RESULTS AND DISCUSSION
HPV ANALYSIS
Fig. 8 - PCR Product of HPV L1 Gene Amplification. Lanes 2, 4, 6, 8, 10, 12, and 14 of Rows (a) and (b) Represents L1 Gene Product of 14 DNA Samples. Row (b) Lane 10 And 12 are Negative for L1 Gene. Lane 1 - Marker, Lanes 3, 5, 7, 9, 11, 13 and 15 Represent House Keeping Gene β - Globin.
Fig. 9 - DNA Sequencing Chromatogram of PCR Product for HPV - L1 Gene
لا يوجد نص يمكن قراءته بشكل طبيعي من الصفحة المقدمة.
The prevalence of other types was as follows: HPV 18, 12.5%, HPV 33, 10%, HPV 58, 7.5%. We also found 7 (17.5%) of 40 positive samples with multiple infections with 4-6 types of HPV (Table 7).

**Table 7: HPV Typing Results**

<table>
<thead>
<tr>
<th>Sample Nature</th>
<th>HPV status (L1 gene)</th>
<th>Infection type</th>
<th>No. of samples</th>
<th>HPV Type</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive</td>
<td>Positive</td>
<td></td>
<td></td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Cancer</td>
<td></td>
<td></td>
<td></td>
<td>18</td>
<td>05</td>
</tr>
<tr>
<td>Cervix</td>
<td></td>
<td></td>
<td></td>
<td>33</td>
<td>04</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>58</td>
<td>03</td>
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<td></td>
<td></td>
<td>Multiple</td>
<td>07</td>
<td>16/18/31/52</td>
<td>01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16/18/31/52/58</td>
<td>02</td>
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<tr>
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<td></td>
<td>16/18/52/58/68</td>
<td>01</td>
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<td></td>
<td>16/18/33/58/66</td>
<td>01</td>
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<td>16/18/31/33/52</td>
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<td>Negative</td>
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</table>
DISCUSSION

Epidemiological and molecular studies over the past two decades have demonstrated convincingly that certain types of human papillomaviruses (HPVs) are etiologically related to the development of most cases of cervical cancer [18,91]

Phylogenetically, the genital HPVs belong to the alpha genus and those associated with cutaneous epidermodysplasia verruciformis to the beta genus. Clusters of lower order are known as species, they are closely related phylogenetically and have similar biological properties. The taxonomic status of papillomavirus types, subtypes, and variants remains unchanged and is based on the traditional criteria that the sequence of their L1 genes should be at least 10% in case of two types, 2-10% in subtypes and maximally 2% dissimilar from one another in variants [92]

Infection with oncogenic high-risk HPV types, of which HPV 16 the most prevalent type, was a major risk factor for the development of pre-invasive CIN and invasive cervical carcinoma. HPV DNA, originating from oncogenic types, was detected in up to 95% of the cervical carcinomas [93]

Although HPV has been known as a necessary cause for cervical cancer, it is not a sufficient cause. Other established cofactors are necessary for progression from HPV infection to cancer such as long-term use of hormonal contraceptives, high parity, tobacco smoking. Co-infection with Chlamydia trachomatis (CT) and herpes simplex virus type-2 (HSV-2), immunosuppression.
and certain dietary deficiencies are genetic and immunological host factors and viral factors other than type, such as variants of type, viral load and viral integration, are also important but have not been clearly identified [94]. The eight most common HPV types detected, in descending order of frequency, were HPV-16, -18, -45, -31, -33, -52, -58, and -35 were documented in recent IARC monograph.

A rapid PCR-based test for HPV DNA was developed to accurately investigate the natural history of HPV infections. Amplification of human papillomavirus (HPV) DNA by L1 consensus primer systems (e.g., MY09/11 or GP51/61) can detect as few as 10 to 100 molecules of HPV targets from a genital sample [95].

Our results are in agreement with many studies wherein HPV 16 was reported as the most predominant type followed by HPV 18 in invasive cervical cancer patients, this type was more prevalent in adenocarcinoma than squamous cell carcinoma. We observed a total of 9 high-risk HPVs prevalent in our population viz. HPV 16, 18, 31, 33, 45, 52, 58, 66 & 68. The distribution of HPV types found in our study is quite similar to a recent large scale study reported from India and is also consistent with the most common types found in South East Asia [96,97,98,99].

Various studies demonstrated the presence of integrated HPV-16 and 18 genomes in the vast majority of cancers and in cell lines isolated from cervical malignancies [100,101]. Our findings reconfirm that infection with high-risk HPV and thereafter integration of viral DNA into host genome as a major event leading to malignant transformation. Park et al concluded that integrated
HPV DNAs were detected in patients with far-advanced stage of cervical cancer, with no episomal forms. This group further observed that pure integrated HPV DNA, mixed forms and episomal forms in HPV16 containing cancers, whereas all HPV 18 containing cancers revealed only integrated form [102]. However, there have been several studies reporting only the presence of episomal forms of HPV16 DNA in preneoplastic and neoplastic cervical lesions. For example, Matsukura et al. detected exclusively episomal forms of HPV16 DNA in infected cervical epithelium, albeit this form was present in at least 70% of investigated cervical malignancies [103]. Similarly, Fuchs et al. and Das et al. reported the presence of viral HPV16 DNA in up to 30% of cervical carcinomas, and again, viral DNA was exclusively present in the episomal form [104,105]

The integration of HPV DNA into the host genome leading to loss of function of E1/E2 proteins and subsequent constitutive expression of the oncoproteins E6 and E7 represent two activation mechanisms for the progression of preinvasive lesions to cervical carcinoma [106]. Integration of viral genomic sequences cause deletions and/or a disruption of the E2 gene and results in loss of its function as a regulator of viral gene expression. This event is followed by up-regulation of E6/E7 gene transcription. E6 and E7 proteins deregulate cell-cycle control through interaction with different cell proteins, for example, tumor suppressor gene products such as p53 and retinoblastoma protein (Rb), therebyinitiating the transformation and immortalization of HPV-infected cells [27].

We made an interesting observation of multiple infections with 4-6 HPV types in 7 (17.5%) of HPV patients. Similar observations previously made wherein 5-22% of multitype
infections have been reported A previous study found that infection with multiple HPV genotypes was a factor for persistent HPV infection in healthy young women [107,108,109]. A recent study also indicated that the presence of multiple HPV infections might contribute to the development or progression of cervical dysplasia [110]. A study with patients from Indonesia reported 14% of their specimens to be positive for multiple infections. Infection with 4 different types has been reported in invasive cancers; however we observed 4-6 types in our patients. The true biological properties of multiple HPV genotypes in cervical carcinoma are still to be clarified. It would be interesting to study the physical status of the papillomavirus in multitype infection and the role of E6, E7 and E2 gene expression in these patients. Multitype infections were common among high-risk populations and conferred greater risk for untoward outcomes [111]. The complex interaction of concomitant HPV infections in cervical carcinogenesis remains to be investigated in the future.

The natural history of HPV infection varies from spontaneous regression to persistence. The most important mechanism for wart regression appears to be cell-mediated immunity. CD8+ positive CTLs and CD4+ T-helper type 1 cells (Th1) are the main components of a cell-mediated immune (CMI) response. Activated Th1 cells produce cytokines, including interferon (IFN)-γ and Interleukin (IL-2). IFN-γ acts directly to eliminate virus by inducing an antiviral state in cells while IL-2 acts indirectly by assisting the activation of CTL precursors into an effector population. Both IFN-γ and IL-2 activate NK cells that are important in the first few days of infection until a specific CTL response develops. Cytokines released by keratinocytes or cells of the immune system play a part in the
induction of an effective immune response against HPV infection and the subsequent regression of lesions [106]. There is much focus on stimulating cellular immunity to HPV oncogenes for immunotherapy in cervical cancer.

It is now fairly well established that infection with "high-risk or carcinogenic" types of HPV is an essential step in the pathogenesis of cervical cancer. Incorporation of HPV tests into screening programs might identify women at risk for developing invasive cervical cancer. Several studies have shown the potential relevance of HPV testing in cervical cancer screening program and management of patients with atypical squamous cells of undetermined significance [112]. In low-resource settings, HPV based "screen and treat" strategies can be a highly effective and inexpensive approach to cervical cancer prevention and work is currently underway to determine how best to introduce these programs [113]. In some countries, a combination of both cervical cytology and HPV DNA testing are used to screen all women.

Vaccines for preventing human papillomavirus (HPV) infection are being developed and tested that hold great promise for reducing HPV infections and HPV-associated diseases. Several reports showed the efficacy of prophylactic vaccines for human papillomavirus (HPV) infection [114]. Effective preventive and therapeutic vaccines may become vital in the management of cancer cervix.
CYTOKINE PROFILE
RESULTS

RNA Extraction from Cervical Tumor Biopsies, Cervical Cell Scrapings and Blood Samples

Total RNA was extracted from tumor biopsies, cervical cell scrapings and blood samples from patients. Cervical scrapings were collected from patients where biopsy was difficult or not feasible for eg, in patients undergoing radiotherapy or in follow-up patients. As collection of biological material is the limiting factor, hence obtaining maximum information from the material available is extremely important. Isolation of RNA for gene expression studies is a very cumbersome and time consuming process and is difficult to isolate from cervical cell scrapings due to three reasons: a) The amount of cells obtained from scraping are low b) High levels of endogenous RNase c) contamination with DNA and proteins. To overcome these problems, we developed a simple and efficient RNA isolation procedure by modifying the original method described by Chomczynski and Sacchi, which improved recovery of total RNA from small quantities of cells suitable for gene expression studies (PCR) (annexure -1) [85]. The total RNA recovery and purity was reasonably good.

The quality of the total RNA isolated was assessed by performing agarose gel electrophoresis and analyzed by UV transilluminator. Distinct 28s (50 kb) and 18s (19 kb) RNA bands were visualized. Fig 10a, b and c represents RNA isolated from untreated tumor biopsy, cervical cell scrapings and blood samples respectively.
Fig. 10 - RNA profile of a) frank tumor biopsies b) cervical cell scrapings and c) blood samples
Concentration and purity of RNA was determined by UV Spectrophotometry. Optical density readings were taken at three wavelengths viz., 280, 260 and 230. A solution of 40 μg/ml gives an OD of 1 at 260 and a pure RNA will exhibit an A260/A280 of 2.0. A known amount of RNA is taken to perform downstream applications.

RT-PCR converts RNA molecules into their complementary DNA (cDNA) sequence followed by the amplification of the newly synthesized cDNA by standard PCR amplification. This is an extremely sensitive method to detect and quantify transcripts present in exquisitely low abundance [115]. We optimized conditions for Reverse Transcription - Polymerase Chain Reaction (RT-PCR).

This is a two-step process, the first step being cDNA synthesis by enzyme reverse transcriptase hence referred to as reverse transcription as DNA copy is obtained from RNA. This reaction was performed using oligo (dT)15 as primer in presence of deoxynucleotides triphosphates (dNTPs), magnesium ions and enzyme AMV reverse transcriptase (AMV RTase).

In the second step, cDNA acts as template to amplify the gene of interest by standard Polymerase chain reaction. This reaction is performed in presence of dNTPs, magnesium ions, primer (short nucleotide sequence of gene of interest), a thermostable DNA Polymerase - Taq Polymerase which supports primer extension at elevated temperatures. The amplification product were analyzed by performing agarose gel electrophoresis and visualized using UV trans-illuminator Bio-Rad Gel.
Documentation system 2000, where band density is measured in a semi-quantitative manner to study the mRNA expression levels of a gene.

Results are presented by dividing the patients into two groups viz., patients with disease free status and patients with recurrent disease (residue/lymph node involvement) depending on the outcome of the patient after radiation treatment. The mRNA expression levels of various cytokine markers viz., Interleukin (IL-6), CD3+ T cell (CD3δ), CD4+, CD8+ T cells (CD8 β) and Interferon γ (IFN γ) are studied at tumor site (local) and in blood (systemic) samples at four time intervals i.e pre treatment, 3 week of radiation treatment and 6 week of radiation therapy (end of RT) and follow-up (during review 6 week after completion RT as outpatient) Fig 11a, b, c and d Consolidated results of the patients are presented in Table 8

Out of the 43 patients under study 34 (79.0%) patients responded well to radiation treatment and were disease free in follow up review however 9 (20.9%) patients showed residual disease or nodal involvement during the follow up review

**Expression of IL-6 levels in cervical cancer patients through treatment**

Important immunological mediators of cell-mediated defenses against tumors are cytokines T helper (Th) cells can be distinguished into Th1 and Th2 cells by the type of cytokines they produce
Fig. 11 - RT-PCR Profile of Cytokines. Lane 1 (Marker), Lane 2 (Negative Control), Lane 3 (β-actin), Lane 4 (IL-6), Lane 5 (CD3), Lane 6 (CD4), Lane 7 (CD8) and Lane 8 (IFN γ). Gel (a) Represents cytokine Profile of Tumor Biopsy Specimen Before Treatment (b) After 3 Weeks of RT (c) After 6 Weeks of RT (d) Follow Up (6 Weeks after Completion of RT)
Th1 cytokines are proinflammatory or immunostimulatory cytokines that boost the cellular immune response, whereas Th2 cytokines are anti-inflammatory or immunoinhibitory cytokines with the capacity to subvert the cellular immune response. IL-6 is expressed by Type 2 T cells and provides efficient help for B cell activation. All the 43 tumor tissue samples collected before radiation treatment amplified for IL-6. Out of 34 disease-free patients, 9 showed increased IL-6 expression in cervical cells scrapings, whereas 12 had decreased levels, and 9 patients showed no change in levels of IL-6 expression after 3 weeks of radiation treatment. In 4 scraping samples, IL-6 levels were below detection. In cervical samples collected at the end of 6 weeks of RT, 7 samples showed increase and 17 showed decreased IL-6 levels. Whereas, samples did not show any change and below detection levels in 5 samples for IL-6. In the case of follow-up samples, 15 cases showed decreased expression, and in 11 samples no change was noticed in IL-6 levels. 7 samples showed an increase in levels.

Recurrent disease was observed in 9 of 43 patients taken for study, of these, 7 showed increased expression, and 2 samples showed no change in the expression levels after 3 weeks of RT when compared to untreated samples. In 6 weeks RT samples, 5 showed increased expression, whereas 3 samples showed no change, and 1 showed decrease in IL-6 expression. In case of follow-up samples in recurrent disease group, 6 samples still showed increased levels, 1 sample showed no change, and 2 samples had levels below detection for IL-6 mRNA.
Interleukin - 6 expression in blood samples collected prior to RT in disease free group, showed that out of 29 out of 34 patients amplified for IL-6 5 samples had levels below detection 10 samples showed decrease and 19 showed no change in blood levels of IL-6 after receiving 3 weeks of RT Only one sample showed slight increase in IL-6 levels when compared to untreated samples In samples collected after completion of 6 weeks of RT, a similar trend of IL-6 expression was observed as in after receiving 3 weeks of RT Among follow up samples 1 showed mild increase, 10 showed decrease and 9 samples had no change in IL-6 expression levels 14 samples had levels below detection (Fig 12)

Increased blood levels of IL-6 in untreated was observed in all patients Overall view of IL-6 expression in tumor tissue samples reveals that post treatment sample levels of expression varied with the outcome of patient In the case patients who responded to treatment the IL-6 expression levels showed a decline trend when compared to patients who did not respond to radiation therapy In non-responding patients expression levels of IL-6 remained elevated or showed a trend of progressive increase through out the study time intervals However, recurrent disease group showed a similar trend as that of disease free group in expression of IL-6 levels in blood

We observed that the tissue expression of IL-6 acted as a better prognostic marker when compared as blood even though both the sites showed a similar trend Thus, target site levels of IL-6 expression have an advantage over systemic levels of IL-6 to predict the outcome of the patient and response to radiation therapy
Fig. 12 - Graphic Representation of IL-6 Expression Levels after 3 Weeks, 6 Weeks of RT and Follow up (6 Weeks after Completion of RT)

Disease Free a) cervix b) blood and Recurrent Disease c) cervix and d) blood samples
Expression levels of Interferon γ in cervical cancer patients through treatment

Interferons are a family of secreted polypeptides with distinct biological effects. These effects include the regulation of expression of specific cellular genes, antiviral properties, and inhibition of cell growth and proliferation [116]. Very low levels of IFNγ expression were observed in 7 untreated cervical biopsy tissue samples. The remaining 36 samples either had no or undetectable levels of IFNγ expression in the tumor tissue samples. Low expression was observed in untreated samples of patients from early cancer stage (IA/B) and not the advanced stage cancer patients. These levels further decreased during radiation treatment and only 4 samples showed very faint amplification in 3 week RT samples (Fig 13). There was no difference in the expression levels between disease-free and recurrent disease group patients. 3 blood samples collected before treatment had very low levels of IFNγ amplification. None of the blood sample collected during RT and post RT showed any expression of IFN γ.

T cell profile in uterine cervix patients through radiation treatment

Defective immune responses are thought to play a role in cancer development, and especially in neoplasias with a probable viral etiology [117]. The control of viral infections, which may be crucial in the etiology of preinvasive and invasive cervical neoplasia, depends largely on cellular immunity mediated by T-lymphocytes. Immunosuppressed individuals show an increased incidence of such lesions [118].
Fig. 13 - Graphic Representation of IFN-γ Expression Levels after 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)

Disease Free a) cervix b) blood and Recurrent Disease c) cervix and d) blood samples
Immunosuppression, as manifested by reduced lymphocyte numbers and responses, or by aberrations in T-helper (CD-4) cell and T-suppressor/cytotoxic (CD-8) cell distribution, has been reported in cervical cancer [119,120]

Here we made an attempt to analyze mRNA expression of levels of T cells viz the helper/inducer subset of T cells (CD4+), suppressor/ cytotoxic T cells (CD8+) and CD3+ T cells (representing the total T-cell population) in cervical tissue biopsies before treatment and in cervical cell scrapings during and post treatment. Blood samples were also analysed for T cells at all the four time intervals.

**CD4+ T cells:**

The expression of the helper/inducer subset of T- cells (CD4+) was seen in both in cervix tissue and blood samples. The expression levels of CD4+ cells were slightly higher in blood than in infiltrated tumor tissue. 41 out of 43 cervical biopsies collected before treatment showed CD4+ T cell expression. Of this, 33 belonged disease free patients and 8 were those of recurrent disease group. In disease free group cervical cell scrapings collected at the end of 3 weeks and 6 weeks after RT showed sharp decrease in the expression levels in 22 and 23 samples respectively. However, 10 samples after 3 weeks and 6 samples after 6 weeks of RT showed no change in expression levels (Fig 14). In follow up samples 14 cases still showed decreased levels of this marker expression and 4 samples showed slight improvement in expression levels. 10 samples showed no change and 6 had levels below detection in disease free group.
Fig. 14 - Graphic Representation of CD4+ T Cell Expression Levels after 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)

Disease Free a) cervix b) blood and Recurrent Disease c) cervix and d) blood samples
The recurrent group disease also followed similar trend where expression levels fell drastically during and post treatment samples. Only 1 out of 9 samples showed slight increase in expression levels post treatment.

Blood levels of helper T cells (CD4+ T cells) in tumor tissue before treatment showed that total of 36 samples showed CD4+ expression. Blood samples collected at the end of 3 weeks and 6 weeks after RT in diseases free group showed that 21 and 19 samples respectively showed sharp decrease in the expression levels. However, 4 and 3 samples showed no change with RT. In follow up samples 18 cases still showed decreased levels of this marker expression and three samples showed slight improvement in expression levels. 5 samples showed no change and 8 had levels below detection. After radiation therapy, the fall in recurrent group was greater than that of disease free group. The disease free patients showed a faster recover by showing increase in expression levels in blood however, the recurrent group showed a reduced tendency to recover. Tissue expression levels of CD4+ T cells were lower than blood levels in post treatment samples.

**CD8+ T cells**

The expression levels of suppressor/cytotoxic T cells (CD8+ T cells) were slightly higher in tumor tissue than in blood before treatment. 40 untreated tumor tissue samples amplified for CD8β+T cells, out these 31 samples were from disease free patients and 9 were from residual/ recurrent disease patients. CD8+ T cells expression levels were found to be independent of stage of disease. Decreased expression levels of CD8+ T cells were seen in both
tumor tissue and blood samples after 3 weeks and 6 weeks of treatment and also in post radiation samples. In samples after 3 weeks of radiation treatment 17 samples showed decreased expression, 2 samples did not show any change in expression levels of CD8β+ T cells. However, in 14 samples the levels were too low to be detected. A similar trend was observed in samples after 6 weeks of radiation therapy and post treatment samples. Expression levels showed no improvement in post treatment samples when compared to end of treatment samples. We observed a similar trend in non-responding cases were a sharp fall in the level of expression was noticed in samples collected during and post RT (Fig. 15).

CD8β+ T cell expression in blood revealed decrease in levels with radiation exposure that did not improved in samples collected after 6 weeks post RT. 26 of disease free patients and 6 of recurrent disease patients were positive for CD 8 +T cell marker in blood samples collected before treatment. A drastic decrease in the expression levels was noticed in blood samples collected after 3 weeks and 6 weeks of radiation exposure. In disease free group 15 patients, showed decrease levels of expression after 3 weeks of RT when compared to before treatment samples. 7 samples showed no change and 10 samples had levels below detection. After 6 weeks of RT, blood samples further showed decreasing levels of CD8+ T cells. In 12 samples expression levels were below detection for this marker in blood. Only one blood sample showed a slight improvement in expressing CD8β+ T cell marker among follow up samples. In case of recurrent disease group none of the samples showed any increase. However, a consistent decrease in the levels of CD8+ T cells was noticed thorough out treatment and this saw no improvement in blood samples collected post treatment.
Fig. 15 - Graphic Representation of CD8+ T Cell Expression Levels after 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)

Disease Free a) cervix b) blood
Recurrent Disease c) cervix and d) blood samples
CD3+ T cells

The levels of total T cell expression (CD3δ+ cells) were examined in cervical tissue and blood samples in patients before radiation therapy. The higher levels of CD3+ T cell expressions were found in advanced disease (stage II and III) than that of initial stages of disease i.e., Stage I. Frank tumor biopsy samples collected prior to radiation treatment 39 out 43 cases were positive for CD3δ+ T cells as determined by expression of CD3δ, marker of T cell infiltration. 4 samples were negative for amplification as levels could be below detection. The CD3+ T cells (representing the total T-cell population) fell drastically after radiation therapy in cervical tissue. 25 samples showed drastic decrease in the expression levels of CD3δ marker after 3 weeks of radiation therapy. Out of this, 19 samples belonged to disease free group of patients and 6 were that of recurrent disease group. A total of 4 samples did not show any expression of this marker. After 6 weeks of RT, 27 samples showed further decrease in levels CD3δ marker and 12 had levels below detection. 4 samples however did not show any change in the expression levels. In follow up patients cervical cell scrapings collected at the time of review showed that 25 patients still had decreased levels of CD3δ expression whereas, only 2 samples showed slight increase in expression levels one each from disease free and recurrent disease group. 10 samples had levels below detection for the marker (Fig 16).

Blood levels of CD3+ T cells were also found to fall sharply during radiation and did not show much improvement in post RT samples.
Fig. 16 - Graphic Representation of CD3+ T Cell Expression Levels after 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)

Disease Free a) cervix b) blood and Recurrent Disease c) cervix and d) blood samples
A notable depression in the counts of CD3+ cells was evident in both disease free and recurrent disease group patients immediately after irradiation. CD3+ cells expression levels showed a slight increase in post treatment samples from disease free subjects when compared to recurrent disease status.
DISCUSSION

Squamous cell carcinoma is thought to be an immunogenic tumor. Solid tumors consist of malignant cells and stroma. Malignant cells elicit stroma formation that is essential for neoplastic growth [121]. Premalignant lesions can regress spontaneously, and if progression to cervical tumors occurs, in the stroma, a significant number of infiltrating host leukocytes consisting of CD4+ T cells, CD8+ T cells, monocytes, macrophages, and granulocytes are seen [122]. Also, these cells obtained from neoplastic effusions are extremely useful in evaluating the interactions between immune and cancer cells in the tumor microenvironment [123].

The interaction between tumor cells and the nearby environment affects the initiation and progression of cancer. Host-tumor relationship results in the production of pro-inflammatory cytokines and chemokines that promote the recruitment of leukocytes within and around developing neoplasms.

Immunologic surveillance, as first described by Burnet in 1967, states that the immune system has evolved to distinguish self from non-self. Tumors are transformed cells bearing non-self antigens. The role of the immune system is to propagate the reaction between host and tumor cells and thus act as a primary mechanism for the natural defense against neoplasia. The cell-mediated arm of the immune surveillance plays a major role in the destruction of neoplastic cells [4].
Escape from immunologic surveillance accompanies the rapid progression of several cancers. In patients with advanced cervical carcinoma, depressed systemic immune responses have been observed [124,125]. Human cervical carcinoma has a characteristic stepwise progression, the anticancer immune reactions are especially important for localizing the spread of this malignancy [126]. Various immune escape mechanisms of cancer have been proposed. Among this failure of the anti-tumor immune response can be related to defective immune regulation [127]. In addition, certain cells may secrete immunosuppressive factors to modify the host immune responses [128,129]. Infiltrating immunocytes (TILs) within the cancer milieu of cervical carcinoma may play an important role in anticancer immunity [130,131]. Cancer cells, together with newly recruited infiltrating cells, can also activate fibroblast and vascular responses, thus resulting in a chronic microenvironment perturbation. In this complex scenario, interactions between innate and adaptive immune cells can be disturbed, leading to a failure of immune-mediated recognition and destruction.

Human papilloma virus (HPV) is detected in 90% of patients with cervical squamous carcinoma predominantly HPV type 16 and 18 [93]. T-cell-mediated protection from viral infection as well as control of tumors is promoted by type 1 cytokine responses and impaired by type 2 cytokine responses.

From radiation therapeutic point of view, the role of immune system against cells causes concern because of the detrimental effect of irradiation on the immune system. It is associated with lymphopenia [132] and depressed T cell function as assessed by
standard *in vitro* lymphoproliferative assays with mitogens and allogenic. The deleterious effects of radiation therapy seem to relate directly to the volume of blood, lymph nodes, or bone marrow within the irradiated volume, and changes have been observed after radiation therapy to the head and neck region, mediasterinum, pelvis, and skull [133]. Immunosuppressive effects of irradiation tend to be prolonged, and patients who are clinically in remission continue to show immune abnormalities for years after completing radiation therapy.

Assessment of expression levels of cytokines viz Interleukin-6 and interferon γ and T helper (CD4+ T cells), T suppressor (CD8+ T cells) and total lymphocytes (CD3+ T cells) as a result of cancer per se and effect of radiation treatment on their levels would give valuable information. There have been only few reports on the population of lymphocytes present in cancer cervix patients. Studies done so far have either concentrated on lymphocyte subpopulation in peripheral blood or tumor infiltrating lymphocytes. There is little of information on the effect of radiation treatment on both local and systemic expression of cytokines and lymphocyte population has not been studied in detail so far.

**Interleukin-6 expression:**

Interleukin-6 (IL-6) plays a central role in defense mechanisms, including the immune response, acute phase reaction and hematopoiesis. On the other hand, IL-6 has a potent anti-tumor activity against certain types of tumors. This anti-tumor effect is mediated by in vivo induction of specific cytotoxic T cells and in part by a growth inhibitory activity of IL-6 [134]. There are
reports of analysis of several human clinical situations in which the intratumoral production of certain cytokines is clearly associated with the clinical evolution of the disease. Interleukin-6 (IL-6) has received particular attention in the pathogenesis of cervical cancer, although the underlying mechanism remains elusive.

We observed expression of IL-6 in all the 43 frank tumor biopsies. We also noticed that the expression of IL-6 was consistently high in both pre and post treatment samples of recurrent disease subjects when compared to disease free patients. Studies have shown that cervical cells were capable of expressing, at transcriptional level, cytokine mRNA for IL-6, IFN-γ and TNF-α and found significant increase in expression of IL-6 gene in invasive cervical carcinoma as compared to CIN and normal cervix [135]. Our observations are in agreement with studies by Pages et al, where IL-6 levels were found to be associated with tumor invasiveness [136]. Higher expression of IL-6 in cancerous tissues than in adjacent tissues in early stage cervical cancer patients was observed by wei et al. Patients with a deeper stromal invasion, vaginal invasion, lymphovascular emboli, or lymph node metastasis appeared to have high intratumoral IL-6 levels [137].

IL-6 performs various biological functions through binding to α-chain (IL-6-R, gp80) and subsequently recruiting the β-chain (gp130) of the receptor. Specifically, the IL-6/IL-6R complex initiates homodimerization of gp130, activates a cytoplasmic tyrosine kinase bound to gp130 [138], and then triggers signaling cascades through the Jak/STAT, Ras/MAPK, and PI 3-K/Akt pathways [139]. The diversity of IL-6 signaling mediated via gp130 explains its functional pleiotrophy. IL-6 regulates inflammatory
reactions, immune responses, hepatic acute-phase protein synthesis, and several other important physiological processes [140] Interestingly, the influence of IL-6 in human cancers is varied depending on the cell types. For example, IL-6 has been demonstrated to promote growth of multiple myeloma, Kaposi's sarcoma, and prostatic cancer cells, while inhibiting the proliferation of lung and breast cancer cells [141,142]

The in vivo angiogenic assays showed that IL-6 increases angiogenic activity in human cervical cancer cells, an effect that is specifically associated with upregulation of vascular endothelial growth factor (VEGF). IL-6 gene is also expressed by the keratinocytes [143] IL-6 expression in tumor tissues was high and correlates with the severity of cervical cancer [135,137] Also, IL-6 may contribute to a local immunosuppressive effect that protects the tumor cells from the host immune system [144] These data support the hypothesis that IL-6 promotes the development of cervical cancer, although the underlying mechanism remains unclear.

We observed fall in IL-6 levels in blood of few patients undergoing RT where as many patients did not show any change in expression levels post treatment. We did not find any difference in serum IL-6 expression levels between responding and non-responding patients. This could be because brachytherapy induces small inflammatory reactions and radiotherapy is less invasive than surgery from the point of view of cytokine related inflammation hence it would be expected to cause no significant change in blood IL-6 levels in post treatment samples. Tang et al who studied serum IL-6 levels after radiation therapy in sixteen patients with cervical
cancer also observed that RT did not cause much change in expression levels [145] Another study indicated that radiation therapy tend to raise levels of plasma IL-6 in breast and prostatic cancer patients suggestive of monocyte activation [146]

To sum up, we observed that consistent overexpression of IL-6 in cervical tissue through radiation treatment indicates poor prognosis and that expression level of this marker reflects aggressiveness of tumor Tissue (local) IL-6 levels have a better marker potential when compared to blood (systemic) levels

**Interferon γ expression:**

IFN-γ, secreted by Th1 cells, cytotoxic T cells, and stimulated natural killer cells, is a major contributor to an effective Th1-type cellular immune response against HPV infections [116] Inappropriate cytokine synthesis may direct the local immune response away from a type-1 (cellular) pattern and may subsequently contribute to the development and progression of precancer Woodworth et al reported that IFN-γ transcriptionally repressed HPV type 16 gene expressions in HPV 16-immortalized cell lines and inhibited the cell growth [147]

Our results indicated that IFN-γ expression is highly depressed in advanced cancer cervix We observed low levels of IFN-γ expression in 16.2 % cervical tumor tissue before treatment whereas 83.7 % of patients had no expression or levels below detection These levels fell down further during and post treatment samples Blood expression levels of IFN-γ were almost undetectable in all the samples irrespective of treatment [117]A significant
decrease in circulating IFN-γ concentrations in women with severe dysplasia and invasive carcinoma was observed. Defective IFN-γ production may be associated with persistent HPV infection and the development of HPV related neoplasia [74]Tartour et al. have recently shown that poor prognosis and tumor recurrence in cervical cancer patients was associated with detection of low numbers of IFN-γ mRNA copies in fresh biopsy specimens, suggesting a role for type 1 cytokine secretion in the control of cervical growth [148].

Based on the reports of downregulation of IFN-γ on HPV gene expression, Poa et al, reported reduced expression of IFN-γ in CIN and cervical cancer [119]. This reduction in IFN-γ levels in cervical tissues may contribute to a local environment which favors the persistent propagation of HPV in these tissues. Such persistent HPV infection may in turn facilitate the development of malignancies in these tissues [117]

Various studies have also demonstrated a decrease in the expression of IFN-γ and IL-12, an IFN-γ inducer, in invasive carcinoma compared with premalignant biopsies or normal cervix [118]. A defect in IFN-γ expression at the site may therefore promote progression, as in vitro, IFN-γ inhibits expression of HPV Type 16 and 18 genes in immortalized cell lines and inhibits the growth of most cervical carcinoma cell lines [147]. A role for IFN-γ in rejection has also been demonstrated in many murine models [120]. The absence of IFN-γ mRNA expression cannot be explained by a defect of T cell recruitment inside the, as biopsies from patients with no IFN-γ expression did not appear to show less T cell infiltration than control biopsies with measurable IFN-γ gene
expression [149]. In cervical carcinoma biopsies a decline of interferon-γ (IFN-γ), interleukin-12 (IL-12), and monocyte chemoattractant protein (MCP)-1 mRNA expression in comparison with normal cervix tissue was observed [118,148]. Majority of ovarian carcinoma expressed TGF-β and IL-10, with absence of expression of IFN-γ [150]

Bais et al, showed IFN-γ concentrations in plasma from patients with cervical cancer and controls were similar [151], whereas in a study by Lebrecht et al observed IFN-γ concentrations were below the detection limit in all groups [152] IFN-γ production was decreased by radiation Radiation had significant effects on cells and cytokines that can influence angiogenesis, growth and immune status [153]

The analysis of cytokine expression by reverse transcriptase polymerase chain reaction (RT-PCR) showed that tumor associated macrophages (TAMs) had reduced expression of genes for IL-2, IFN-γ and IL-4, and inversely, had increased IL-10 gene expression relative to normal PBMCs [154] Taken together, these results suggest that the incomplete activation of TAMs in vivo may be due to the accumulation of Th2 cells instead of Th1 cells, and it is plausible that the increased IL-10 contributes to down regulation of the Th1 cytokines TAMs produced appreciable amounts of IL-6 and spontaneously released significantly higher amounts of IL-8, compared to PBMCs These differences in cellular composition and the variable prognostic significance of leukocytes that infiltrate many human tumors suggest that different types of interactions are possible between and host cells, possibly resulting in heterogeneous responses [155]
**T-Cell profile:**

In neoplastic lesions, immune cells are recruited by soluble chemotactic factors originating from carcinoma cells, and the fate of the lesions is eventually determined by a complex interaction between the attracted immune cells and the carcinoma cells. Although the role of infiltrating cells in malignant tumors is controversial, a likely stimulus for their presence is the local production of chemokines, so that the leukocyte content of a tumor may depend on the expressed cytokines [156].

Local radiation therapy is a widely accepted treatment for various types of neoplasia. The lymphopenic effect of irradiation has been recognized for many years, and the cellular depletion, which mainly involves recirculating lymphocytes, may possibly explain, at least in part, the increased incidence of infections in some patients and the impairment of delayed type hypersensitivity responses [157]. It has also been shown that the various immunologic functions of peripheral blood lymphocytes measured by in vitro assays are affected to differing extents. Quantitative analysis of TILs in the cancer milieu may provide clues for elucidating the possible cancer-host immune interactions in human cervical carcinoma. Thus, the findings relating to the behavior of the T-cell subsets, namely the helper/inducer T-cell (CD4+) and the suppressor/cytotoxic T-cells (CD8+) would be of significant value.

CD4+ T cells play a central role in orchestrating the immune response to cancer. Essentially, CD4+ T cells recognize peptides presented on MHC class II molecules expressed primarily on antigen-presenting cells. Although most tumor cells do not express
MHC class II molecules, CD4+ T cells can effect an antitumor response in the absence of CD8+ T cells by secreting cytokines, such as interferon-γ [158], or by activation and recruitment of effector cells such as macrophages and eosinophils [159]. However, the main role of CD4+ T cells in the immune response to cancer is to prime CD8+ cells and maintain their proliferation.

In the immune response to cancer cells, tumor-infiltrating CD8+ T cells play an essential role, recognizing tumor-associated antigens presented on MHC class I molecules expressed on the cancer cell surface and directly lysing cancer cells expressing the same antigens. In a variety of cancers, larger numbers of tumor-infiltrating CD8+ T cells usually signified a stronger immune reaction against the cancer and indicated a better prognosis.

We observed slightly higher levels of CD4+ and CD8+ T cell expression in frank tumor tissue of disease free patients when compared to patients with recurrent disease. However, both the group of patients showed decreased expression levels during and post RT. Our result is in agreement with other studies where in the presence of a relatively high level of CD4+ T-cell infiltration, patients with a sufficient number of tumor-infiltrating CD8+ T cells demonstrated a significantly better prognosis. A similar synergistic effect between tumor-infiltrating CD8+ T cells and CD4+ T cells in oesophageal squamous cell carcinoma and pancreatic adenocarcinoma has been demonstrated [160,161]. Hiraoka et al. suggested that cooperation between infiltrating CD4+ T cells and CD8+ T cells in tumors might be important in the suppression of the progression of Non small cell lung carcinoma (NSCLC) [162]. Previous studies have demonstrated that activation of CD4+ T cells
is required for immunisation of CD8+ T cells against cancer. For activation and maintenance of tumor-infiltrating CD8+ T cells, CD4+ T cells play an important role by secreting cytokines such as interleukin-2, which is required for CD8+ T cell growth and proliferation [163]. Reduction of CD4+ T lymphocytes by administration of anti-CD4 antibody allowed human lung cancer xenografts to form orthotopically in immuno-competent mice. As CD4+ T cells are necessary for the full antitumor activity of CD8+ T cells, this may explain why a high level of CD8+ T-cell infiltration alone in this study did not correlate with a more favorable prognosis. Neither CD8+ T cells within cancer cell nests nor those in cancer stroma had a significant impact on patient survival. The reasons for this discrepancy were difficult to explain, because the antitumor effect of CD8+ T cells may be circumvented by various mechanisms in the tumor cells [162].

Despite the presence of a lymphocytic infiltrate, many human tumors including cervical cancers grow relentlessly; suggesting that TIL populations may eventually become functionally anergic in vivo. The factors responsible for this suppression are not known but may include the release of immunosuppressive cytokines, such as TGF-β, IL-10, or vascular endothelial growth factor by infiltrating mononuclear cells and cells [164]. These data allow speculation that absence or reduced levels of costimulatory molecules on cervical cells could eventually lead to a reduction in the local secretion of cytokines (i.e., IL-2) by the chronically activated TIL [165,166] and, eventually, to a progressive deterioration in their function, as demonstrated by CD25 down-regulation and reduced expression of CD3-ζ. Studies by Rabinnovich et al showed that TAMs had reduced expression of genes for IL-2, IFN-γ and IL-4, and
inversely, had increased IL-10 gene expression relative to normal PBMCs in ovarian cancer [154] Taken together, these results suggest that the incomplete activation of TAMs in vivo may be due to the accumulation of Th2 cells instead of Th1 cells, and it is plausible that the increased IL-10 contributes to downwards regulation of the Th1 cytokines

Studies have shown that lymphocytes infiltrating invasive carcinoma of the cervix were of CD8 phenotype and infiltrating CD8+ and memory CD45RO+ cells were more numerous in invasive cancers than in dysplastic lesions [126,167] Massive infiltration of CD8+ and CD45RO+ cells in the stroma and epithelium of invasive carcinoma with lower CD4+ cell counts and decreased CD4/CD8 ratios have been demonstrated by some researchers [168] Such observations raise the question why infiltrating memory CD8+ cells are not functional This could be because the mucosal HR-HPV has a battery of immune evasion mechanism

Tumor cells may obtain the ability to evade immune surveillance by several strategies, including a lack of adequate T-cell costimulation [169], downregulation of cell-surface MHC class I expression [170], dysfunction of Fas (CD95/APO1)-mediated apoptosis [171] and secretion of immunosuppressive factors, such as transforming growth factor-β [172] or interleukin-10 [173] Thus, the efficiency of the immune reaction against cancers can be evaded by a variety of mechanisms used by tumor cells, and these can vary, depending on the individual cancer

IL-2Rα is pivotal for the proliferation and differentiation of functional T cells, depressed expression in the tumor milieu may
result in rendering the TILs into an anergic state, which may alter the composition of anti tumor effector cells. Generally, the generation of tumor specific responses is believed to depend on the help of activated CD4+ T cells [174] The tumor-reactive CD4+ T cells can produce lymphokines that amplify the cytotoxic activity of CD8+ T cells and other inflammatory cells and activation of both CD4+ and CD8+ T lymphocytes will lead to an efficient immune response to destroy tumor cells [175] It has been shown that the inability of the host to reject a tumor may be due to insufficient generation of specific CD4+ T cells. It is possible that CD4+ T cells are not necessary for inducing, but serves to amplify, the cytotoxic T-cell response. Accordingly, low proportions of CD4+ T cells may correlate with poor antitumor response. The relatively low CD4+ T cells with reversed CD4/CD8 ratios in tumors from lymph node positive patients support this view. Decreased tumor-infiltrating CD4+ T cells with further reversed CD4/CD8 ratios also were observed in bulky tumors from patients with cervical carcinoma. Overgrowth of tumor may be the consequence of poor regional immune response. As growth progresses, tumor-associated antigens may be taken up by antigen-presenting cells to activate the specific CD4+ T cells through the MHC Class II pathway. Failure of the antitumor immune response, as discussed above, can result from inadequate activation of specific CD4+ T-helper cells [176]

A recent study by Steinbrink et al, showing that IL-10 treated human dendritic cells can induce anergy in tumor specific cytotoxic CD8+ T cells and result in failure to lyse tumor cells, further elucidates the down-regulatory role of IL-10 in tumor-mediated immunosuppression [177] Activated T cells in human
cervical carcinoma milieu predominantly expressed the Th2/Tc2 phenotype. Cancer cells could drive the encountered T cells toward Th2/Tc2 polarity, which attributed to the prominent IL-10 and TGF-α expression in cervical carcinoma cells [178].

Despite the fact that no prognostic significance of high CD4+ T-cell infiltration alone was observed, a synergistic effect of simultaneous high CD4+ T-cell and CD8+ T-cell infiltration in cancer stroma as a favorable prognostic factor in Non Small Cell Lung Carcinoma (NSCLC) was seen[162]. Evaluations on the prognostic significance of infiltrating lymphocytes (TIL) in other human cancers revealed that especially intraepithelial infiltrating CD3+ CD8+ T cells contributed to better prognosis [179]. On the other hand, the infiltration by CD3+CD4+ T cells or a subpopulation of CD4+ T cells with immunosuppresive properties, so called regulatory T cells that were detected was reported to counteract the beneficial effect of CD8+ T cells [180]. High ratios between CD8+ T cells and the other cells types were associated with improved survival.

We observed that CD3+ T cell (total T cell population) expression levels did not show any difference between responding and non-responding patients in tissue biopsy samples collected prior to treatment. All the subsets of T cell population showed a drastic decrease in the expression levels during and post treatment. A significant decrease in CD3+ cells counts has been reported [181] reflecting the nonspecific lymphodepleting effect of radiation therapy.
Our studies confirm that CD8+ T cell recruitment is necessary for antitumor immunity. Increased expression levels of CD8+ T cells were found in cervical tissue than in peripheral blood. Higher CD8+ T cell levels in the tumor tissue prior to treatment and post treatment resulted in better prognosis. Inefficient antitumor response by tumor infiltrating CD8+ and CD4+ T cells results in residual disease in some patients, this may be due to tumor-mediated immunosuppression or anergy of T cells due microenvironment of the tumor milieu. Increased expression of cytokine IL-6, greater infiltration of tumor with CD8+ T cells and decreased expression of IFN-γ suggests that the human cancer cells may alter the functional composition of antitumor effector cells towards Th2/Tc2 polarity. However, we also noticed in few patients the above said trend did not hold good and demonstrated variable outcome. The implication of such findings is at present unknown, but previous reports have clearly shown that patients belonging to similar FIGO stages are often heterogeneous in their immunological competence. It is therefore possible that these differences may represent different “immunologic stages” of the interactions between the host immune system and tumor cells. Radiation-induced T-cell lymphopenia was observed in patients during and post RT. The levels of T cells did not show much improvement in post treatment samples collected 6 week after completion of RT, suggesting that radiation induced lymphopenia requires longer time for recovery.
DNA ADDUCTS
RESULTS

Markers of oxidative stress are known to be useful in evaluating tumor burden and response to therapy. In this chapter we aim to assess oxidative damage caused by both exogenous and endogenous sources, to DNA in terms of modified deoxynucleotides i.e., DNA adducts. We also studied the influence of radiation therapy on the levels of these adducts. 43 invasive cervical cancer patients were taken to study the levels of polar (P-1, P-2, 8-oxodG) and lipophilic (L-1) adducts. From each patient cervical samples were collected at four time intervals namely, untreated cervical biopsy, at the end of 3 weeks of radiation treatment (30 Gy), at the end of six weeks of radiation treatment (i.e., completion of radiation treatment with (60 Gy) and during follow up review (6 weeks after completion of radiation therapy). Consolidated results are presented in Table 9. Samples were analysed for normal nucleotides level simultaneously for all samples (Fig 17).

Estimation of 8-Oxodeoxyguanosine Levels:

All the samples analyzed at different stages of treatment showed the presence of 8-oxodG adducts (Fig 18). There was significant increase in the levels of 8-oxodG adducts levels between untreated and 3 weeks of radiation therapy (p=0.003). A decrease in the levels of 8-oxodG adducts was observed from 3 weeks of radiation to 6 weeks of radiation but was not statistical significant (p=0.529). A slight increase in the levels of 8-oxodG adducts was seen from end of treatment to follow up samples. However, this increase was not statistical significant (p=0.384).
Fig. 17- Scanned Image of Autoradiogram of Normal Nucleotides
Fig. 18 - Scanned Image of Autoradiogram of
a) 8-OxodG Adduct b) control - no DNA
8 OxodG adduct

(a)

- DNA

(b)
Fig. 19 - Graphical Representation of 8- OxodG Adduct Level in a) Stage I, b) Stage II and c) Stage III Patients at four Time Intervals viz., Before Treatment, After 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)
When a comparison was made between untreated and follow up samples we observed an increase in adduct levels that reached statistical significance (p=0.006). Total 8-oxodG adducts formed at four intervals namely, untreated samples, 3 weeks of radiation treatment, 6 weeks of radiation treatment and in follow up (post treatment) samples were, 2,393, 4,134, 4,675, 3,772 adducts/10⁹ N respectively.

Highest levels (2,985 adducts/10⁹ N) of 8-oxodG were found in untreated Stage I samples when compared with Stage II (2,226 adducts/10⁹ N) and Stage III (1,969 adducts/10⁹ N) samples (Fig.19). However, Stage II samples showed greater increase in 8-oxodG levels (5,485 adducts/10⁹ N) than that of Stage I (3,857 adducts/10⁹ N) or Stage III (3,059 adducts/10⁹ N) samples after 3 weeks of radiation treatment. The levels of 8-oxodG adducts were found highest in stage III samples (5,353 adducts/10⁹ N) at the end of 6 weeks of radiotherapy when compared to Stage I (4,328 adducts/10⁹ N) and Stage II (4,343 adducts/10⁹ N) samples. In follow up samples the lowest levels were found in stage I (3,166 adducts/10⁹ N)

**Estimation of Polar Adducts:**

We analyzed polar and lipophilic adducts in our study samples. These adducts are designated as P1, P2, and L1 based on their mobility, derived by subjecting to varying concentrations of sodium phosphate.
P1 and P2 adducts were resolved using lower concentrations of sodium phosphate. P1 and P2 adducts patterns are shown in Fig 20.

P1 adducts showed significant increase after 3 weeks of radiation treatment when compared to untreated samples (p=0.0007) whereas, increase in levels of adducts from 3 weeks to 6 weeks of treatment did not reach statistical significance (p=0.618). We observed decrease in the adduct levels at end of treatment compared to follow up samples, it reached statistical significance (p=0.007) No significant increase in adducts levels was observed when a comparison was made between untreated samples with that of follow up samples (p=0.978). Total P1 adducts found at four study intervals were as follows, 4,280, 27,273, 20,952, and 6,446 adducts/10^9 N respectively.

Stage I showed lowest (3,259 adducts/10^9 N) P1 adduct levels in untreated sample whereas highest levels were shown by stage II samples (5,463 adducts/10^9 N). Untreated Stage III samples had P1 adducts level of 4,118 adducts/10^9 N. We observed 4-11 fold increase in the adduct levels after 3 weeks of radiation therapy. Stage I samples showed the highest increase in adduct levels (36,113 adducts/10^9 N) followed by Stage III (19,029 adducts/10^9 N) and Stage II (26,677 adducts/10^9 N) samples. A trend of decrease in P1 adducts levels was seen in Stage I (16,104 adducts/10^9 N) and III (12,197 adducts/10^9 N) samples whereas, Stage II sample showed increase (34,554 adducts/10^9 N) in adduct level after 6 weeks of radiation therapy. Stage I samples showed the highest P1 adduct levels (12,939 adducts/10^9 N) in the follow up samples and the lowest levels were seen in Stage II (3,765 adducts/10^9 N) samples (Fig 21).
Fig. 20- Scanned Image of Autoradiogram of P-1 (green arrows), P-2 (purple arrows) and L-1 (yellow arrow) adducts at four Time Intervals viz., Before Treatment, After 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)
Fig. 21 - Graphical Representation of P-1 Adducts Level in a) Stage I, b) Stage II and c) Stage III Patients at four Time Intervals viz., Before Treatment, After 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)
We observed significant increase (p<0.0001) in the levels of P2 adducts in samples after 3 weeks of radiation treatment from that of untreated samples. There was slight decrease in the P2 adduct levels in the 6 weeks of treatment samples, however this was not statistically significant (p=0.854). A further decrease in levels of adducts were observed in post treatment samples (follow up), which was significant (p=0.006) when compared with 6 weeks treatment samples. There was a significant increase (p=0.006) in the levels of P2 adducts between untreated samples from that of post treatment samples. The total P2 adducts found in untreated, 3 weeks treatment, 6 weeks treatment and follow up (post treatment) samples were 2,847, 20,692, 15,418 and 5,931 adducts/10^9 N respectively.

Lowest levels of P2 adducts in untreated samples were observed in Stage II (2,993 adducts/10^9 N) whereas, highest levels were found in stage I samples (3,049 adducts/10^9 N). Stage III samples had an average of 2,499 adducts/10^9 N. After 3 weeks of radiation therapy Stage I and Stage II sample showed higher P2 adduct level of 26,320 adducts/10^9 N and 24,220 adducts/10^9 N respectively when compared to Stage III sample (11,537 adducts/10^9 N) (Fig 22). A similar trend was found after 6 weeks of radiation therapy where Stage I (17,525 adducts/10^9 N) and Stage II (19,196 adducts/10^9 N) sample showed higher adduct levels than Stage III samples (9,534 adducts/10^9 N). All cancer stages however, showed lower level of P2 adducts in 6 weeks radiation treatment samples than 3 weeks radiation samples. In case of follow up samples Stage III had the lowest levels of P2 adducts (3,938 adducts/10^9 N) when compared to Stage I (6,333 adducts/10^9 N) and Stage II (7,522 adducts/10^9 N).
Fig. 22 - Graphical Representation of P-2 Adducts Level in a) Stage I, b) Stage II and c) Stage III Patients at four Time Intervals viz., Before Treatment, After 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)
Estimation Of Lipophilic Adducts

A group of more lipophilic adducts were resolved along with P2 adducts. These adducts were marked from 70 mM sodium phosphate polar maps and were designated as L-1 adducts. We found a significant increase (p=0.005) in the L-1 adduct levels in 3 weeks of treatment samples when compared to untreated samples. 6 weeks of treatment samples showed a slight decrease in adducts levels that was not significant (p=0.727) when compared with 3 weeks treatment group. We observed a slight increase in L-1 adduct levels in post treatment group of samples when compared with 6 weeks treatment samples however, it did not reach statistical significance (p=0.144). Follow up samples showed a remarkable increase in adduct levels when compared to untreated samples and was significant (p=0.0002).

Among untreated samples highest L-1 adducts were found in Stage I (1,902 adducts/10^9 N) when compared to Stage II (1,537 adducts/10^9 N) while, lowest levels were observed in Stage III (1,335 adducts/10^9 N) samples. Lowest L1 adducts after 3 weeks of radiation treatment were found in Stage III samples (2,131 adducts/10^9 N) whereas highest levels were seen in Stage I (9,794 adducts/10^9 N) followed by Stage II (8,285 adducts/10^9 N). All the stages showed an increase in L1 adduct levels in follow up when compared to 6 weeks of radiation treatment. Stage II had highest levels of L1 adducts (6,746 adducts/10^9 N) while lowest were observed in Stage I samples. Stage III samples showed an average level of 6,600 adducts/10^9 N. Fig 23 and Table 9.
Fig.23- Graphical Representation of L-1 Adduct Level in a) Stage I, b) Stage II and c) Stage III Patients at four Time Intervals viz., Before Treatment, After 3 Weeks, 6 Weeks of RT and Follow Up (6 Weeks after Completion of RT)
Total Adducts

Total adducts at four study interval were compared to get overall picture of the adduct levels during study period. We observed significant (p<0.0001) increase in total adduct levels in 3 weeks radiation treatment samples (48,333 adducts/10⁹ N) when compared with untreated samples (11,335 adducts/10⁹ N) from the same patients. 6 weeks of radiation treatment samples showed a slight decrease in total adduct level (45,857 adducts/10⁹ N) that was not statistical significant (p=0.7241) when compared to untreated samples. We observed a further decrease in total adduct levels in 6 weeks radiation therapy samples (21,185 adducts/10⁹ N) and this was significant (p=0.0329) when compared with 3 weeks radiation treatment samples. Significant increase (p=0.0092) in total adduct levels were found in post treatment samples when compared with untreated samples (Table 10). Fig. 24 represents adducts levels in scatter plot with statistical significance.

Table 10: Total Adducts

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>3rd wk RT</th>
<th>6th wk RT</th>
<th>Follow up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total adducts</td>
<td>11,335 ± 1,321</td>
<td>48,333 ± 6,428</td>
<td>45,857 ± 6,109</td>
<td>21,185 ± 2,472</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM
Fig. 24 - Scatter Plot Representation of Adduct Levels (a) 8-OxodG, (b) P-1 Adducts, (c) P-2 Adducts (d) L-1 Adducts and (e) Total Adducts.
COCHROMATOGRAPHY:

Cochromatography was performed to confirm that adducts seen in samples from different time points are actually the same with different intensity though and to rule out any new adducts formed, if any due to radiation

DNA samples were taken from two time intervals namely untreated and 3 weeks of radiation treatment and were labeled. Samples were analyzed for polar adducts DNA samples were diluted appropriately before performing cochromatography.

No additional spots /new adducts were formed in radiation treated samples when compared with untreated samples. However, 3-weeks radiation treated specimens showed many fold increase in the levels of adducts. In summary, radiation by itself doesn't lead to the formation of new adducts, however could cause many fold increase in levels of the existing adducts in vivo Fig 25.
Fig. 25 - Scanned Image of Autoradiogram of Cochromatography.
DISCUSSION

Several analytical methods are currently used to identify endogenous DNA adducts, such as immunoassay, gas chromatography-mass spectrometry and $^{32}$P-postlabeling $^{32}$P-postlabeling assay, is standard method used for measuring adducts from endogenous carcinogens in humans and has advantage of high sensitivity allowing detection of $1/10^8$ to $10^8$ nucleotides. This method requires as little as 10μg of DNA [182].

We had earlier analyzed DNA adducts elaborately in cancer cervix patients at various stages of disease and the levels were compared with normal, inflammatory cervix, dysplastic and invasive cancer specimens and identified novel DNA adducts. These adducts were classified based on their chemical nature from polar to lipophilic adducts viz, P-1, P-2, PL-1, PL-2 and L-1 adducts [183]. Present study was designed to assess DNA adducts levels in the same patient undergoing radiation therapy at three time intervals viz, before, during and post- treatment with RT. We intend to recognize the role of radiation on adducts levels. Ionizing radiation is known produce wide spectrum of DNA damage ranging from strand breaks to different types of chemical changes of the deoxynucleotides [80]. This damage to DNA is responsible for some unwanted acute or late radiation induced reaction of normal tissues. We observed that total DNA adducts levels were independent of the stage of cancer in pre and post treatment samples. Our observation suggests that adducts levels are more patient dependent than on clinical stage of disease. We observed that all the four types viz, polar P-1 & P-2, 8-oxodG and lipophilic (L-1) increased many folds in 3 weeks and 6 weeks of radiation.
treatment samples when compared to the baseline levels prior to RT

8-OxodG DNA adducts is the most extensively studied oxidative damage in DNA, whose mechanism of formation, metabolism and elimination is well known. It is a typical form of ROS-induced DNA damage and reported to be the key biomarker relevant to carcinogenesis [184] and to cause mainly G to T transversions. We observed a significant increase in the levels of 8-OxodG DNA adducts in patients exposed to therapeutic irradiation doses at 3 weeks and 6 weeks of treatment when compared to samples before treatment, and the levels stayed higher in follow up samples also. Although there are only a few clinical studies on this subject, one study showed increased 8-oxo-dG excretion in urine of cancer patients [185] reflecting increased 8-OxodG DNA adducts formation due to radiation treatment. Our result correlates with that done by Bialkowski et al., where they observed significant increase in 8-OxodG over control value of DNA isolated from lymphocytes [186]

Polar (P-1) adducts were increased to the highest at 3 weeks of treatment and decreased in post treatment sample significantly. Polar (P-2) adducts also showed the same trend. However, lipophilic adducts (L-1) attained to maximum levels at 3 weeks of radiation therapy and did not show much decrease in levels in post treatment (follow up) samples. The polar and lipophilic DNA adducts analyzed in this study represent a group of structurally unelucidated adducts in human cervix tissue. Even though underestimated due to lack of adequate methodologies for validation, the levels of these adducts in human cervix tissue have been found to be so high that
projection of their etiologic role in cancer progression is an interesting exercise.

Several studies have shown association of DNA adducts of smoking with cervical cancer. Cotinine, a metabolite of nicotine, has been detected in the cervical mucus demonstrating that the cervical epithelium is exposed to components of tobacco smoke [187]. Elevated levels of bulky/ aromatic DNA adducts were detected in cervical epithelium of smokers by Ali et al. [188] and also elevated levels of PAH-DNA adducts were detected of smokes by immunohistochemical analysis [189] suggesting that cervical tissue of smokers suffers damage from carcinogens present in tobacco smoke. Interestingly, HPV infection appeared to enhance induction of DNA adducts by BPDE in cervical epithelial cells [190] suggesting a mechanism for the interaction of the HPV and smoking in cervical cancer risk. In Indian women smoking is not a common practice however chewing tobacco & betel nut (paan) is widespread and exposure to passive smoke is prevalent. Our study samples constituted these nonsmoking women. Many studies observed detectable levels of adducts in non-smokers and smokers and suggested that levels reflected passive exposure to tobacco smoke carcinogens and/or carcinogens from other environmental sources [191]. As for passive smoke, studies have demonstrated that the involuntary exposure to tobacco smoke was significantly associated with DNA and protein adducts in nonsmokers [192]. McCann et al., reported much higher levels of nicotine and cocaine in women with smokeless tobacco use than other nonsmoking women [193]. In a study from our center, paan chewing showed a dose-dependent direct association with invasive cervical cancer (ICC). The adverse influence of paan chewing on ICC risk was
attributable to a higher prevalence of cervical HPV infection in women who chewed [194]

Damage to DNA from endogenous exposures may be several folds greater than those from exogenous sources including exposure to tobacco/ environmental carcinogens [195] However, endogenous agents do play a role in tobacco carcinogenesis Tobacco exposure can directly and indirectly increase oxidative stress In animals, cigarette smoke exposures were shown to enhance endogenous DNA adducts by several fold in different tissues [90,196] Significant progress has been made in characterizing carbonyl-containing products of lipid peroxidation (e g malondialdehyde, 4-hydoxy-2-alke-nals) and the adducts they form with DNA [197] as well as the contributions of physiologic mediators such as nitric oxide in these reactions [198] The DNA adducts formed in reactions with carcinogenic aldehydes may produce structurally unique adducts [199], these hold promise as sensitive markers of oral cancer risk associated with tobacco use [200] Some endogenously derived DNA adducts are thought to increase with age [201] Several reviews have been presented on endogenous DNA adducts and cancer risk [202] This may lead to genetic susceptibility studies

We observed that increase in adduct levels due to irradiation varied among patients despite similar doses and conditions of irradiation, possibly due to interindividual differences We could divide our patients irrespective of the cancer stage into two groups, namely, high adducts forming group, how had higher adduct levels post treatment samples and low adduct forming group, those who showed lower levels of DNA adducts in follow up samples when
compared with their respective untreated samples. Individuals vary in their radiation sensitivity and repair capacity of DNA damage, which in turn may affect the extent of modification of DNA [186]. Our results suggest that the difference in response to radiation depends on the patient rather than on the applied dose or the stage of cancer.

Higher adduct levels in post-treatment samples suggest that either the cell repair machinery is compromised due to the cancer load and/or radiation therapy might have made the cell functionary inefficient to fix the lesions caused by radiation. We also observed that there were no new or additional adducts formed due to the exposure to radiation and was proved by TLC cochromatography wherein a mixture of before and after treatment samples were analyzed. However, there was many fold increase in the levels P-1, P-2, and L-1 adducts in samples after 3 weeks or 6 weeks of radiation treatment when compared with their respective to before treatment counterparts reflecting the radiation induced damage to the tissue.

The interplay between radiation exposure, oxidative stress, and the biological consequences of exposure such as lethality and mutagenesis is intriguing. Measurement of DNA adducts holds promise in assessing the state of oxidative damage of DNA in patients suffering for cancer but also reflect the outcome of the patient post radiotherapy. One principle application of DNA adduct detection to molecular epidemiology studies on human may be useful in assessing carcinogenic risk. The aim of modern radiotherapy is to optimize the probability of tumor control while minimizing the unwanted acute or late radiation induced damage of
normal tissues. Detection and monitoring DNA adduct levels in patients undergoing radiation therapy would reveal the patient response to radiation so that suitable interventions in terms of radiation dose is made for patients with radioresistant tumors but at the same time regulating the dose of exposure in radiosensitive patients. Further, studies with larger population would allow the majority of the cancer patients to get optimized radiation and hopefully better tumor control.