CHAPTER-V

RESULTS
RESULTS:

5.1: Enrolment of patients:
Clinically correlated and proved cervical cancer cases (N=102) were enrolled from Department of Obstetrics and Gynecology under clinical supervision and informed consent. Selection of the patients for cervical biopsy was done following doing VIA and VILLI positive test.

Fig 5.1: showing site of (VIA & VILLI positive, cancer) areas from where cervical biopsy was taken
5.1.1: Demographical profile:
The complete demographical characteristics of the patients were tabulated at the time of enrolment of the patients. The patients’ age was grouped within range distribution of 10 years. The age ranges was from 30 years to 70 years of age. The mean age of patients was 43.68 years, and the highest number of cases belonged to patients aged 41-50 years (n=53/102, 51.96%) followed by 31-40 years age group (n=36/102, 35.29%) which signifies that HPV infection happens in the early and early-middle phases of life. This also indicates that the chances of carcinoma cervix are higher in perimenopausal age then at postmenopausal age.

Fig 5.2: Showing age distribution of patients

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-40</td>
<td>36</td>
</tr>
<tr>
<td>41-50</td>
<td>53</td>
</tr>
<tr>
<td>51-60</td>
<td>7</td>
</tr>
<tr>
<td>61-70</td>
<td>3</td>
</tr>
<tr>
<td>71-80</td>
<td>3</td>
</tr>
</tbody>
</table>

5.1.2.: Social factor:

5.1.2. A: Religion:
It was found that the occurrences of carcinoma cervix are more in Hindu population and significantly lower incidence in Muslim population. It was also found that the incidence is more in patients who are illiterate or received little educational training.
5.1.2. B: Socioeconomic status and locality:

In our study 79.41% patients is from lower socioeconomic status. It has been shown that women who belong to the lower social classes are more likely to contact and sustain cervical cancer. Patients in the lower socioeconomic status had significantly higher rates of late stage cancer diagnosis and lower rates of cancer survival.

Maximum number of patients was from rural background. Women in the rural area were less likely to be screened and often proper screening procedure is not available.
5.1.3: Clinical risk factors:

5.1.3. A: Age of marriage and age of first pregnancy:
70.57% patients got pregnant before the age of 20 years and only 29.4% patients got their first child above the age of 20 years. The risk of cervical cancer increases with the first onset of sexual activity. In addition, the incidence of cervical cancer declines as the age of marriage increases.

![Fig 5.5: Relation of age of marriage and first pregnancy to cervical cancer](image)

5.1.3. B: Relation with parity:
It has been seen that cervical cancer development is high as parity is more. 57.84% cases were parity 4. The risk increases with multiple child birth. In the present study had 96.05% of females had with multiple childbirth.

![Fig 5.6: Relation of parity to cervical cancer](image)
5.2.: HPV screening analysis

5.2.1: HPV prevalence:
Total DNA was extracted from the cervical tissue samples of the affected and the adjoining non-neoplastic control area using standard phenol chloroform method. The DNA thus collected was checked by agarose gel electrophoresis. The DNA was then used for HPV detection by using consensus primer MY09/11. Overall, out of the 102 cases screened, HPV infection was observed in 85 (83.33%) and 83 (81.37%) of affected and non-neoplastic control areas respectively. Wilcoxon signed rank test based analysis shows that HPV infection was significantly associated with cervical cancer development (p<0.001).

5.2.2: HPV Genotyping:
The HPV positive cases were further genotyped for HPV16 and HPV18 by using type specific PCR primers, and were categorized under three categories, viz; HPV16 genotype positive, HPV18 genotype positive, and non-16 non-18 HPV positive cases.

Fig 5.7: Representative photograph of agarose Gel electrophoresis picture of PCR amplification of HPV (product size 450bp) and of HPV16 (119bp Size)
HPV-16 was the predominant genotype in our cohort (Table no: 6.1). Wilcoxon signed rank test showed that HPV16 was:

(i) Significantly associated with cervical cancer (p<0.001).
(ii) Significantly predominant genotype in the HPV infected cervical cancer cases (p=0.003).

<table>
<thead>
<tr>
<th>Cases</th>
<th>N=102</th>
<th>Affected area</th>
<th>Non-neoplastic control area</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV positive</td>
<td>85</td>
<td>85 (83.33%)</td>
<td>83 (81.37%)</td>
</tr>
<tr>
<td>HPV 16 positive</td>
<td></td>
<td>76 (89.41%)</td>
<td>75 (90.36%)*</td>
</tr>
<tr>
<td>HPV 18 positive</td>
<td></td>
<td>3 (3.53%)</td>
<td>3 (3.61%)</td>
</tr>
<tr>
<td>Non16 non18 HPV positive</td>
<td>6</td>
<td>6 (7.06%)</td>
<td>5 (6.02%)*</td>
</tr>
<tr>
<td>HPV negative</td>
<td>17</td>
<td>17 (16.67%)</td>
<td>17 (16.67%)</td>
</tr>
</tbody>
</table>

*in one case HPV positivity was found to be present in affected area only by PCR.

Table 5.1: Screening results of HPV detection and HPV genotypes distribution in the Cancer cervix cases

5.2.3.: Relation of age and detection of HPV infection and its genotype to the occurrence of Carcinoma cervix:

![Fig 5.8: Relation of age and detection of HPV genotype to the occurrence of carcinoma cervix](image-url)

Fig 5.8: Relation of age and detection of HPV genotype to the occurrence of carcinoma cervix
In India average menopausal age is fifty (50) years. In the present study, majority of cervical cancer cases 89(87.25%) were below 50 years of age, which was found to be statistically significant by virtue of Wilcoxon signed rank test based analysis (p ≤ 0.001). Mann-Whitney statistical analysis based results shows that HPV infection was significantly associated with the development of cervical cancer in the early reproductive phase ≤ 50 years of life (P ≤ 0.001) thus; indicating premenopausal patients are mostly suffering.

5.3: Differential Th1/Th2 modulation in the development & progression of cervical cancer at serum level:
The differential expression of Th1/Th2 modulation of the collected samples in control versus HPV positive cervical cancer patients was studied by the Magpix multiplex ELISA method using customized magnetic bead based kit and Xpotent software based analysis (Merck Millipore). From the data obtained, it was seen that expression of Th1 and Th2 are lower in the affected areas than the control ones and thus the Th1:Th2 ratio also decreases in the affected areas. Thus, it can be inferred that the modulation of Th1 and Th2 expression plays a major role in predisposing the patient to the development of cervical cancer.

5.3.1: IL-12 / IL-10:
In affected patients IL-10 response is more than the control one thus it shows inhibition of immune responses. The ratio of IL-12/IL-10 decreases in cancer, suggestive of the fact that Th1 type response is a prerequisite in limiting the progression to cervical cancer from normal.

<table>
<thead>
<tr>
<th>CASES</th>
<th>IL-12 (Th1) (pg/ml)</th>
<th>IL-10 (Th2) (pg/ml)</th>
<th>IL-12 / IL-10 (Th1/Th2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.718711</td>
<td>0.091162</td>
<td>18.853370</td>
</tr>
<tr>
<td>Affected</td>
<td>1.295103</td>
<td>0.275243</td>
<td>4.705307</td>
</tr>
</tbody>
</table>

Table 5.2: Showing the modulation of IL-12 & IL-10 in affected and control

5.3.2: TNF-α (Th1) and IL-10 (Th2):
TNF-α level is quite high in control patients than affected one whereas IL-10 production is high in affected patients than control one. Thus, indicating that Th2 production is
comparatively more than Th1 so, the ratio TNF-α / IL-10 is also less in cancerous than non-neoplastic control patients.

<table>
<thead>
<tr>
<th>CASES</th>
<th>TNF-α (Th1) (pg/ml)</th>
<th>IL-10 (Th2) (pg/ml)</th>
<th>TNF-α / IL-10 (Th1 / Th2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.633±3.607</td>
<td>0.091162</td>
<td>105.669028</td>
</tr>
<tr>
<td>Affected</td>
<td>5.535±3.509</td>
<td>0.275243</td>
<td>20.109503</td>
</tr>
</tbody>
</table>

Table 5.3: showing TNF-α & IL-10 modulation in affected and control areas

5.3.3: Cumulative Th1/Th2 expression:
Cumulative expression shows that there is up regulation of Th1 (IL-12, TNF-α, IFN-γ) in control patients and down regulation of Th2 (IL-10). The IFN-γ level is comparative. The ratio of Th1 & Th2 is down regulated in affected patients in comparison to control one. There is increased level of type 1 cytokines in controls which are immune stimulatory and are thus capable of limiting tumor growth. In our case, the production of IL-10 is abnormally elevated as compared to production of IL-12 and TNF-α in HPV infected patients and that represents suppressing immunity due to HPV infection.

<table>
<thead>
<tr>
<th>Cases</th>
<th>IFN-γ(pg/ml) (Th1)</th>
<th>IL-12 (Th1) (pg/ml)</th>
<th>TNF-α (Th1) (pg/ml)</th>
<th>IL-10 (Th2) (pg/ml)</th>
<th>IL-12/IL-10 (Th1/Th2)</th>
<th>TNF-α/IL-10 (Th1/Th2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.20</td>
<td>0.091162</td>
<td>9.633±3.607</td>
<td>0.091162</td>
<td>18.853370</td>
<td>105.669028</td>
</tr>
<tr>
<td>Affected</td>
<td>3.06</td>
<td>0.275243</td>
<td>5.535±3.509</td>
<td>0.275243</td>
<td>4.705307</td>
<td>20.109503</td>
</tr>
</tbody>
</table>

Table 5.4: Showing cumulative Th1/Th2 expression:
5.3.4: Altered Th1 (IFN-γ and TNF-α) cytokine modulation analysis:

Since HPV was found to be significantly associated with the pathogenesis of cervical cancer, the cases in our studied cohort showing positivity for presence of HPV were included for the analysis. The differential serum IFN-γ and TNF-α expression levels in HPV infected cervical cancer cases (n=83; the two cases in which HPV infection was confined to affected area only were excluded) compared to controls was analyzed by Magpix multiplex ELISA method using customized magnetic bead based kit and Xpotent software based analysis (Merck Millipore).

While the IFN-γ levels was found to be comparative between the control and cervical cancer cases (p=0.734); the serum TNF-α levels were found to be down regulated in the cervical cancer cases compared to controls (p=0.162). The serum IFN-γ and TNF-α level in HPV cases were found to correlate significantly.
Cytokine levels

<table>
<thead>
<tr>
<th>Cytokine levels</th>
<th>IFN-γ (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>Correlation analysis in HPV infected cervical cancer cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=50)</td>
<td>8.184 ± 4.589</td>
<td>9.633 ± 3.607</td>
<td>Pearson correlation: 0.466*, P value: 0.011; spearman’s rho: 0.369*, P value: 0.038</td>
</tr>
<tr>
<td>HPV infected Cervical cancer cases (n=83)</td>
<td>9.259 ± 3.927</td>
<td>5.535 ± 3.509</td>
<td></td>
</tr>
</tbody>
</table>

Cytokine expression levels represented as Average ± Standard Dev; *correlation was significant at the 0.05 level (2-tailed).

Table 5.5: Serum based differential expression and correlation analysis of IFN-γ and TNF-α

5.4: Differential Th1/Th2 modulation in the development & progression of cervical cancer at transcript level:

5.4.1: Differential IL-10 expression at mRNA level in cervical tissue:
Differential fold change in IL-10 mRNA expression between HPV infected affected and paired non-neoplastic control areas of cervix was studied by real time PCR using β-actin for internal normalization control. The mRNA based expression analysis showed up regulation in IL-10 expression (1.608±0.944) in affected area compared to non-neoplastic control areas in the HPV positive cases.

5.4.2: Differential IL-12 expression at mRNA level in cervical tissues:
Study of the mRNA expression of IL-12 was done using IL-12 mRNA specific primer by Real Time PCR method using cDNA which was prepared from extracted RNA of the samples from both cancerous and paired non neoplastic control area. The expression of IL-12 was found to be comparative between control and affected area (1.015±0.623 folds).

5.4.3: Differential TNF-α expression of cervical tissue at mRNA level:
Differential fold change in TNF-α mRNA expression between HPV infected affected and paired non-neoplastic control areas was studied by real time PCR using β-actin for internal normalization control. The mRNA based expression analysis showed down regulation in TNF-α expression in affected area (0.28958 ± 0.273198 folds) compared to non-neoplastic control areas in the HPV16 positive cases (n=76).
Fig 5.10: Quantization report in the Real Time RT PCR-Linear Amplification plot showing Ct curve/values of IL-10, IL-12, and TNF-α. The curve also analyses the Ct-values of β-actin (internal control).

5.5: Expression of pro-inflammatory cytokine, TNF-α: Differential TNF-α expression in cervical tissue at protein level:

Further, CIN I-III grade cases (n=25) and randomly selected HPV-16 infected squamous cell carcinoma (SSC) cases along with control areas (n=35) were taken for immunohistochemical study of TNF-α protein expression in cervical tissues using the Super Sensitive™ One-Step Polymer-HRP Detection System (Biogenex). Slides with 3-5 µ tissue segments were prepared for the test. Since HPV-16 was the predominant genotype in our cohort, therefore CIN cases (n=25) who were screened positive for HPV-16 genotype were only included for immunohistochemistry based analysis. From the results obtained, it was found that TNF-α expression was significantly higher in the non-neoplastic control regions than the adjacent affected regions in majority of the cases studied (p=0.036). The expression of TNF-α was found to be decreasing gradually from CIN I to CIN III through to cervical carcinoma affected areas, with almost no expression in SSC cases; therefore indicating that TNF-α plays a defensive role in development of tumor and thus helps in controlling the disease.
Fig 5.11: Representative panel of IHC results showing gradient down regulation of TNF-α expression during the progression to HPV related cervical cancer through the CIN stages, compared to the normal control cervix.

5.6: Expression of pro inflammatory cytokine, NF-kB in cervical tissue:
PCR amplification of the cDNA product of the cervical tissue sample was done using specific primer for NF- kB of the HPV positive and HPV negative cases compared to its adjacent control non neoplastic areas was done by semi-quantitative RT-PCR using β- actin as an internal control.

5.6.1.: Expression of NF-kβ in HPV-16 positive cases:
Semi-quantitative RT-PCR based expression analysis showed, that NF-kβ expression was higher in the non-neoplastic control areas compared to the affected areas, except for one case,
in which expression of NF-κβ was found to be higher in the affected region than its adjacent non-affected one.

Fig 5.12 (a): Representative photograph of agarose gel electrophoresis of PCR amplified products (205 bp size) showing expression of NF-κβ in HPV-16 positive cases. (b) Representative photograph of agarose gel electrophoresis of PCR amplified products (148 bp size) showing the expression of β-actin in the above HPV-16 positive cases.

Note: N → non-neoplastic controls, A → affected area

5.6.2.: Expression of NF-κβ in HPV negative cases:

In HPV negative cases, the NF-κβ expression analysis was inconclusive, with different sets of cases and their adjacent controls showing different patterns of NF-κβ expression.
5.6.3.: NF-kB expression and its correlation with TNF-α level:
The protein level data was in accordance with the mRNA based NF-kB data, with high expression of total NF-kB (0.813±0.109μg/ml) was observed in HPV infected non-neoplastic control areas compared to HPV infected affected area (0.517±0.143μg/ml), the difference in expression being statistically significant (p=0.002); but negligible difference in the NF-kB expression was seen in the HPV negative cases (p=0.836). Therefore, the data indicates the specificity and role of differential NF-kB modulation in HPV related cervical cancer pathogenesis.

5.6.4. Comparative analysis of NF-kB and TNF-α expression:
Since, down regulation of NF-kB expression was found to be associated with cancer cervix development, therefore its further correlation with TNF-α expression levels at mRNA level was screened by Real time PCR for HPV-16 cases. In HPV-16 infected paired samples, sharp up regulation in NF-kB mRNA expression was noticed in the non-neoplastic control areas compared to affected areas (p=0.096); whereas the NF-kB mRNA expression in non-HPV paired cases were comparative. The statistical correlation of NF-kB mRNA expression indicated strong positive correlation with TNF-α mRNA expression {Pearson correlation=0.796, p=0.093; and spearman’s rho= 0.843, p=0.067}.
When the NF-kB tissue protein expression was correlated with Magpix ELISA based serum TNF-α levels in HPV-16 positive cases, the correlation analysis was again indicative of positive correlation between the two \( \text{Pearson correlation}=0.568, p=0.138; \) and \( \text{spearman's rho}=0.612, p=0.116 \). Hence, the present data shows that the TNF-α levels are dependent on the NF-kB expression levels.

![Boxplot analysis for comparative analysis of NF-kB and TNF-α in controls and HPV infected cervical cancer cases showing significant association and correlation of down-regulation in both NF-kB and TNF-α in the pathogenesis of cervical cancer](image)

**Fig 5.14:** Boxplot analysis for comparative analysis of NF-kB and TNF-α in controls and HPV infected cervical cancer cases showing significant association and correlation of down-regulation in both NF-kB and TNF-α in the pathogenesis of cervical cancer