroying aberrant cells by promoting apoptosis (Darnton, 1998). Overexpression of bcl-2 specifically prevents cells from initiating apoptosis in response to a number of stimuli (Kernohan and Cox, 1996; Crawford et al., 1998). All these reports suggest that both p53 and bcl-2 are antagonistic in their function and also interregulated in normal cell cycle. Therefore, one may expect an inverse relationship between their expression level in cancer as evidenced in most of the malignancies (Haldar et al., 1994; Henriksen et al., 1995; Nguyen et al., 1996; Popescu et al., 1998). But this may not
be true in the case of cervical cancer because of the influence of HPV infection.

Since p53 is a negative regulator of proliferation (Soddu and Sacchi 1997; Darnton, 1998) there must be a strong association between these apoptosis regulatory proteins and proliferation marker (Pradip Nair et al., 1999b). The finding that p53 induces the decrease in bcl-2 and increase in bax expression may have important implications for the mechanism of p53-induced apoptosis and this hypothesis in turn may have an effect on the uncontrolled proliferation. Based on this hypothesis, the present study was designed to analyse the interrelationship between the factors involved in HPV-associated cervical carcinogenesis.

STUDY DESIGN

The analysis of an interrelationship between the oncogenic factors in cervical cancer has been achieved by statistical correlation analysis of the data given in the chapters IV and V. The study population is also the same as given in previous chapters. Spearman’s and Pearson’s correlation tests were applied for correlation analysis using SPSS software system.

RESULTS

Interrelationship between the Apoptosis Regulatory Proteins, p53 and bcl-2 Expression

The previously given data of immunoreactivity for p53 and bcl-2 were correlated in the same 125 cases in order to see whether there is
any functional association between these two proteins. Statistical analysis showed a positive relation between p53 and bcl-2 expression in relation to histopathologic stages and a highly significant correlation has been seen between these two regulatory proteins \( r=0.83925; \ p=0.00001 \) (Fig.15).

Statistical analysis of association between p53 and bcl-2 has been summarised in Table 14. Out of 34 cases with p53 negative expression, 33 cases were also negative for bcl-2 \( (97.1\%; \ 33/34) \). In the 13 cases with mild p53 expression, 5 were positive with mild expression \( (38.4\%) \) and 8 were with moderate expression \( (61.6\%) \). Out of 52 cases with moderate p53 expression 36 \( (69.2\%) \) also showed moderate expression for bcl-2 and 11 \( (21.1\%) \) cases showed intense expression for bcl-2. Out of 26 cases with intense expression of p53, 21 showed moderate and 5 showed intense expression for bcl-2. Our results suggest the co-overexpression of both the regulatory proteins.

**Association between Apoptosis Regulatory Proteins and Proliferative Marker**

**Correlation between PCNA Expression and p53 Expression**

A correlation analysis has been made between p53 expression and tissue proliferation marker, PCNA in the same 125 cases. Out of 34 cases with negative p53 expression, 22 showed moderate expression and 12 showed mild expression of PCNA. Among the 13 cases with mild expression for p53, 5 were moderately stained for PCNA and 8 \( (61.6\%) \) were intensively stained for PCNA. Ninetyeight percent \( (51/52) \) of p53
Table 14: Association between p53 Expression and bcl-2 Expression

<table>
<thead>
<tr>
<th>Study population (n=125)</th>
<th>Negative expression (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>Negative expression</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(n=34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild expression</td>
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<td>5</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Moderate expression</td>
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<td>5</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>(n=52)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intense expression</td>
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<td>0</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>(n=26)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure 15  Correlation between p53 Expression and bcl-2 Expression in Cervical Cancer

* Immunoreactivity
  - Negative expression  – Values < 10
  - Mild expression   – Values 11-25
  - Moderate expression – Values 26-50
  - Intense expression – Values > 50
moderately positive cases showed intense expression of PCNA (51/52) and the remaining one had moderate expression. All the 26 intensively stained p53 positive cases also were intensively positive for PCNA. The data are summarised in Table 15.

As shown in Fig.16, a highly significant correlation was observed between p53 and PCNA expression \((r=0.81858; p=0.00001)\)

**Correlation between PCNA Expression and bcl-2 Expression**

There was a highly significant positive correlation between bcl-2 expression and PCNA expression and the results were more or less similar to the relationship between p53 and PCNA expression as shown previously. Out of 33 bcl-2 negative cases, 12 were positive with mild expression of PCNA and 21 were moderately positive for PCNA. In the 65 cases with moderate expression of bcl-2, 64 showed intense expression for PCNA (98.4%) and remaining showed moderate expression. All the cases with intense expression of bcl-2 also showed intense expression of PCNA (Table 16).

A strongly positive correlation was observed between the expression of bcl-2 and PCNA \((r=0.85171; p=0.000001)\) which is shown in Fig.17. The interrelationship between HPV infection and altered cell cycle markers such as p53, bcl-2 and PCNA are depicted in Fig.18.
### Table 15: Association between p53 Expression and PCNA Expression

<table>
<thead>
<tr>
<th>Study population (n=125)</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>p53 negative (n=34)</td>
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</tr>
<tr>
<td>Mild expression (n=13)</td>
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<td>8</td>
</tr>
<tr>
<td>Moderate expression (n=52)</td>
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<td>51</td>
</tr>
<tr>
<td>Intense expression (n=26)</td>
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<td>0</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure 16  Correlation between PCNA Expression and p53 Expression in Cervical Cancer

* Immunoreactivity as given in Figure 15.
Table 16: Association between bcl-2 Expression and PCNA Expression

<table>
<thead>
<tr>
<th>Study population (n=125)</th>
<th>Mild expression (values 11-25)</th>
<th>Moderate expression (values 26-50)</th>
<th>Intense expression (values above 50)</th>
</tr>
</thead>
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<tr>
<td>bcl-2 negative (n=33)</td>
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<td>0</td>
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<tr>
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<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Moderate expression (n=65)</td>
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<td>64</td>
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<tr>
<td>Intense expression (n=16)</td>
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<td>16</td>
</tr>
</tbody>
</table>
Figure 17  Correlation between PCNA Expression and bcl-2 Expression in Cervical Cancer

* Immunoreactivity as given in Figure 15.
Figure 18  HPV Infection in Relation to p53, bcl-2 and PCNA Immunoreactivity

1.00 – HPV negative cases
2.00 – HPV 6 positive cases
3.00 – HPV 11 positive cases
4.00 – HPV 16 positive cases
5.00 – HPV 18 positive cases
DISCUSSION

Association between p53 and bcl-2

The significance of the apoptotic pathway in the development and progression of human malignant tumours has become a major topic of discussion during the last few years (Tjalma, 1999a). Since p53 gene inactivation occurs in over half of human cancers, it is possible that loss of p53-mediated repression of bcl-2 gene expression accounts for at least in part for the frequent abnormalities in bcl-2 protein production seen in tumours (Miyashita et al., 1994).

In the present study, we have observed a direct significant correlation between p53 and bcl-2 expression as shown in Table 14 and Fig.15 (r=0.83925; p=0.00001) which is in agreement with the reports of Liang et al. (1995) and Pradip Nair et al. (1999b), where they have shown a positive correlation between p53 and bcl-2. The data presented in Table 14 show the concomitant co-overexpression of p53 and bcl-2. Out of 34 p53 negative cases, 33 also showed negative expression for bcl-2, suggesting the strong association between them. The present study is therefore in good agreement with the hypothesis that the p53 inactivation releases the repression of bcl-2 gene which leads to the over-expression of both bcl-2 protein and nonfunctional p53 protein in cervical cancer (Sidransky and Hollstein, 1996).

Certain other studies have shown an inverse correlation (Rajkumar et al., 1998) and/or no correlation (Dimitrakakis, 2000) between p53 and bcl-2 expression in cervical cancer, and which also indicated the bcl-2 as
an independent prognostic marker. This may support the view of Wang et al. (1993) in which gene transfer experiments indicated that over production of bcl-2 protein can abrogate p53 induced cell death, suggesting that if high enough levels of bcl-2 proteins are made, then p53 can no longer induce apoptosis (Wang et al., 1993). This also suggests that bcl-2 expression is not a reflection of apoptosis (Tjalma et al., 2001).

Association between Apoptosis Regulatory Proteins p53, bcl-2 and Proliferation Marker PCNA

It has been generally accepted that the potential of tumour progression is associated with the balance between cell death and cell proliferation. It is also accepted that apoptosis plays an important role in the maintenance of this balance (Williams, 1991; Kerr et al., 1994). Among the apoptosis regulatory proteins, the tumour suppressor protein, p53 has major direct influence on cell proliferation (Sidransky and Hollstein, 1996; Soddu and Sacchi, 1997; Darnton, 1998). There is a good correlation between tumour cell-kinetics measured by the PCNA index and the p53 gene in several malignancies (Bourhis et al., 1994). A relatively higher proliferative activity has been observed in p53 positive tissues than in p53 negative epithelia of oral cancer, suggesting that the p53 stabilization may confer proliferative advantage to a tissue (Warnakulasuriya and Johnson, 1994). However, they also suggested the possibility that p53 stabilization may be a result rather than the cause of rapid cell proliferation in aggressive neoplasms. A positive association between PCNA and p53 over expression was observed in squamous cell
carcinoma of the cervix (Steinbeck et al., 1995). The present study shows a highly significant positive correlation between p53 and PCNA expression (r=0.8158; p=0.00001) (Table 15 and Fig.16). Since p53 is a negative regulator of proliferation, this also could explain the higher rates of tumour proliferation as reflected by PCNA observed in the present study and which may be the reflection of p53 inactivation. Increased tumour cell proliferation was also associated with decreased apoptosis which was evidenced by the negative correlation between apoptotic index and cell proliferation index in terms of ki-67 and cydin D1 respectively (Pradip et al., 1999b).

The present study also analysed the association between anti-apoptotic protein bcl-2 expression and proliferation marker PCNA. A highly significant correlation has been observed between bcl-2 expression and cervical cancer progression in this present study as well as in earlier studies (Brychotova et al., 2000; Dimitrakakis et al., 2000). Although it has been stated that bcl-2 over expression inhibits apoptosis without promoting cell proliferation on cell cycle progression, the present study found a strong association between bcl-2 expression and PCNA over expression with a significant correlation r=0.85171; p=0.000001 (Table 16 and Fig.17). Recently, two other studies also found a strong association between the presence of bcl-2 in pathological epithelium and PCNA expression (Brychotova et al., 2000; Tjalma et al., 2001). However, the mechanism underlying this association is not yet known. Our result suggests that the influence of HPV on bcl-2 might have directed the bcl-2 protein to enhance cell proliferation which is also shown to be an essential process for viral replication, in vivo.