CHAPTER-VI

DISCUSSION
Discussion:

Cervical cancer is one of the most common cancers among women and is the 2nd most cause of cancer death among women in India (Benjamin E, et al, 2010; Chopra, 2001). The cases of cervical cancer remain high despite the availability of HPV vaccine and it has been seen that the cervical cancer cases are increasing being the second highest incidence rate in the world among women after breast cancer (Ferlay et al., 2015). Infection of the cervix with human papilloma virus (HPV) has been established as a key determinant of cervical carcinogenesis (Castellsague, 2008). It is reported that more than 200 different HPV genotypes have been identified, and about 40 oncogenic subtypes are associated with the majority of cases of cervical cancer (Munoz et al., 2003). There is a wide variation in geographical distribution of HPV types in the world (Bosch et al, 1995).

The present study was planned to evaluate the epidemiology of development of cervical cancer. Patients who were diagnosed and clinically correlated for cervical cancer by the registered clinician in the outdoor of Obstetrics and Gynecology department, Guwahati Medical College & Hospital, were enrolled in the present study with patients’ informed consent.

One of the most important information from the present study was the apparent strong correlation between the ages at marriage with the chances of incidence of cancer cervix. About 70.36% women in our study got pregnant before the age of 20 years and only 28.16% of patients got their first child above the age of 20 years. Patients who had conceived before 20 years, incidence of carcinoma cervix were found to be high. In consistence with the correlation between chance of occurrence of carcinoma cervix and the age of marriage, similar relation was also observed with the age at first pregnancy by J.T. Boyd and co-workers who showed similar result with increased risk of developing carcinoma cervix in women who got pregnant before the age of 18 years (Boyd, J. T., Doll, R.1964). In a case control study by Biswas L N et al, showing maximum risk in women who reported their first intercourse at less than 12 years of age (Biswas LN et al, 1997).
In our study, incidence of cervical cancer was less in Muslim population than Hindus. It may be due to circumcision statuses which lead to less HPV virus colonization in penis (Castellsagué X et al, 2003, Louie KS 2009; S B Paul 2011).

Studies have shown that women who belong to the lower social classes are more likely to contact HPV infection and subsequently progress to cervical cancer. It has been seen that patients in the lower socioeconomic class had higher prevalence of cervical cancer. Incidence of cervical cancer also increased in illiterate or patients who received little to no educational training (S Malik; 2005). Furthermore, lower socio economic classes are less likely to be screened and at times health care facility is not reachable even if they did contact with HPV with presence of sign, symptoms (S Malik, 2005). Eventually, lower socioeconomic status is observed to be the confounding factor in higher cervical cancer rates in our study, similar finding was found in other study (Juneja, 2003).

It was observed that during personal interviews many of the patients were unable to understand what can be defined as infection and failed to seek any medical opinion for their infection related symptoms. This lack of awareness may have root in the fact that 77% of the patients were educated up to primary level which was correlating with other study by D Das et al, 2013. It is well recognized that cervical cancer risk is associated with a low socio-economic status, as defined by education or income levels.

In the present study had 96.22% of females had with multiple childbirth. Study by E.L. Wynder and his co-workers showed that increased risk is directly proportional to parity (Wynder, E L et al, 1954).

The mean age of patients was 43.68 years and the most vulnerable age group of cervical cancer in the present study was 41-50 years followed by the age group 31-40 years which signifies that HPV infection happens in the early and early-middle phases of life. This indicates that the chances of carcinoma cervix are higher in peri menopausal age then at postmenopausal age. In our study, cervical cancer cases mostly found below 50 year of age and HPV infection significantly associated with the development of cervical cancer (p value is <0.001) which is similar with other studies like Munoz N et al, 2009. It is already documented that the majority of HPV infections are cleared spontaneously from cervical area by cell mediated immunity and lower rate of clearance of HPV16 infection.
in women less than 30 years of age was observed. Viral load is the main determinant of persistence, and persistence of HPV16 infections carries a higher risk of CIN2/3, which is inversely associated with clearance (Munoz N, 2009). Spontaneous clearance of the HPV is one of the important contributing factors for negative result of HPV infection in non-cancerous area in our study.

The majority of the study in India shows that HPV 16 and 18 are prevalent in 80-90% cases, so our study was focused mainly in evaluation of HPV 16 and 18 only. Although there is a variation in HPV genotype geographically but different studies indicates that high risk HPV 16 and 18 are found to play an aggressive role in a large amount case of cervical cancer (Howleya, P et al., 2009). Our study observed, HPV infection was in 83.33 % (85/102) case, HPV 16(89.41%) being the most predominant genotype followed by HPV 18 (3.53%). The prevalence rate is comparable with the study reported from Indonesian population where the most prevalent genotype was HPV 16 (90%) (Maringan DL Tobing et al, 2014). 84.1% of invasive cervical cancers are attributed to HPVs 16 or 18 (Ferlay J, 2010). Another study from India has mentioned that HPV type 16 alone is found to be 70 to 90% prevalent while occurrence of HPV type 18 varies from 3 to 20% (Franceschi S, 2003).

Although HPV-16 has been documented as the most prevalent strain from different studies in India and globally, but results from other regions adjoining the northeast Indian territory like Thailand shows that difference in HPV genotype prevalence rates do exist, where high prevalence of HPV types like HPV 52 (17.6%), HPV 58 (13.89%), HPV 33 (11.11%), HPV 51 (11.11%), and HPV 56 (9.26%) has been found along HPV 16 (14.81%) (Kannika Paengchit et al., 2014). Another North East India related study by Ghosh SK et al. (2011) reported that HPV-18 compared to HPV-16 is the prevalent genotype in cervical cancer patients from southern Assam. The disagreement in our findings with Ghosh et al data could be attributed to the variation in patient population.

Available literature is suggestive that, other than HPV infection are host genetic and immunological factors the most specific factor(s) responsible for cervical cancer development (Deng CX et al, 2009). Study shows that neoplastic diseases of human are usually associated with deregulations of the equilibrium between the production of certain type 1 and type 2 cytokines (Bais AG, et al; 2005). Analysis of T helper type 1
(Th1) and type 2 (Th2) cytokine profiles has been used to characterize the immune response in several human diseases, including HPV-associated diseases (Clerici M, et al; 1997). In our study, differential Th1 and Th2 modulation study in the serum level it was found that production of type 2 cytokines (IL-10) was comparatively higher with down-regulation of Th1 in cancer cervix cases than controls. With respect to the Th1/Th2 status, the ratio of IL-12/IL-10 decreases in cancer compared to non-neoplastic control cases, suggestive of the fact that Th1 type response is a prerequisite in limiting the progression to cervical cancer. Thus, the differential modulation of Th1 and Th2 expression plays a major role in predisposing the patient to the development of cervical cancer. There is increased level of type 1 cytokines in controls indicating that Th1 are immune stimulatory and are thus capable of limiting tumor progression. But in cancer cases, the production of Th2 (IL-10) is abnormally elevated as compared to production of IL-12 and TNF-α in HPV infected patients. Reports from other research group has stated that both IL-12 and TNF-α are the potent activator of cellular immunity and has antitumor and anti-metastatic against tumors (M Clerici, 1998; X Zhu et al, 2013), thus decreasing production of IL-12 and TNF-α indicating suppression of immunity due to HPV infection in our study. IL-10 down regulates tumor specific immune response by directly suppressing interferon gamma and IL-12 production thereby preventing the activation of CTLs and Natural Killer Cells which indicates a Th2 skewed status predisposing to HPV infection and resulting progression to cancer cervix in our case, similar to reports from other groups (Mosmann and Coffman, 1987). Similar finding was reported by Giannini et al, 1998 and Mota et al, 1999, where they have mentioned that peripheral blood mononuclear cells (PBMC) from patients with both squamous intraepithelial lesions (SIL) and cervical cancer produced decreased amount of IL-12 and IFN-γ and increased level of IL-4 and IL-10. An increased number of IL-10-positive cells were detected in the cervix of patients with cervical intraepithelial neoplasia (CIN) (Mindiola R et al, 2008). Jacobs N et al, 1998 has also demonstrated that basal levels of IL-10 are augmented in the peripheral blood mononuclear cell of patients with SIL. Serum levels of IL-10 were observed to be significantly higher in women with invasive cervical cancer and high grade CIN as compared to controls (Feng et al., 2012).

In differential mRNA expression profile of Th1 and Th2 makers the data clearly indicates an up-regulation of IL-10 (Th2) and either down-regulation (TNF-α) or non-sufficient stimulation (IL-12) of Th1 response in the pathogenesis of cervical cancer. Similar to the
Th1/Th2 profile at serum level; we also found that TNF-α differential expression at mRNA level, and was directly associated with the severity of the disease. Scientific evidence shows that during the process of cancer development in the epithelium of cervix there is shifting of cytokines from Thl to Th2 cytokines (Dinashaw KA et al, 2010), which supports our data. It has been demonstrated that IL-10 is highly expressed locally in biopsies from patients with premalignant lesions and cervical cancer and may encourage a confined state of immune suppression (Vidal AC et al., 2015). Whereas, other studies have suggested that cervical cancer progression is associated with specific immune failure in response to HPV-16 and that it is not related to a shift to a Th2 cytokine profile (Tsai HT et al; 2007). Earlier studies have also reported that the immune responses against tumor were mainly mediated by Thl cells. Imbalance of the Th1 /Th2 ratio with increase of Th2 cells, the immune response of the body against the tumor would be severely destabilized which will propagate to the cancerous growth of the tumor (Z Chen et al, 2013) which might be the underlying factor in our case cohorts. De Gruijl et al. (1999) studying the expression of cytokine mRNA transcripts at the site of HPV infection in relation to development of cervical neoplasia found a reduced type 1 immunity correlating with HPV-induced invasive cervical carcinoma. HPV-induced immunity is crucial for clearance of the infection.

In the immunohistochemical study for differential TNF-α expression at protein level, it was found that TNF-α expression was higher in the non-affected control regions than the adjacent affected regions. Expression of TNF-α was down-regulated in a gradient pattern, the least expression being observed in squamous cell carcinoma (SCC) cases, which is indicative of its immune-protective role and prognostic significance. Our result was found to be concordant with previous findings that tumor necrosis factor is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity and is a member of a group of cytokines that stimulate the acute phase response. TNF-α and its implication in both direct and indirect control of HPV infection reported by Malejczyk J et al (1992), its expression by keratinocytes is enhanced in response to injury to tissue, inflammation and viral infection (L Termini, 2008) and others holds true with data from our study cohort where we found out that down regulation of TNF-α expression gradient correlates with the progression of the disease, signifying its prognostic character in cancer cervix development through different CIN stages, thereby making it a molecular target. The specific role of TNF-α in
cancer progression remains to be solved, given that different results with several tumor types have shown that this pro-inflammatory cytokine may present both tumor necrotic and tumor-promoting behavior (Eksteen JA et al, 2001; Pillai S et al, 1989).

IFN-γ production is important for antiviral activity leading to protection from neoplastic predisposition (Matthias Nees et al, 2001), but in our study the IFN-γ expression was comparative to the control group (p=0.734). Up regulation of IFN-γ production is also done by IL-12 (M Clerici et al, 1998) which was also found to be down regulated in our cancer cases. The statistical analysis for correlation in serum IFN-γ and TNF-α level in HPV cases showed, that statistically significantly correlated with the serum lower TNF-α levels. IFN-γ is also important in activation of macrophages to produce TNF-α, which then acts together with IFN-γ to increase macrophage phagocytosis and microbicidal activity (Robinson CM, 2005). Thus, higher TNF-α level in control area indicated that it has a potent anti-proliferative effect on normal primary human keratinocytes.

NF-κB, the central pro-inflammatory molecule plays a key role in regulating the immune response to infection. Imprecise production of NF-κB has been linked to cancer development, inflammation, viral infection, and improper development of immunity (Emily L. et al, 2013). Rising evidences support a major role of NF-kB in cancer development. The expression of genes which is regulated by NF-kB plays a key role in the proliferation of cell, or migration of cell and apoptosis (X Dolcet et al, 2005). Thus NF-κB used to be a preliminary responder to harmful cellular stimuli. Recognized inducers of NF-κB activity are very much unpredictable like tumor necrosis factor alpha (TNF-α) (Chandel NS et al, 2000). Activation of NF-kB promotes malignant development and progression in several animal models (Erez et al., 2010; Greten et al., 2004; Pikarsky et al., 2004). It has been seen that NF-kB is activated in many human malignancy including cancer of the cervix (Karin, 2006; Prusty BK et al, 2005; Xavier Dolcet et al 2005). NF-kB has been identified to be a vital link between chronic inflammation and cancer (Karin, 2009). Thus, activation of the transcription factor NF-kB is an important barrier against persistent HPV infection and cervical cancer.

In the present study, NF-kβ expression was higher in the non-neoplastic control areas compared to the affected areas, it can be inferred that HPV induced pro-inflammatory cytokine NF-kβ activation in the non-affected areas may be associated with chronic
inflammation which may further stimulate the progression of the cervical cancer lesions to the adjoining areas. Available literature also shows that, NF-κB has been found to be an important modulator of the expression of various genes contributing to the malignant phenotype in human squamous cell carcinoma (SCC). NF-kB is the molecule whose role in immune modulation and carcinogenicity has been established, with data being equivocal; thereby NF-kB has been rightly stated as a two edge sword. Further, recently it has been established by works using in vitro systems that TNF-α induction is dependent of NF-kB activation and expression (Wang X et al, 2008).

Thus, TNF-α is conclusively illustrated to show an anti-tumourogenic role through our data, and since NF-κB to be a first responder to harmful cellular stimuli and had been reported to be induced by TNF-α, the positive correlation of NF-kB expression may be beneficial component.

In HPV negative cases; the NF-kβ expression analysis was inconclusive in our study. Comparative analysis of NF-kB and TNF-α level in controls and HPV infected cervical cancer cases showed significant association and correlation of down-regulation in both NF-kB and TNF-α in the pathogenesis of cervical cancer. In our study, there was down regulation of NF-kBp65 in HPV positive cervical cancer cases. Study shows that down regulation of NF-kB might represent a unique mechanism by which HPV interferes with innate immunity and promotes persistent infection and progress to cancer (Erik R. Vandermark et al, 2012). TNF mediate tumor initiation via activating NF-κB dependent pathway. NF-κB in human carcinomas frequently demonstrates activation and initiation role (Suzukawa K et al, 2004). In early stage of carcinogenesis NF-κB may play an inhibitory rather than promotional role (Arnott CH et al, 2002). Inhibition of NF-κB in human keratinocytes with activation of oncogenic ras in human keratinocytes was reported to promote development of malignant human epidermal lesions resembling squamous cell carcinoma in mice (M Brown, 2008; Dajee M et.al 2003), and a similar situation may also be existing in our cases since all the cases of cervical carcinoma in our studied cohort were squamous cell carcinoma.

However, others have reported that inhibition of NF-κB inhibits murine and human squamous cell carcinoma tumorigenesis in mice, or cholestatic hepatitis associated hepatocellular carcinomas in Mdr2 knockout mice. These contradictory results suggest that the cancer promoting role of NF-κB in certain contexts may be tissue dependent, or may involve additional steps that are not well understood (M Brown et al, 2008).
Persistent infection with high-risk HPVs and immortalization of epithelial cells are important early events in the development of cancer cervix. NF-κB activity is inhibited by HPV-16 E6 and E7 proteins in cells cultured from the transformation zone of cervix, the site of most of the cancer cervix. Thus, NF-κB has the potential to act as a tumor suppressor in cervical cells (Georgopoulos, Proffitt, and Blair, 2000). Decreased NF-κB activation might represent a unique mechanism for the virus to interfere with the host immune response and promote persistent infection (KS Ahn, 2005).

Conflicting evidence exists about whether HPV-16 stimulates or suppresses activation of NF-κB (ER Vandermark, 2012; James et al., 2006). NF-κB initiate induction of various classes of genes; genes that have products involved in negative-feedback run of NF-κB. These genes present as regulators of the immune system, encourage proliferation of cell and inhibit programmed cell death (apoptosis). These classes of genes can contribute to tumor genesis. Therefore, NF-κB plays a crucial role in cancer development, and is being proposed as a probable target for cancer treatment. It is also been publicized that NF-κB act as a tumor suppressor under some conditions, indicating paradoxical function (Perkins 2006).

We also show that down regulation of NF-κB stimulates growth and immortalization of cervical epithelial cells. Our observations raise concerns about chronic suppression of NF-κB in patients who have an increased risk for cervical cancer.

NF-κB activity has been reported to enhance tumor cell sensitivity to apoptosis and senescence. In addition, it has been shown that canonical NF-κB is a Fas transcription activator and the alternative NF-κB is a Fas transcription repressor [Liu F et al, 2012].

TNF-mediated cell proliferation inhibition of PHKs and HPV16-immortalized keratinocytes was earlier reported to be associated with NF-κB activation [Termini L et al, 2008].

TNF-α induced NF-κB activity has contradictory effects in organs with different regenerating rates like in rapid regenerating liver it has got anti-tumorigenic while pro-tumorogenic in slow regenerating colon (Karin M, Lawrence T, 2006) whether anticancer immunity is involved may also contribute to the discrepancy (Nakagawa J, Saio M, 2007;
Dace DS, Chen PW, 2007; Zhang B, Karrison T, 2008). The contradictory roles of TNF-α in tumor genesis has raised the questions regarding use of TNF-α as an anti-inflammatory or anti-TNF agents for cancer prevention. More studies of TNF are required to clarify the mechanism behind the development of various types of cancers before TNF modulating approaches can be used for cancer prevention. Therefore, the balance of TNF-α induced survival and death-signaling is pivotal in determining the fate of TNF-α responding cell. Modulating this balance could help to prevent cancer development and facilitate using TNF-α for cancer therapy.

As the affected areas of the study samples show lower expression of these host immunological factor than the non-affected areas, it may be suggested that under expression of key mediators of Th1 cytokine profile is detrimental for the pathogenesis of HPV16 infection mediated cervical cancer development. Although there are many limitations of the study, but this first study from NE India clearly illustrates the prognostic significance of specific Th1 markers like TNF-α and its upstream effectors such as IFN-γ and NF-kB in the pathogenesis of cervical anomalies, and its significance as molecular targets.