CHAPTER V

SIGNIFICANCE OF CELL PROLIFERATION MARKER IN CERVICAL CANCER

INTRODUCTION

Apoptosis and cell proliferation are the two factors which are critical in tissue homeostasis. One of the hallmarks of a cancer cell is its inability to homeostatically control the mechanisms that regulate cell growth or to completely differentiate into the terminal cell type of its lineage (Trosko and Goodman, 1994). Thus, the disruption in this homeostatic control accounts for the basic molecular mechanism involved in carcinogenesis (McDonnell, 1993) and this can be brought about either by increasing cell proliferation or by decreasing cell death. Hence, equal importance should be given to the involvement of cell proliferation in addition to the apoptosis regulatory system in cancer.

Normal cell growth regulation is the result of both positive and negative growth factor-mediated signalling pathways, which converge in the nucleus and induce target genes involved in cell cycle control. The proto-oncogenes, including peptide growth factors, peptide growth factor receptors and intracellular signalling proteins, enhance growth by facilitating the entry of cells into S-phase. In contrast, growth inhibition is mediated by growth inhibitory factors such as, TGF-β, and tumour suppressor gene products like Rb and p53.
Thus, the delicate balance of peptide growth factors and proto-oncogenes versus growth inhibitors and tumour suppressor genes direct the cell cycle and regulate cellular proliferation. In contrast to normal cells, cancer cells typically have diminished requirements for positive growth factors, such as EGF during G₁ phase and have lost the capacity to arrest growth in response to inhibitory signals such as TGF-β₁ (Steiner et al., 1995). It is therefore necessary to determine the factors involved in the disruption of homeostatic control in cancer and is of fundamental importance in understanding the process of oncogenic transformation. Hence the present study was evolved to analyse the effect of proliferation fraction in the course of cervical carcinogenesis and its association with HPV infection.

**Role of Proliferation Marker in Cervical Cancer Progression**

Cancer cells are characterized by limitless proliferative autonomy and immunity to inhibitory and apoptotic signals, thus ensuring growth and metastasis (Hanahan and Weinberg, 2000). In olden days the tumour proliferation was assessed by counting the number of mitotic figures (Baak, 1990; Tjalma et al., 1999). Only in recent years, the immunocytochemical assessment of proliferation cell nuclear antigen (PCNA) has been evolved as a potential marker for the assessment of cell proliferation (Wada et al., 1994; Steiner et al., 1995; Tjalma et al., 2001).

PCNA is a 36 kDa nuclear protein that functions as an auxilliary protein of DNA polymerase-delta (δ) (Bravo et al., 1987) and is essential for the synthesis of DNA during cell proliferation (Jaskulski et al., 1988).
Synthesis of PCNA begins in the late G₁ phase and peaks during the S phases of the cell cycle (Bravo et al., 1987) and it therefore reflects the proliferation state of a cell population. Immunocytochemical expression of PCNA may therefore be an useful parameter of the biological malignant potential in many malignancies. The expression of PCNA has been studied in various malignancies using anti-PCNA monoclonal antibodies. A significant correlation was indicated between the PCNA expression rate and degree of malignancy in breast carcinoma (Dawson et al., 1990), malignant lymphoma (Kamel et al., 1991), haemangiopericytoma (Yu et al., 1991) and in bladder cancer (Wada et al., 1993). But in cervical cancer, there are very few studies on the involvement of PCNA in histopathologic progression. A statistically significant correlation between PCNA expression and CIN grades has been reported by Shurbaji et al. (1993). Some recent studies have also shown the PCNA expression as a diagnostic marker (Maeda et al., 2001; Tjalma et al., 2001). But none of the studies have shown the association of PCNA expression with histopathologic progression of cervical cancer. So, the present study was also extended to analyse the cervical cancer proliferation status in the course of progression by assessing the PCNA immunoreactivity.

**Association of PCNA Expression with HPV Infection**

DNA tumour 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 involved in cell cycle regulation
(Helland et al., 1993). The association of high-risk HPV infection with decreased apoptosis and increased cell proliferation has been reported (Pradip Nair et al., 1999b). PCNA was also induced in primary human keratinocytes immortalized by HPV-18 or in acutely infected cells with retroviruses expressing the HPV-16 E7 gene (Halbert et al., 1992; Merrick et al., 1992), suggesting a strong association between PCNA expression and HPV infection in cervical cancer. A report of Penneys et al. (1992) has suggested that the aberrant expression of PCNA may be related to HPV infection. But it is not clear whether the HPV infection has a direct role on host cell proliferation or not. The loss of p53 function by HPV can also increase both cell proliferation and the probability of their neoplastic transformation (Harris and Hollstein, 1993). Further, it has been postulated by a study that the HPV gene products induce the expression of PCNA and other components of the host DNA replication machinery in differentiated cells of squamous lesions to facilitate vegetative viral replication (Demeter et al., 1994). However, the role of PCNA expression in the progression of HPV associated cervical cancer in relation to the oncogenic potency of HPV is still unclear.

WORKING HYPOTHESIS

Since a balance between tumour cell proliferation and programmed cell death (apoptosis) theoretically represents an important characteristic of malignant growth, alterations in apoptotic regulatory proteins may in turn affect cellular proliferation rate. It is therefore necessary to determine the factors involved in the disruption of homeostatic control
in cancer and is of fundamental importance in understanding the process of oncogenic transformation. It has been hypothesized that cervical intraepithelial neoplasia involves a progressive dysfunction of proliferation activity of cervical epithelial cells, whereby, higher levels of proliferating cells will be present with increasing CIN grades (Shurbaji et al., 1993). It has also been postulated that the HPV gene products induce the expression of PCNA and other components of host cell DNA replication machinery to facilitate viral replication (Demeter et al., 1994). The E5 gene product of HPV also has been shown to synergise with EGF in the stimulation of epithelial cell proliferation (Leechanachi et al., 1992; Bouvard et al., 1994), which may enhance the cell growth signal pathway and lead to uncontrolled proliferation. The present study was raised based on this hypothesis to analyse the association between cell proliferation in terms of PCNA expression and HPV infection. The purpose of this investigation was to obtain information for a better understanding of the genesis of uterine cervical carcinoma in order to improve the diagnosis of premalignant and early malignant cervical lesions.

STUDY DESIGN

This study was carried out in one hundred and twenty five cervical specimens as given in Chapter IV. The PCNA immunoreactivity was analysed in the same biopsy tissues in which the p53, bcl-2 and HPV status were analysed. The data obtained were subjected to correlation
analysis with cancer progression and HPV status. The details of the cases are also given in Chapter IV.

METHODOLOGY

Immunocytochemistry for PCNA

Sample collection and processing are given in Chapter III and paraffin tissue sections were prepared as given in Chapter IV. The duplicate serial sections were used for immunocytochemistry for PCNA expression using standard method (Hsu et al., 1981). The detailed protocol is given in appendix. The primary antibody used was anti-PCNA monoclonal antibody (Clone PC 10, DAKO A/S, Denmark; Dilution 1:50, gifted by Prof. Radhakrishna Pillai, Division of Laboratory Medicine, RCC, TVM). The reaction products were visualised using secondary antibody complex (DAKO, Strept ABC complex/HRP duet, Mouse/Rabbit; Dilution 1:300) with DAB (DAKO). The sections were then counter stained with haematoxylin.

Sections of normal lymph node tissue were used as positive control, and sections without primary antibody treatment were used as negative control for PCNA immunoreactivity.

Assessment of Immunoreactivity

Immunocytochemical staining for PCNA was regarded as positive whenever the characteristic nuclear staining (brown colour) was observed. The intensity of immunoreactivity was graded by evaluating
100 cells for both positive and negative cells. A grade of 0 was assigned if no PCNA staining was detected or if the PCNA positive nuclei were limited to the basal layer. Proliferating cell nuclear antigen expression grades 1, 2 and 3 were assigned when the highest PCNA-positive nuclei fell in the lower, middle or upper third of the epithelium and in the range 11-25, 26-50 and above 50. The grade 1 can also be considered as normal PCNA expression. The immunoreactivity index is given in Table 11.

Data Analysis

Correlation Analysis was carried out between the PCNA expression and histopathologic grades using Spearman’s and Pearson’s correlation test. The association between PCNA expression and HPV status was also analysed by univariate statistical correlation analysis.

RESULTS

PCNA Expression in Relation to Histopathologic Stages of Cervical Lesions

We have analysed the association of PCNA expression with progressing histopathologic stages of cervical lesions in a 125 study population. As the lesion progresses from mild dysplasia to invasive cancer, the intensity of nuclear immunostaining for PCNA was increasing. The nuclear expression of PCNA in different histopathologic stages of cervical lesions are shown in Plate 5. Sixty percent (12/20) of normal sample showed moderate expression and in mild dysplasia cases,
Table 11: Immunoreactivity Index for PCNA Expression in Cervical Cancer

<table>
<thead>
<tr>
<th>Grade</th>
<th>Immunoreactivity(%)</th>
<th>Expression Index</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0-10</td>
<td>Insignificant</td>
<td>(-)</td>
</tr>
<tr>
<td>1</td>
<td>11-25</td>
<td>Mild (Normal)</td>
<td>(+)</td>
</tr>
<tr>
<td>2</td>
<td>26-50</td>
<td>Moderate</td>
<td>(++)</td>
</tr>
<tr>
<td>3</td>
<td>Above 50</td>
<td>Intense</td>
<td>(+++)</td>
</tr>
</tbody>
</table>
Plate 5. Nuclear expression of PCNA in normal and different histopathologic lesions of the uterine cervix

a. Normal PCNA immunoreactivity in basal layer of normal cervical epithelium (40 X )

b. Negative control showing negativity for PCNA expression (40 X)

c. PCNA immunoreactivity in dysplastic lesion of cervical epithelium (40 X)

d. PCNA immunoreactivity in invasive cervical cancer epithelium (40 X)
80% (16/20) showed moderate expression of PCNA. Interestingly in the case of severe dysplasia (25 cases) and invasive cancer (60 cases), 100% of intense expression was observed. The data are given in Table 12.

A highly significant correlation was observed between histopathologic stages and PCNA expression (r=0.85775; p=0.00001) (Fig.13).

**HPV Status in Relation to Proliferative Activity in Cervical Cancer**

In this we have analysed whether there is any correlation between HPV infection and proliferative activity by measuring the nuclear PCNA staining in the same 125 cases, in which the HPV status were analysed. Normal tissues will have little expression of PCNA and hence the over expression of PCNA was taken into account and were typed into three groups. They are mild expression (values 11-25), moderate expression(values 26-50) and intense expression(values above 50). Our observation showed over expression of PCNA in HPV positive cases. Out of 42 HPV negative cases, 11 showed normal mild expression of PCNA (26.2%) and 13 showed intense expression. All the 3 low-risk HPV 6 infected cases showed moderate expression and the one and only HPV 11 infected case analysed showed intense expression of PCNA. In high-risk type HPV 16 infected cases, 89.5% were intensively stained for PCNA (60/67) and the remaining 10.5% (7/67) were moderately positive. HPV 18 infected cases showed 91.7% (11/12) intense expression and 8.3% showed moderate expression. The data are summarized in Table 13.
Table 12: Association between PCNA Expression and Histopathologic Grades of Cervical Lesions

<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>Normal (n=20)</td>
<td>8</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Mild dysplasia (n=20)</td>
<td>4</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Severe dysplasia (n=25)</td>
<td>0</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Invasive cancer (n=60)</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
</tbody>
</table>
Figure 13  Correlation between PCNA Expression and Histopathologic Progression in Cervical Cancer

PCNA immunoreactivity

Histopathology

1.00 - Normal
2.00 - Mild dysplasia
3.00 - Severe dysplasia
4.00 - Invasive cancer
PCNA immunoreactivity is in mean expression grouped in a range
Table 13: Association between HPV Status 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>HPV negative (n=42)</td>
<td>11</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>HPV 6 positive (n=3)</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>HPV 11 positive (n=1)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HPV 16 positive (n=67)</td>
<td>0</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td>HPV 18 positive (n=12)</td>
<td>0</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>
Thus we found a highly significant correlation between HPV status and PCNA expression in tissue samples ($r=0.61494$; $p=0.00001$) (Fig. 14).

**DISCUSSION**

**PCNA Expression in Relation to Histopathologic Progression**

PCNA has been shown to be a cell cycle-regulated protein (Celis and Celis, 1985) and its levels were reported to increase two to three fold between the early G1 and early S-phases, plateau through G2 and begin to decline from G2/M to G1 (Morris and Mathews, 1989; Bolton et al., 1992; Linden et al., 1992). Monoclonal anti-PCNA antibodies have been demonstrated to be useful as S-phase probe in the assessment of cell proliferation in formalin-fixed, embedded tissues (Garcia et al., 1989). In the present study we have used commercial anti-PCNA monoclonal antibody, PC10 (Waseem and Lane, 1990) to assess the cell proliferation. Shurbaji et al. (1993) has demonstrated that the immunoreactivity to anti-PCNA antibody in dysplastic cervical epithelium was different from that seen in the normal cervix and they also found a significant correlation between PCNA expression and CIN grades.

In the present study, we observed an abnormal nuclear PCNA immunoreactivity above the basal layer of uterine cervix epithelium and the intensity of which increased in relation to the cancer progression as shown in Plate 5 and Fig 13. Surprisingly, the normal cervical biopsies also showed moderate (12/20; 60%) PCNA immunoreactivity. Aberrant PCNA localization, however, has been previously reported in normal tissues (Hall et al., 1990). Hall and co-workers (1990) also observed weak
Fig. 14 Association of HPV with PCNA Expression in Cervical Cancer
PCNA immunoreactivity above the generally accepted zone of proliferation in gastro-intestinal crypt which may be due to the relatively long half-life of PCNA (~20 hrs) in an *in vivo* system (Bravo and Macdonald-Bravo, 1987). In the present study, all the 25 severe dysplastic and 60 invasive cancer cases showed intense PCNA immunoreactivity (Table 12), suggesting the role of PCNA expression in cancer progression. The statistical correlation analysis also has shown a significant correlation between PCNA expression and histopathological progression (*r*=0.85775; *p*=0.00001) (Fig.13). A similar pattern of significant positive correlation between cell proliferation rate in terms of Ki-67 which is also a proliferation marker and histopathologic progression has been reported by Pradip Nair *et al.* (1999b).

The present study therefore suggests that the level of PCNA expression can be used as a marker for cancer progression. Charuruks and co-workers (1998) also suggested the PCNA expression as a potential marker for tumourigenesis and prognosis of cervical cancer. Another study has shown the PCNA expression as a marker for vascular proliferation in cervical intraepithelial lesions (Soini *et al.*, 1996).

**Association of PCNA Expression with HPV Infection**

High-risk HPV infection has been reported to be associated with decreased apoptosis and increased human cell proliferation (Pradip Nair *et al.*, 1999b). Penneys and colleagues (1992) have reported that the PCNA expression in cutaneous squamous cell carcinoma *in situ* frequently involved the nuclei of all keratinocytes within the lesions. From the pattern of PCNA immunoreactivity the authors concluded that
the PCNA expression may be related to HPV infection (Penneys et al., 1992). In the present study we have analysed the association between cell proliferation in terms of PCNA assessment and HPV infection by statistical correlation analysis. Our result has shown a significant correlation between PCNA expression and HPV infection (r=0.06676; p=0.000001) (Table 13 & Fig.14). Among the 42 HPV negative cases only 13 (31%) showed intense PCNA expression, whereas the majority of high-risk HPV infected cases such as HPV 16 (60/67;90%) and HPV 18 (11/12;92%) showed intense PCNA immunoreactivity and the remaining showed moderate expression of PCNA (Table 13). This suggests a strong association between the PCNA expression and HPV infection.

During the integration of HPV genome into host cellular genome, the E5 gene product, a hydrophobic protein was found to be released and bound to the host cell membrane (Halbert and Galloway, 1988). This E5 protein synergizes with EGF in the stimulation of epithelial cell proliferation (Leechanachi et al., 1992; Bouvard et al., 1994). It is also indicated that the E5 expressing cells showed increased turnover and phosphorylation of EGFR and therefore leads to enhanced response to positive extra cellular growth signals (Straight et al., 1993). HPV E5 protein can also interact with cellular receptors for platelet derived growth factor and the 16 KD integral membrane subunit protein of vacuolar ATPase and probably enhances transformation through constitutive activation of growth factors response pathway (Petti et al., 1991; Goldstein et al., 1992a,b; Petti and DiMaio, 1992). All these mechanisms suggest the direct role of HPV in the activation of cell proliferation by influencing growth signal pathways. Our result also suggests that the role of HPV E5 may be one of the reasons for the highly significant correlation observed between HPV infection and PCNA
expression. Another reason could be the inactivation of p53 protein by complex formation with HPV E6 protein which in turn may enhance the cell proliferation.