Chapter - II
CHAPTER - II

ORGANIZATION AND STUDY DESIGN

RATIONALE FOR THE PRESENT INVESTIGATION

In India, with change in life expectancy and life styles, the incidence of cervical cancer has increased steadily. Chennai is the area in India with highest prevalence of cervical carcinoma associated with HIV and HPV infection. The risk factors for cervical cancer are poorly understood, although a number of risk factors have been suspected world-wide: sexual behaviour or exposure to sexually transmitted disease (Brinton et al., 1987; Herrero et al., 1990a; Williams et al., 1994), low socio-economic status (Williams et al., 1994), number of deliveries (Brinton et al., 1987; Williams et al., 1994), smoking (Barnum and Greenberg, 1993), hormonal contraception (Herrero et al., 1990b). Cervical cancer is a typical, preventable, detectable and treatable cancer. Yet, it is one of the most prevalent cancers in developing countries. Although recent research developments on cervical cancer have shown HPV as an etiological factor, the most prevalent HPV subtypes in Chennai have not yet been identified and established. It is not understood how HPV causes the premalignant changes in its infected host system. A more thorough understanding of the molecular mechanisms responsible for viral oncogenesis will facilitate the development of novel preventive and therapeutic strategies to prevent and treat papilloma virus-associated cervical neoplasias (Hines et al.,
Since HPV infection is transient by published data, it is also necessary to evaluate the prevalence of HPV subtypes in different histopathological grades of cervical cancer progression. So we paid attention to find out the most prevalent subtype responsible for invasiveness and metastasis of cervical cancer, particularly in Chennai, India.

The association between HPV infection and development of cervical cancer is not absolute, because the majority of women infected with HPVs do not develop cervical cancer. A previous study of college-age women in USA demonstrated that approximately 40% of this population had evidence of HPV infection but the majority were able to eliminate HPV infection within 6 months. (Ho et al., 1998a). This suggests the involvement of host cellular factors for the progression of HPV infection to cervical cancer. The clinical and histopathological study to establish the role of HPV in cervical cancer development in association with the host cell oncogenic factors was focussed in the present study.

Recent research has centered on identifying the host genes which are upregulated in association with HPV infection, determining their suitability as "Surrogate markers" for HPV infection to identify HPV associated epithelial lesions in tissue or cytologic specimens (Keating et al., 2001). A better understanding of the molecular pathology of disease may provide us with the ability to improve prognosis. This study thus focused on cell cycle associated factors at the protein level along with HPV status to have a better understanding of the tumourigenesis at the
molecular level. In order to determine the effect of HPV on apoptotic mechanism, we made an attempt to analyse the interrelationship between factors involved in tumourigenesis.

Recent evidence has shown that p53 inactivation is a relatively late event in the progression of cervical cancer (Boabang et al., 2001). Studies on bcl-2 expression in cervical cancer have suggested the strong association between the expression of bcl-2 in pathological epithelium with both the degree of dysplasia and the proliferative activity. But this study could not find any significant correlation between the bcl-2 oncoprotein expression and the HPV infection.

Apoptosis and proliferation are interrelated and interregulated processes in a cell (Trosko et al., 1994). But the interrelationship between proliferation marker and apoptosis regulatory factors are not yet clearly understood. Attempts were made to analyse the association between p53, bcl-2 and PCNA during the course of cervical carcinogenesis at different stages of progression. This study may provoke oncologists to develop new approaches for cervical cancer therapy by modulating the expression of the proteins involved in the tumourigenesis.

Thus the objective of the study is to reveal the prevalence of HPV subtypes in cervical cancer in Chennai, India which may help in development of vaccines against that particular HPV subtype in future. Further, the focus of this study on changes in cell cycle associated proteins upon HPV infection may evoke a better understanding of the interlinked complex mechanism involved in cervical carcinogenesis. It is
hoped that the data presented in this study will further stimulate investigations on the interaction of HPV infection with cellular oncogenes at the gene level (molecular level). The findings thus may help to improve the early detection and prevention strategies which in turn improve the health care of the patients.

STUDY DESIGN

The present study was designed to analyse the prevalence of HPV infection in cervical cancer in Chennai, India and its interactions with other oncogenic factors to exert its effect. We have selected few such cofactors associated with HPV influenced cervical carcinogenesis for our study. They are apoptosis regulatory proteins such as tumour suppressor protein p53, anti-apoptotic protein bcl-2 and epithelial cellular proliferation marker called proliferation cell nuclear antigen (PCNA). In order to understand the mechanism involved in HPV induced cervical carcinogenesis, we have analysed the abnormal expression of oncogene products such as p53, bcl-2 and PCNA in various stages of tumour progression in cervical epithelium. This study has been carried out in the same study subjects in which the HPV status has been analysed already. Finally the data obtained on the study of oncogene expression and HPV status were subjected to correlation analysis in order to analyse the interaction of these parameters in the critical process of cervical carcinogenesis.

STUDY GROUP

The study subjects involved both normal subjects with complaints other than cervical cancer as well as cervical cancer patients ranging
from low grade to invasive squamous cell carcinoma of the uterine cervix. Cancer tissue samples for this study were obtained as punch biopsies from patients attending the Gynecologic OP of the Institute of Obstetrics and Gynecology (IOG), Research Institute of Madras Medical College, Chennai. Normal cervical epithelium were obtained from patients undergoing hysterectomy for various non-malignant diseases.

Approval for this study was obtained from the Institute’s ethical board. The biopsy samples were collected in 10% buffered formalin and embedded in paraffin within 24 hrs for long term preservation. This study was carried out in four study groups based on Bethesda histopathological classifications as given below.

Group I : Age matched normal subjects
Group II : Mild dysplasia cases
           (Low grade SIL, CIN I & CIN II)
Group III : Severe dysplasia cases
           (High grade SIL, CIN III & Carcinoma Insitu)
Group IV : Invasive Cancer.

PARAMETERS SELECTED FOR ANALYSIS

1. Analysis of low and high-risk HPV subtypes status in cervical biopsies.
2. Role of apoptosis regulatory proteins
   a. Analysis of tumour suppressor protein, p53 expression
   b. Analysis of anti-apoptotic protein, bcl-2 expression

3. Analysis of epithelial cell proliferation marker (PCNA)

JUSTIFICATION FOR THE CHOICE OF SELECTED PARAMETERS

Even though diagnostic and therapeutic advances have been made for cancers in recent years, cervical cancer still ranks top with high morbidity and mortality. Therefore further development in early diagnostic and mainly prevention strategies are needed to improve the cancer control program.

In order to achieve this, a clear and concise understanding of the cellular processes involved in the development of cervical cancer is an important pre-requisite. Although the association between HPV and cervical cancer has been proven, the potentiality for the development of cervical cancer varies among the HPV subtypes with regions. Also it is not understood how the particular HPV subtype exerts its effect in a cell with higher potency. The prominent parameters involved in such HPV induced cervical cancers are tumour suppressors like p53, oncogenes which are involved in apoptosis regulatory pathways like bcl-2 and cell proliferation associated factors like proliferation cell nuclear antigen (PCNA). The study on the interactions of these factors in HPV induced cervical cancer may help to understand the mechanism in depth.
WORKING HYPOTHESIS

The oncocenic HPV subtypes are classified into low-risk and high-risk based on their potential oncogeneity. The prevalence of a particular HPV subtype with higher oncogenic potential may account for the highest incidence of cervical cancer reported in Chennai (Cancer Incidence, 1995). It is also believed that the prevalence of specific HPV subtypes in cervical carcinomas vary according to the geographic origin of the specimen (Bosch et al., 1995). The role of these HPV subtypes in the progression of cervical cancer from mild dysplasia to invasive cancer is not yet clear. Therefore, an attempt was made to study the prevalence of selected HPV subtypes in the development of cervical cancer and their involvement in tumour progression.

HPV infection appears to be an early event in cervical carcinogenesis with additional abnormalities being required for biological transformation (Giannoudis et al., 2000). One of the important aspects of the transforming and immortalizing activities of HPVs is their cooperation (association) with oncogenes. Several previous studies have reported a number of contradictory statements regarding the involvement of such oncogenes like apoptosis regulatory proteins such as p53, bcl-2 and cell proliferation marker PCNA in HPV-associated cervical cancer (Bravo et al., 1987; Merrick et al., 1992; Radhakrishna Pillai et al., 1996). Although various reports have depicted the expression of apoptosis regulatory proteins in cervical cancer, the co-overexpression of both p53 and bcl-2 in HPV-induced cervical cancer is of considerable controversy. It is also important to note that a balance of tumour cell proliferation and programmed cell death (apoptosis) theoretically represents an
important characteristic of malignant growth and hence alteration in apoptosis regulatory proteins may in turn affect cellular proliferation rate. However, the role of PCNA expression in the progression of HPV-associated cervical cancer in relation to the oncogenic potential of HPV is still unclear. The interrelationship between the HPV infection and host cellular factors is illustrated in Fig.4.

We believe that the documentation of any changes in the above said parameters occurring in the cervical epithelium might have parallel morphological changes at various stages of neoplastic development. These findings may be helpful in diagnosis, prognosis and aid our understanding of cervical carcinogenesis which may help to prevent such cancer at an earlier stage.

SPECIFIC AIMS

1. Analysis of the prevalence of high-risk HPV types (HPV 16/18 and HPV 6/11) in cancer biopsy tissue, using type-specific primers by sensitive PCR technique to find out whether a particular subtype of high-risk HPV can predict the risk of cervical neoplasia in Chennai, India. This study also focussed on the analysis of HPV sub types in progression during the course of cervical cancer.

2. As the development of cervical cancer is a multistep process, the progression of HPV infection into cervical carcinomas requires certain cofactors at the molecular level. So the present study was also aimed at evaluating the involvement of apoptosis regulatory proteins such as p53, bcl-2 and the proliferating marker, PCNA in HPV-associated cervical cancer. This study was also extended to
Fig. 4 Hypothetical Representation of HPV Induced Cervical Carcinogenesis

Susceptible basal cells of epithelium

High-risk HPV subtypes 16/18

Transformation zone

Cellular proto-oncogenes

Tumor suppressor Anti-apoptotic factor cell growth signal events

Integration into cellular genome by HPV E1 and E2

Reprogramming host cell for HPV replications

Viral oncoproteins E5, E6, E7

Cellular oncoproteins p53, bcl-2, EGFR

Other cofactors

Cellular Transcription factor, AP-1

Cellular Transformation

PCNA overexpression in nucleus

Immortalization

Uncontrolled proliferation

Cervical cancer
analyse the interactions between these parameters in order to understand the mechanism involved in HPV-induced cervical carcinogenesis by correlation analysis.

**DATA ANALYSIS**

Statistical analysis was carried out to evaluate the following:

a. Univariate analysis of HPV and expression of markers were studied in relation to clinical and histologic diagnosis in each group of study subjects.

b. Assessing the odds ratio (OR) for HPV was carried out using the Fisher's exact test. ORs were calculated as estimates of the relative risk to test for any significant association between the factors studied.

Statistical analysis was done utilizing the SPSS and INSTAT statistical software. Frequency tables were tested for association using chi-square and Spearman's correlation.