Alterations in cytokine levels in cervical carcinoma patients through radiation therapy

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Carcinoma cervix accounts for 86–90% of all genital cancers in Indian women. This is presented with prolonged infection and impairment of immune response even after completing the radiation therapy protocol. Increase in expression of *IL-6* gene in invasive cervical carcinoma was observed against CIN and normal cervix. Evaluation of infiltrating lymphocytes TIL showed considerable $CD3^+$ and $CD4^+$ expression which predicted better prognosis and improved survival that was negated by increased IL-6.

Keywords: Carcinoma cervix, cytokine, HPV, impaired immunogenic.

CARCINOMA of the cervix is the second most common cancer in women worldwide. It accounts for 86–90% of all genital cancers in Indian women. In India, the incidence of carcinoma of cervix is estimated to be 130,000 new cases every year. Almost 250,000 women die each year worldwide as a consequence of this disease. Human papilloma virus (HPV) is implicated as a major risk factor in the development of both cervical dysplasia and cancer. More than 80 types of human papilloma viruses (HPVs) are known today, and they are generally classified according to their potential to induce malignant transformation. Among the high-risk strains, HPV 16 and 18 are the most closely associated with cervical carcinoma[1]. HPV is detected in 90% of patients with cervical squamous carcinoma, predominantly HPV type 16 and 18. T-cell mediated protection from viral infection and control of tumours is promoted by type 1 cytokine responses and impaired by type 2 cytokine responses. Sharma *et al.*[2] reported the prognostic significance of cytokines in CIN and cervical carcinoma and immunological disfunction through shift from type 1 to type 2 cytokines.

Squamous cell carcinoma is thought to be an immunogenic tumour. Solid tumours consist of malignant cells and stroma[3]. Cervical stroma has a significant number of infiltrating host leukocytes consisting of $CD4^+$ T cells, $CD8^+$ T cells, monocytes, macrophages and granulocytes. These cells from neoplastic effusions are extremely useful in evaluating the interactions between immune and cancer cells in the tumour microenvironment. The interaction between tumour cells and the nearby environment affects the initiation and progression of cancer. Host–tumour interactions result in the production of pro-inflammatory cytokines and chemokines that promote the recruitment of leukocytes within and around developing neoplasms[4].

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[5]. 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. 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 behaviour of the T-cell subsets, namely the helper/inducer T-cell ($CD4^+$) and the suppressor/cytotoxic T-cells ($CD8^+$) would be of significant value. We report here the immunological profile of cancer cervix patients vis-à-vis the levels of cytokines through treatment by RT–PCR and determine the prognostic value of cytokines in patients undergoing radiation treatment.

Fresh biopsy tissues from 43 patients with frank tumour, who attended the Cancer Institute Out Patients Division were collected and stored at –70°C until use. A written consent was taken from all the patients willing to participate in the study. Patients were treated with the standard protocol of 6 MeV X-ray beam therapy to deliver 50–60 Gy to the pelvis followed by ICA to deliver 16 or 18 Gy to point A. Cells were scraped from patients who underwent primary treatment at three intervals, i.e. on completion of three weeks radiation (30 Gy), end of treatment (60 Gy) and during the follow-up (during review 6 weeks after completion of RT as outpatient). Here biopsy is not feasible.

A modified method single-step acid guanidine thiocyanate-phenol-chloroform (AGPC) by Chomczynski and Sacchi was used to isolate total RNA from tissue biopsy, cell scrapings and blood[6]. Briefly, tissue/scrapings were homogenized and treated with guanidinum thiocynate and phenol followed by chloroform–isoamyl alcohol mixture (49 : 1). RNA was precipitated from the aqueous layer with isopropyl alcohol. The RNA thus obtained was washed with 75% ethanol to remove salt contamination and finally precipitated with absolute alcohol. This RNA was used to carry out RT–PCR amplification.

RNA was initially denatured to remove secondary structures at 95°C for 5 min, to obtain linear RNA molecule for cDNA synthesis. We used the protocol according to manufacturer's guidelines (Roche) to prepare the master mix for cDNA synthesis. The master mix was distributed equally into each tube, briefly vortexed and centrifuged. cDNA synthesis conditions: 30°C for 5 min for anneal-

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Table 1. Primer sequences for RT–PCR amplification of cytokines

	Primer sequence (5′–3′)	Length	Product size (bp)
β-actin	(fw) 5′ TCG TCG ACA ACG GCT CCG GCA TGT GC 3′	26	688
	(rv) 5′ TTC TGC AGG GAG GAG CTG GAA GCA GC 3′	26	
IL 6	(fw) 5′ ACG AAT TCA CAA ACA AAT TCG GTA CA 3′	26	335
	(rv) 5′ CAT CTA GAT TCT TTG CCT TTT TCT GC 3′	26	
CD 4	(fw) 5′ GGA GCT CCT TTT AGG CAC TTG C 3′	22	652
	(rv) 5′ GAA CTC CAC CTG TTC CCC CTC 3′	21	
CD8 β	(fw) 5′ CTC CTC CTG GCC GCG CAG CTG 3′	21	567
	(rv) 5′ GCC GGG CTC TCC TCC GCC G 3′	19	
CD3 δ	(fw) 5′ TGT CTG AGA GCA GTG TTC CCA C 3′	22	220
	(rv) 5′ CCA GGC TGA TAG TTC GGT GAC C 3′	22	
IFN γ	(fw) 5′ GGT TCT CTT GGC TGT TAC TGC C 3′	22	340
	(rv) 5′ GTT GGA CAT TCA AGT CAG TTA CC 3′	23	

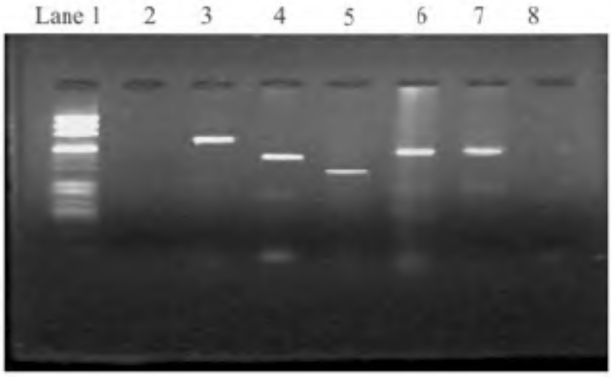

Figure 1. RT–PCR profile with cytokine primers. Lane 1, Molecular marker; lane 2, Blank; lane 3, β-actin; lane 4, IL-6; lane 5, CD3; lane 6, CD4; lane 7, CD8 and lane 8, IFNγ.

ing of oligo primer, 42°C for 60 min for synthesis and 90°C for 5 min for enzyme-denaturation.

2 µl of cDNA synthesized above was used for each PCR reaction using primer pairs (Table 1). Master mix was prepared with components of PCR kit according to manufacturer's instruction (Roche). Master mix was thoroughly mixed and centrifuged briefly. To each tube 18 µl of master mix and 2 µl cDNA were added. The PCR conditions were: 94°C for 2 min, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 29 cycles and final extension at 72°C for 5 min. The amplification products were analysed by performing agarose gel electrophoresis and visualized using UV trans-illuminator Bio-Rad Gel Documentation system 2000.

The patients are divided into two groups, viz. patients with disease-free status and patients with recurrent disease (residue/lymph node involvement) depending on the outcome after radiation treatment. Of the 43 patients under study, 34 (79.0%) patients responded well to radiation treatment and were disease-free in follow up review; however, nine (20.9%) patients showed residual disease or nodal involvement during the follow up review. The mRNA expression levels of various cytokine markers, viz. interleukin (IL-6), $CD3^+$ T cell (CD3δ), $CD4^+$, $CD8^+$ T cells (CD8β) and interferon γ (IFN γ) were studied at tumour site (local) and in blood (systemic) samples at four time intervals, i.e. pre-treatment, after 3 weeks and 6 weeks of radiation therapy and follow-up (Figure 1). Consolidated results of the patients are presented in Table 2.

IL-6 is a proinflammatory protein and affects defense mechanism. There are reports of analysis of several human clinical situations in which the intratumoral production of certain cytokines is clearly associated with the clinical outcome. 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 tumour biopsies and 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[7]. 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. IL-6 may contribute to a local immunosuppressive effect that protects the tumour cells from the host immune system. These data support the hypothesis that IL-6 promotes the development of cervical cancer[8].

Table 2. Cytokine expression levels in cervical tissue of patients through treatment

	Disease-free patients (34)				Recurrent disease patients (09)			
	⇑	⇓	⇔	Below detection	⇑	⇓	⇔	Below detection
IL-6								
BT		34		0		9		0
3 Wk	9	12	9	4	7	0	2	0
6 Wk	7	17	5	5	5	1	3	0
FU	7	15	11	1	6	0	1	2
CD3								
BT		32		2		7		2
3 Wk	2	19	4	9	0	6	0	3
6 Wk	1	23	2	8	0	4	1	4
FU	1	21	5	7	1	4	1	3
CD4								
BT		33		1		8		1
3 Wk	1	22	10	1	0	4	2	3
6 Wk	1	23	6	4	0	6	1	2
FU	4	14	10	6	1	5	0	3
CD8								
BT		31		3		9		0
3 Wk	1	17	2	14	0	7	0	2
6 Wk	1	16	6	11	0	6	1	2
FU	0	15	4	15	1	6	0	2
IFNγ								
BT		6		28		1		8
3 Wk	0	1	2	31	0	1	0	8
6 Wk	0	0	1	33	0	0	0	9
FU	0	0	0	34	0	0	0	9

⇑ = Increased expression; ⇓ = Decreased expression; ⇔ = No change in expression.

We observed a fall in IL-6 levels in blood of few patients undergoing RT, whereas many patients did not show any change in expression levels post treatment. These results are in agreement with those of Tang *et al.*[9]. 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. Another study indicated that radiation therapy tend to raise levels of plasma IL-6 in breast and prostatic cancer patients suggestive of monocyte activation.

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[10]. 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. 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 tumour tissue before treatment whereas 83.7% of patients had no expression or levels below detection. These levels declined further during and post-treatment samples. Blood expression levels of IFN-γ were almost undetectable in all the samples irrespective of treatment. A significant decrease in circulating IFN-γ concentrations was observed in women with severe dysplasia and invasive carcinoma. Defective IFN-γ production may be associated with persistent HPV infection and the development of HPV-related neoplasia[11]. Tartour *et al.*[12] have recently shown that poor prognosis and tumour 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. The absence of IFN-γ mRNA expression cannot be explained by a defect of T cell recruitment, 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[13]. IFN-γ production was decreased by radiation as it had significant effects on cells and cytokines that can influence angiogenesis, growth and immune status[14].

We observed slightly higher levels of $CD4^+$ and $CD8^+$ T cell expression in frank tumour 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 wherein the presence of a relatively high level of $CD4^+$ T-cell infiltration, patients with a sufficient number of tumour-infiltrating

$CD8^+$ T cells demonstrated a significantly better prognosis. A similar synergistic effect between tumour-infiltrating $CD8^+$ T cells and $CD4^+$ T cells in oesophageal squamous cell carcinoma and pancreatic adenocarcinoma has been demonstrated[15]. Hiraoka *et al.*[16] suggested that cooperation between infiltrating $CD4^+$ T cells and $CD8^+$ T cells in tumours might be important in the suppression of the progression of non small cell lung carcinoma (NSCLC). Other studies have demonstrated that activation of $CD4^+$ T cells is required for immunization of $CD8^+$ T cells against cancer. For activation and maintenance of tumour-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. $CD4^+$ T cells are necessary for the full antitumour 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 favourable 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 are difficult to explain, as antitumour effect of $CD8^+$ T cells may be circumvented by various mechanisms in the tumour cells[16].

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. 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. High ratios between $CD8^+$ T cells and the other cell types were associated with improved survival[17].

Despite the presence of a lymphocytic infiltrate, many human tumours including cervical cancers grow relentlessly, suggesting that TIL populations may eventually become anergic *in vivo*. A recent study by Steinbrink *et al.*[18], showing that IL-10 treated human dendritic cells can induce anergy in tumour specific cytotoxic $CD8^+$ T cells and result in failure to lyse tumour cells, further elucidates the down-regulatory role of IL-10 in tumour-mediated immunosuppression. 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[19]. These results suggest that the incomplete activation of TAMs *in vivo* may be due to 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.

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 count has been reported, reflecting the non-specific lymphodepleting effect of radiation therapy[20].

Our studies confirm that $CD8^+$ T cell recruitment is necessary for antitumour immunity. Increased expression levels of $CD8^+$ T cells were found in cervical tissue than in peripheral blood. Higher $CD8^+$ T cell levels in the tumour tissue prior to treatment and post treatment resulted in better prognosis. Inefficient antitumour response by tumour infiltrating $CD8^+$ and $CD4^+$ T cells results in residual disease in some patients, this may be due to tumour-mediated immunosuppression or T cell anergy due to microenvironment of the tumour milieu. Increased expression of cytokine IL-6, greater infiltration of tumour with $CD8^+$ T cells and decreased expression of IFN-γ suggests that the human cancer cells may alter the functional composition of antitumour effector cells towards Th2/Tc2 polarity. However, we also noticed in few patients that the trend did not hold good and demonstrated variable outcome. The implication of such findings is at present unknown, but 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 interaction between the host immune system and tumour 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 six weeks after completion of RT, suggesting that radiation-induced lymphopenia requires longer time to restore.

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Received 22 September 2007; revised accepted 4 April 2008

Determination of rare earth elements in Indian coastal monazite by ICP-AES and ICP-MS analysis and their geochemical significance

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The performance of inductively coupled plasma-atomic emission spectrometry (ICP-AES) for the determination of fourteen lanthanides and yttrium in monazite was evaluated in comparison with inductively coupled plasma-mass spectrometry (ICP-MS) analysis. Geochemical reference samples were analysed for checking the precision and reproducibility of the methods. Monazite was separated by gravity and magnetic separations from the mineral black sands that were collected from Manavalakurichi (MK) and Chavara (CH) deposits along the southwest coast of India. Effective dissolution was carried out. The rare earth elements were collectively separated from concomitant matrix elements by ion-exchange chromatographic separation using the resin Dowex 50 X-8. Monazite mineral of the SW coast of India, is a light lanthanide-enriched mineral with negative Europium anomaly. The average value of the ratio $(ICP\text{-}AES)_{result}/(ICP\text{-}MS)_{result}$, was 0.933 and 0.897 for MK and CH samples respectively, demonstrating that ICP methods applied under proper working conditions give satisfactory results.

Keywords: Geochemical significance, lanthanides, monazite, rare earth elements.

THE concentrations of rare earth elements (REEs) in many natural substances are much below the detection limits using conventional methods. Most of the matrix elements interfere with the REE determination because of spectral overlaps and background continuum interferences[1]. Accurate and convenient methods of determining REEs are needed for the development of geochemical knowledge and industrial applications. Secondary ion mass spectrometry (SIMS)[2] has the potential to detect REEs up to the sub-ppm level and has a spatial resolution of less than 100 μm. However, the SIMS method is affected by spectroscopic interference of light REE oxides onto the heavier REE, and this method could not be applied for the determination of REEs in monazite as the variation between light REE and high REE is in high magnitude. For many years, neutron activation analysis

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