RESEARCH ARTICLE

An Altered Ratio of CD4+ And CD8+ T Lymphocytes in Cervical Cancer Tissues and Peripheral Blood – A Prognostic Clue?

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Abstract

  Background : Several studies have provided evidence of CD4+ and CD8+ lymphocyte infiltration in various malignancies with probable implications for prognosis. Cervical cancer accounts for a major part of the cancer burden in the developing world. Study of genetically and ethnically diverse Indian cervical cancer patients is necessary to assess effects on lymphocytic infiltration of tumour tissue. **Methods :** This observational study was conducted over a period of 12 months with selected cervical cancer patients meeting inclusion criteria. Samples of cervical cancer tissue and peripheral blood were obtained and tumour infiltration with CD4+ and CD8+ lymphocytes was noted. Cell numbers were quantified by flow-cytometry and proportions compared between tumour and peripheral blood samples. **Results:** Tumour infiltration was noted with both CD4+ (13.93±10.95) and CD8+ (19.5±12.05) lymphocyte subtypes. However, compared to peripheral blood, CD4+ cells were significantly less predominant in tumour tissue (p, 0.0013). There was a statistically significant (p, 0.0004) reversal of the ratio of CD4+ and CD8+ in the tumour tissue (0.68±0.39) compared to peripheral blood (1.5±0.66) with maximal alteration in higher stage disease. **Conclusion :** The study revealed that T lymphocyte infiltration of cervical cancer tissue occurs but the ratio of CD4+ to CD8+ subtypes is sifnificantly lower than in peripheral blood, especially with in advanced stages of disease. The clinical implications of such a reversal of CD4+ and CD8+ ratios is unknown, but might have prognostic significance.

  Keywords: Tumour infiltrating lymphocytes- cervical cancer- CD4- CD8

Introduction

After the successful researches related to immunology by Paul Ehrlich, the newer concepts started evolving with newer researches in this field (Bosch and Rosich, 2008; Turk, 1994). Gradually the term immune surveillance was coined in the field of tumour. Spontaneous regression of certain tumours was probably the eye opener for the scientists to put more stress on the theory proposed by Paul Ehrlich. It is also claimed that immune system may serve to prevent emergence of cancer and cellular immunity may provide a critical role in protection against tumours (Saranchova et al., 2016). The tumour infiltrating lymphocytes are considered to be part of the tumour surveillance system (Umansky et al., 1996). T lymphocytes were found to infiltrate human tumours in many cases and has often been implicated with the prognosis of the disease, both positive and negative (Disis et al., 2009). It was claimed that presence of TIL resulted in antitumour response in several types of cancer like ovarian, pancreatic or colorectal carcinoma; and was correlated with a better prognosis (Fukunaga et al., 2004;

Prall et al., 2004; Ruffini et al., 2009; Sato et al., 2005). Role of CD4+ and CD8+ lymphocytes has drawn attention of the researchers and resulted in controversies due to observed differences in their of action. It is considered that CD4+ T-cell response can elicit both immune stimulatory or immune inhibitory effects (Pardoll and Topalian, 1998). CD8+ T cells of the immune system are able to distinguish between normal cells and cancerous or virus-infected cells by monitoring major histocompatibility complex class I (MHC-I) molecules on the cell surface (Alimonti et al., 2000; Gabathuler et al., 1994; Saranchova et al., 2016).

Considering the controversies about the role of CD4+ T cells, it was described as a 'double-edged immunologic sword' because CD4+ T cells play a central role in initiating and maintaining anticancer immune responses (Bevan, 2004; Shah et al., 2011; Toes et al., 1999). But the CD8+ T Cells can recognise tumour antigens bound in class 1 MHC molecules in the tumour cells and directly kill them (Ye et al., 2007). The generation of tumour-specific cytotoxic T lymphocyte (CTL) responses depend on the help of activated CD4+T cells, which recognize tumoural antigens presented with class II MHC molecules on

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antigen presenting cells (Bevan, 2004). Disis et al., (2009) and also Sato et al., (2005) in their previously mentioned work had shown that, in the absence of CD8+ T cells, CD4+ T cells can eliminate the tumour cells; however, according to Fukunaga et al. (2004) effective tumour elimination needs both CD4+ and CD8+T cells. It is also well known that these subtypes of lymphocytes are also present in the peripheral blood. The establishment of adaptive immune responses to neoplasm involves at least two compartments namely, the peripheral blood, and the tumour tissue. There is evidence for changes in the frequencies of CD4+ and CD8+ T cells in the peripheral blood and the tumour tissue in several types of tumour and this may be related to the overall prognosis of the cancer.

Cervical cancer is the fourth most common malignancy in women and seventh overall worldwide. As per WHO statistics, in 2012, cervical cancer resulted in 7.5% of all female cancer deaths and the worldwide estimated deaths from cervical cancer were 266,000. Around 87% cervical cancer deaths occur in the less developed regions which mean nearly nine out of ten deaths. The same report revealed that India had an incidence of 123,000 cases and 67,000 deaths from cervical cancer in 2012 ("Fact Sheets by Cancer," Globocan). Therefore, there is need to explore any relevant factor which might influence the overall prognosis of cervical cancer. Immune cellular infiltration may provide useful prognostic data and may be a step to develop effective immunotherapy as a modality of management of cervical cancer. As India is a country of a population with varied and diverse genetic and ethnological background, study in the Indian population could be helpful to generate data representative of a diverse community. Therefore, the present study aims to find and quantify T lymphocyte subpopulation in the cervical cancer tissue in a cohort of cervical cancer patients. The study had also aimed to compare the subsets T lymphocyte in the cervical tissue with that of blood.

Materials and Methods

This was an observational study conducted in the Rotary Cancer Hospital of All India Institute of Medical Sciences, New Delhi, India during January 1996 to May 1997. Permission from the Institutional review Board was obtained before starting the study. Written informed consents were obtained from all participating patients.

All the consenting patients between age 18- 65 years, diagnosed to be suffering from Cervical Cancer were screened for inclusion and exclusion criteria during the study period. Random sampling was done for enrollment of the screened patients and serial enrollment was done over a period of 12 months. Histologically, all patients had either non-keratinising or keratinising squamous cell carcinoma of uterine cervix of any size and none of the patients received chemotherapy at the time of study. Patients with positive serology for HIV, hepatitis B, Hepatitis C; patients suffering from other major systemic diseases which might affect immunity, those receiving any immunosuppressive therapy and those with ECOG 3 or more were excluded for this study.

Age of the patient, present symptoms, duration of

symptoms, record of any past illness, menstrual history, obstetrical history, family history of cancer and personal history with particular emphasis on socio economic status, marital status, any addiction and level of genital hygiene were noted. Performance statuses of the patients were noted by using ECOG status. Local disease assessment was done by gentle speculum examination with well lubricated gloved index and middle finger, to assess former volume, consistency, fornicial involvement, and vaginal wall infiltration, Position, size, consistency and mobility of uterus was assessed and parametral evaluation done.

Investigations done were hematological, renal and hepatic function tests along with blood glucose profile. Chest x-ray, urine analysis, intravenous pyclography, ultrasound examination of abdomen and pelvis, CT scan of abdomen and pelvis. Proctosinoidoscopy and cystoscopy were done in patients as and when indicated.

Blood and tumour tissues were collected in the same visit for each patient. Tumour tissue were obtained using cervical punch biopsy forceps. In the next step Single cell suspension were prepared from blood and cervical tissues.

Preparation of single cell suspension from blood and cervical tissue: Single cell suspension from blood and cervical tissues were prepared by enzymatic digestion. Whole blood was collected, 10µl was taken in each tube, treated with specific monoclonal antibody labeled with FITC (flourecein isothiocyanate for CD4+ Cells) and PE (phycoerythrin for CD8+ cells) respectively (Axberg et al., 1991; Imlach et al., 2001; Johnson, 2015; Kenny et al., 2000) . After labeling, red blood cells were lysed by hypotonic shock with lyning solution. About 10,000 events were acquired in FAC SCAN and analysis was done using the lyses II programme. Percent of CD4+ and CD8+ T cells were calculated and ratio of CD4+ : CD8+ was established (Hernandez et al., 2005; Picot et al., 2012).

For TILS, the method was same except preparation of single cell suspension, which was done by the combination of enzymatic and mechanical disaggregation of tumour cells, following the protocol standardized in the Biotechnology Laboratory of the Institute. The result was expressed in terms of percent CD4+, CD8+ as well as ratio of CD4+ with CD8+.

Statistical analysis
Data were analysed by using SPSS vesion 16 and Open EPI version 3 softwares (Dembe et al., 2011; Scotch et al., 2006) . Other than descriptive statistics, the mean values were compared statistically by unpaired t-test. P values of <0.05 were considered significant. Data were expressed as mean and standard deviation (SD).

Results

Total 20 patients were enrolled for the study (n=20) and all of them were suffering from invasive squamous cell carcinoma of cervix. All of them were married female with age between 28-65 years. All of them had history of childbirth, and most of them, multiple numbers of pregnancies (Table 1). Mean age of the enrolled patients were 48 years (SD = 9.98).

All the enrolled patients were having ECOG

Study in the Ethnically Diverse Indian Population

Table 1. Baseline Demographic Parameters

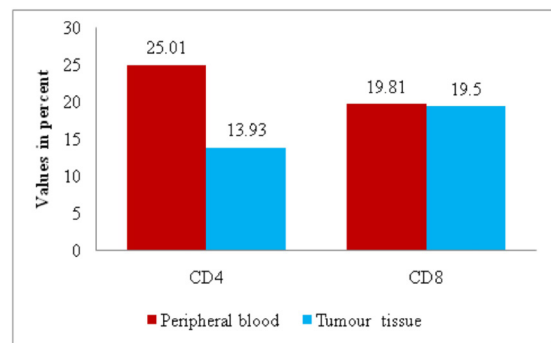|	Number (n=20)	Percent (%)
Age range		
25-35 years	3	15
36-45 years	4	20
46-55 years	11	55
> 55 years	2	10
Menstrual status		
Premenopausal	7	35
Postmenopausal	13	65
Parity		
<4 (1-4)	7	35
>4	13	65
Performance status		
ECOG 1	14	70
ECOG 2	6	30
Symptoms		
Bleeding PV	20	100
Discharge PV	17	85
Abdominal pain	8	40
Low backache	3	15
Duration of symptom		
< 6month	14	70
6-12 months	5	25
>12 months	1	5
Clinical stage		
II B	6	30
III B	14	70
Tumour volume (mean= 121.45 ± 85.36)		
<100cm^2	10	50
> 100cm^2	10	50
Tumour histology		
Non keratinizing SCC	14	70
Keratinizing SCC	6	30

Figure 1. Extent of CD4+ and CD8+ Infiltration in the Tumour Tissue and Mean Value in the Peripheral Blood. CD4+ & CD8+ values are shown as percent values.
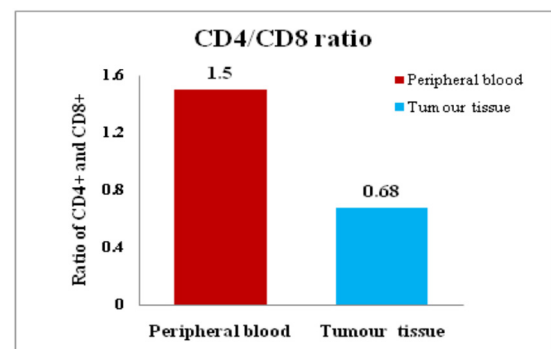

Figure 2. Ratio of CD4+ and CD8+ in Peripheral Blood and Tumour Tissue. Footnote: A reversal of ratio was noted which was significant statistically (p value, 0.00004)

performance status 2 or less. All the patients had the symptom of bleeding per vagina (100%). Majority of them had symptom duration of less than 6 months. Two patients had past history of pulmonary tuberculosis, more than 20 years ago. But they had been adequately treated with anti-tubercular chemotherapy and presently had no sign of disease at the time of enrollment in the study. Three patients had smoking history of bidi (a form of country cigarette in India). All of them were from lower socio-economic status and had poor level of knowledge about genital hygiene.

The tumour volume assessment was done by gentle manual digital examination at the time of initial inspection. The findings were confirmed by CT scan findings later. Tumour size was highly variable between two extremes, the largest becoming 384 cm^2 where the smaller one had a size of 27 cm^2. The mean volume was, however 121.45+85.36 cm^2. Clinical staging was also done for all the patients (Table 1).

The samples of peripheral blood and the cervical tumour tissue were analyzed for lymphocytes including the CD4+ and CD8+ subpopulations. The numbers of tumour infiltrating lymphocytes (TIL) were compared to that of peripheral blood (Figure 1 and 2). Evidence of tumour infiltration with lymphocyte was evident. However, TIL could not be isolated from one patient. Both CD4+ and CD8+ infiltration was noted in the collected tumour samples. CD4+ count was higher in the peripheral blood sample compared to tumour tissue and the difference was statistically significant (p, 0.0013). However, CD8+ count in the tumour and peripheral blood were similar (p,

Table 2. Menstrual Status and Lymphocyte Subpopulation

	Peripheral blood			Tumour tissue		
	Premenopausal (n=7)	Postmenopausal (n=13)	p	Premenopausal (n=7)	Postmenopausal (n=13)	p
CD4+	25.88±7.03	24.69±9.65	0.77	13.63±10.55	14.09±11.58	0.93
CD8+	17.11±2.15	22.12±8.31	0.05	20.68±14.05	18.87±11.4	0.74
CD4+/CD8+	1.54 ± 0.50	1.12 ± 0.79	0.16	0.65 ± 0.41	0.69 ± 0.39	0.83

Values are in number ± standard deviation; CD4+ and CD8+ are in percent

Table 3. Performance Status and Lymphocyte Subpopulation

	Peripheral blood			Tumour tissue		
	ECOG1 (n=14)	ECOG2 (n=6)	p	ECOG1 (n=14)	ECOG2 (n=6)	p
CD4+	25.06±8.68	24.84±9.62	0.96	11.81±10.49	18.86±11.30	0.22
CD8+	19.92±6.75	19.43±7.84	0.97	16.84±10.08	25.72±13.50	0.18
CD4+/CD8+	1.49±0.76	1.48 ± 0.79	0.97	0.62±0.38	0.81±0.41	0.35

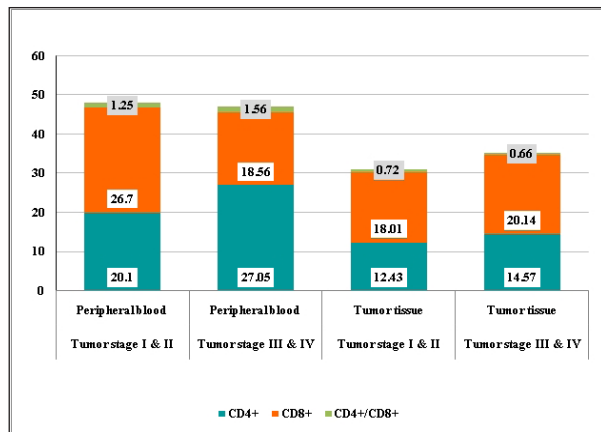Values are in number ± standard deviation; CD4+ and CD8+ are in percent.

Figure 3. Stage of Disease and Lymphocyte Subpopulation. CD4+ & CD8+ values are shown as percent values. CD4+/CD8+ is expressed as ratio. Out of total 20 patients, 13 patients were in stage III and 7 patients were in stage II disease. Note, In lower stage disease, higher count of CD8+ was noted in peripheral blood compared to tumour tissue. However, in higher stage (stage III) diseases, higher count of CD8+ were noted in tumour tissue compared to peripheral blood (p value, 0.67).
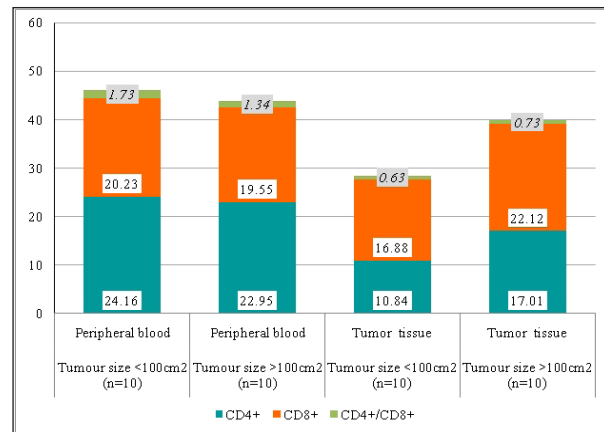

Figure 4. Tumour Size and Lymphocyte Subpopulation. CD4+ & CD8+ values are shown in percent. CD4+/CD8+ is expressed as ratio. Out of total 20 patients, 10 patients had tumour size <100sq.cm and the other 10 patients had tumour size>100sq.cm. Note, Higher counts of CD8+ were noted in tumour tissue compared to peripheral blood in the patients with larger tumour size (p value, 0.47).

0.92). As a result, the CD4+/CD8+ ratio was different in tumour tissue (0.68±0.39) compared to peripheral blood (1.5±0.66). The CD4+/CD8+ ratio was also significantly higher in peripheral blood (p, <0.0001).

Further analysis was carried out to find out any possible relationship of CD4+ and CD8+ subpopulation of lymphocytes in respect to menopausal status, tumour size, ECOG status and stage of the disease. Count of CD4+ cells were almost similar in the peripheral blood in pre and post menopausal patents. But a higher CD8+ cell count were recorded in the peripheral blood in the post menopausal patients compared to premenopausal (p, 0.05). Tumour infiltration with CD4+ and CD8+ were noted irrespective of the menstrual status of the patients. However, the counts of CD4+ and CD+ in the tumour tissue were different from the peripheral blood. The variation in the CD4+ and CD8+ in the tumour tissue in relation to menopausal status were not significant statistically. Reversal of CD4+/CD8+ ratio in the tumour tissue in comparison to peripheral blood were consistent in both pre and post-menopausal patients (Table 2).

Infiltration of the tumour tissue with CD4+ and CD8+ lymphocytes were noted in the patients of different performance ststus. The counts of both lymphocyte subtypes were similar in peripheral blood irrespective of the performance status. Higher amount of infiltration were noted for both the lymphocyte subtypes in patients with poorer performance status. Count of CD8+ in the tumour tissue was higher than that of peripheral blood in the patients with poorer performance status. Reversal of the ratio of CD4+/CD8+ was consistent in both ECOG1 and ECOG2 performance status patients (Table 3).

Tumour infiltration was noted in the patients of all stages of disease. Again, higher amount of infiltration were noted in higher stages of disease. CD8+ count was marginally higher in the tumour tissue of stage III disease in comparison to peripheral blood. Again, reversal of CD4+/CD8+ ratio in the tumour tissue in comparison to peripheral blood were noted and such reversal were consistent irrespective of the stage of disease (Figure 3).

Higher amount of tumour infiltration with both CD4+ and CD8+ were noted in the more voluminous tumours. Such differences were not statistically significant. Again, the mean values of CD8+ were higher in the tumour tissue compared to peripheral blood of higher stage disease (Figure 4).

Discussions

In the present study, the cervical tumour tissue and peripheral blood of the patients of cervical carcinoma patients were obtained at the same sitting and the samples were analyzed to find CD4+ and CD8+ lymphocyte subpopulation. Evidence of lymphocyte infiltrations of the tumour was noted in all the patients and it was positive for both CD4+ and CD8+ subpopulation. CD4+ percent

in the peripheral blood were significantly higher in the peripheral blood compared to tumour tissue though CD8+ percent were almost similar in both. The ratio of CD4+ and CD8+ was reversed in the tumour tissue compared to that of peripheral blood. Further analysis was done to compare the difference of CD4+ and CD8+ percent in the different subgroups made according to the menopausal status, performance status, disease stage and tumour size. Higher amount of infiltration were noted in the patients with higher stage disease, larger size tumour and poorer performance status. Cell count of CD8+ in the tumour was higher than the count in the peripheral blood in higher stage diseases and more voluminous tumours. None of the differences were statistically significant. However, CD4+:CD8+ were consistently reversed in the tumour tissue in comparison to peripheral blood across all the subgroups. This was indicative of higher percent of CD4+ in peripheral blood compared to the tumour tissue.

Tumour infiltration of lymphocytes

Tumour infiltration with lymphocytes was noted by several researchers in different types of cancers including breast, prostate, oesophagus, lung, melanoma or different squamous cell carcinomas (Adams et al., 2014; Itoh et al., 1991; Li et al., 2016; Mishra et al., 2016; Nardone et al., 2016; Semeraro et al., 2016). TIL was also observed in the cervical cancers by several researchers (Brustmann et al., 2015; Hou et al., 2012; Wu et al., 2012). Among the different cells, CD4+, CD8+, CD25, CD4+5 and regulatory T cells (Foxp3) were some significant one (Bedoya et al., 2013; Gorter et al., 2015; Patel and Chiplunkar, 2009). The present study also detected the presence of CD4+ and CD8+ lymphocytes in the tumour tissue from cervix which is an evidence of TIL.

Tumour-infiltrating lymphocytes are considered as manifestations of the recognition and defense against malignant cells by the host immune system. Unfortunately, even with the presence of TIL in the tumour tissues, they often fail to control the growth of tumour (Chiou et al., 2005). However, several researchers commented that immunosurveillance against cancer is possible a implication of TIL and is linked to the prognosis of several types of cancers. In many cases TIL was considered to be associated with a better prognosis (Dieci et al., 2015; Katz et al., 2013; Kim et al., 2016; Li et al., 2016; Liakou et al., 2007). Dysfunction of TILs was also linked to failure of immunosurveillance against cancer (Chiou et al., 2005; Gajewski et al., 2006; Liakou et al., 2007). Immunotherapy was developed to exploit the possible role of such immune defense mechanism against cancer. Cytotoxic T cells were used successfully for this role (Benchetrit et al., 2003; Ye et al., 2007). Tumour-infiltrating lymphocyte therapy has consistently yielded durable clinical responses in selected patients with metastatic melanoma and is now being increasingly applied to treat other solid tumours, including head and neck squamous cell carcinoma, cervical cancer, breast cancer, and lung cancer (Radvanyi, 2015; Sim et al., 2014; Wu et al., 2012). However, the present study did not aim to detect any such findings.

Peripheral blood, CD4+ count and ratio of CD4/CD8

Uppal et al., (2003) reported normal values of CD4+ and CD8+ lymphocyte subsets in the blood of healthy Indian adults by flow cytometry method. The mean values of CD4+ and CD8+ were 40.2 and 31.3% respectively and the CD4/CD8 ratio was 1.7. There was gender variation in the counts and the ratio. The corresponding CD4/CD8 ratio males and females were 1.55 and 1.92. In the males, the corresponding level of CD4+ and CD8+ were 39.09% and 32.41% respectively; in females the CD4+ and CD8+ were 41.71% and 29.71% respectively. There is considerable ethnic variation in the CD4+/CD8+ ratio in the peripheral blood of healthy adults. The CD4+/CD8+ ratio in the peripheral blood of healthy adults of China, USA and UK were 1.49, 1.4 and 1.51 respectively (Bofill et al., 1992; Jiang et al., 2004; Reichert et al., 1991). Though the ratios were different across countries and population subset, the CD4+ level were consistently higher in comparison to CD8+ in the peripheral blood. The differences were statistically significant across all the studies mentioned. The present study also supports the previous findings of higher proportion of CD4+ compared to CD8+ in the peripheral blood. However, the ratio of CD4+/CD8+ was found to be different from the earlier studies. The present study had detected a much lower ratio of CD4/CD8 at 1.5 in comparison to earlier Indian study which estimated the ratio 1.9 for the healthy Indian females. It is unclear that whether the lowered ratio in the present study is attributable to the disease process.

CD4+ count

It was considered that maintaining and initiating anticancer immune response is one central role of CD4+ T cells (Pardoll and Topalian, 1998; Toes et al., 1999). During the primary antigen specific response, CD4+ cells help the CD8+ cells to develop long lasting functional memory cells (Bevan, 2004). Potential role of the CD4+ T lymphocytes were also found important in the anti-tumour immunity resulting from the adoptive transfer of CD8+ T lymphocytes where it helps in persistence of the CD8+ subpopulation (Dudley et al., 2002). However, the presence of subset of CD4+ T cells, referred to as CD4+CD25+ regulatory T cells (Treg), in the TIL suppress the proliferation of effector T lymphocytes (effector function of CD8+ T cells) and has negative influence in the prognosis of certain cancers (Fu et al., 2007; Yu and Fu, 2006). Again, certain other cancers, like oesophageal squamous cell carcinoma and follicular B cell lymphoma, were not found to be associated with the negative prognostic role of Treg. In fact, in these cases, Tregs was associated with either no prognostic role or even better prognosis (Lee et al., 2008; Yoshioka et al., 2008). The present study detects CD4+ infiltration in the TIL and a relatively higher proportion of CD4+ with more advanced disease or larger tumours. However, the CD4+ count in TIL in the present study remained always lower to peripheral blood irrespective of the stage of disease or size of tumour.

Role of CD8 and higher CD8 count in advanced disease

The CD8+ count was found to be linked with invasive cervical carcinoma in a study in high risk human papillomavirus-associated pre-malignant and malignant lesions of the uterine cervix. This study, conducted in a very small sample also found a CD45 were more abundant in such progressing lesions (Monnier-Benoit et al., 2006). A meta-analysis suggested a CD8+ TILs has 'positive effect' on survival. Incidentally, this meta-analysis also reported that the beneficial prognosis were mostly from the studies were on ovarian and colorectal cancers, smaller samples and shorter median follow up (Gooden et al., 2011). Another study on cervical cancer patients suggested that a robust CD8+ response is usually associated with no lymph node metastasis in large early stage cancers (Piersma et al., 2007). The present study also found higher CD8+ count in stage III diseases and larger tumours. However, implication of this finding is unknown.

CD4/CD8 ratio and the possible implications

As the antitumour immune response is mainly attributable to cell medicated immunity, activation of both CD4+ and CD8+ subsets of T lymphocytes are required for the efficient immune response to destroy the tumour cells (Dudley et al., 2002; Ho et al., 2002; Pardoll and Topalian, 1998; Toes et al., 1999). So, in this scenario, Shah et al. (2011) suggested that 'infiltration of the tumour site with high numbers of CD8+ TILs would be desirable (Shah et al., 2011). They had suggested that "the ratio of CD4+/CD8+ T cells is likely a key parameter for appropriate TIL function; this ratio may be different for different types of cancer". They found a lower ratio CD4+/CD8+ in the tumour stroma in higher FIGO stage patients and the patients who died compared to the surviving patients. They suggested a poorer prognosis of the patients with reversed CD4/CD8. Altered CD4+/CD8+ in the TIL, as noted in the present study were also reported in other studies conducted on the cervical cancer patients (Sheu et al., 1999). A similar lowering was noted in the present study also. Though conduction of survival analysis was beyond the objectives of the present study, it could be assumed that the Indian females with cervical cancer suffer from reversed ratio of CD4+/CD8+, similar to their counterparts of the rest of the world with a similar prognostic implication.

Limitations

Small sample size was a limitation of the present study. Also, the study did not separately determine TIL from tumour stroma and tumour nest. The study did not prospectively followed the patients which could further enlighten about prognostic implication of the altered CD4+/CD8+ in the TIL. The status of HPV infections, which is often found in cervical cancer patients, were also not taken in to account in this study. In spite of these limitations, this study aims to determine the TIL in cervical cancer patients in the Indian patients, who might have specific implications due to separate genetic make-up of Indian females compared to other parts of the world.

In Conclusions it could be concluded that there is infiltration of the cervical tissue by the T lymphocytes in the cervical cancer patients. Higher amount of infiltration were noted in the patients with advanced stages of diseases, larger tumour and poorer performance status. The ratio of CD4 and CD8 lymphocytes in the tumour infiltrate is reversed in comparison to peripheral blood. The clinical implication of such reversal of CD4 and CD8 is unknown, but the ratio is more reduced with higher stages of disease. So, alteration of the ratio and higher amount of infiltration might have some prognostic implications. However, larger prospective studies are recommended before considering the findings of this study for decision making in the global scenario.

References

Adams S, Gray RJ, Demaria S, et al (2014). Prognostic value of tumor-infiltrating lymphocytes in triple-negative breast cancers from two phase III randomized adjuvant breast cancer trials: ECOG 2197 and ECOG 1199. J *Clin Oncol Off J Am Soc Clin Oncol*, **32**, 2959–66.

Alimonti J, Zhang QJ, Gabathuler R, et al (2000). TAP expression provides a general method for improving the recognition of malignant cells in vivo. *Nat Biotechnol*, **18**, 515–20.

Axberg I, Gale MJ, Afar B, Clark EA (1991). Characterization of T-cell subsets and T-cell receptor subgroups in pigtailed macaques using two- and three-color flow cytometry. *J Clin Immunol*, **11**, 193–204.

Bedoya AM, Jaramillo R, Baena A, et al (2013). Location and density of immune cells in precursor lesions and cervical cancer. Cancer microenviron. *Off J Int Cancer Microenviron Soc*, **6**, 69–77.

Benchetrit F, Gazagne A, Adotevi O, et al (2003). Cytotoxic T lymphocytes: role in immunosurveillance and in immunotherapy. *Bull Cancer (Paris)*, **90**, 677–685.

Bevan MJ (2004). Helping the CD8(+) T-cell response. *Nat Rev Immunol*, **4**, 595–602.

Bofill M, Janossy G, Lee CA, et al (1992). Laboratory control values for CD4 and CD8 T lymphocytes. Implications for HIV-1 diagnosis. *Clin Exp Immunol*, **88**, 243–52.

Bosch F, Rosich L (2008). The contributions of Paul ehrlich to pharmacology: A tribute on the occasion of the centenary of his nobel prize. *Pharmacology*, **82**, 171–9.

Brustmann H, Igaz M, Eder C, Brunner A (2015). Epithelial and tumor-associated endothelial expression of B7-H3 in cervical carcinoma: relation with CD8+ intraepithelial lymphocytes, FIGO stage, and phosphohistone H3 (PHH3) reactivity. *Int J Gynecol Pathol Off J Int Soc Gynecol Pathol*, **34**, 187–95.

Chiou S-H, Sheu B-C, Chang W-C, Huang S-C, Hong-Nerng H (2005). Current concepts of tumor-infiltrating lymphocytes

in human malignancies. *J Reprod Immunol*, **67**, 35–50.

Dembe AE, Partridge JS, Geist LC (2011). Statistical software applications used in health services research: analysis of published studies in the U.S. *BMC Health Serv Res*, **11**, 252.

Dieci MV, Mathieu MC, Guarneri V, et al (2015). Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. *Ann Oncol Off J Eur Soc Med Oncol*, **26**, 1698–1704.

Disis ML, Bernhard H, Jaffee EM (2009). Use of tumour-responsive T cells as cancer treatment. *Lancet*, **373**, 673–83.

Dudley ME, Wunderlich JR, Robbins PF, et al (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*, **298**, 850–4.

Fact sheets by cancer [WWW Document], n.d. URL http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx (accessed 9.6.16).

Fu J, Xu D, Liu Z, et al (2007). Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology*, **132**, 2328–39.

Fukunaga A, Miyamoto M, Cho Y, et al (2004). CD8+ tumor-infiltrating lymphocytes together with CD4+ tumor-infiltrating lymphocytes and dendritic cells improve the prognosis of patients with pancreatic adenocarcinoma. *Pancreas*, **28**, e26-31.

Gabathuler R, Reid G, Kolaitis G, Driscoll J, JefferiesWA (1994). Comparison of cell lines deficient in antigen presentation reveals a functional role for TAP-1 alone in antigen processing. *J Exp Med*, **180**, 1415–25.

Gajewski TF, Meng Y, Harlin H (2006). Immune suppression in the tumor microenvironment. *J Immunother Hagerstown Md*, **29**, 233–40.

Gooden MJM, de Bock GH, Leffers N, Daemen T, Nijman HW (2011). The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer*, **105**, 93–103.

Gorter A, Prins F, van Diepen M, Punt S, van der Burg SH (2015). The tumor area occupied by Tbet+ cells in deeply invading cervical cancer predicts clinical outcome. *J Transl Med*, **13**, 295.

Hernandez O, Oweity T, Ibrahim S (2005). Is an increase in CD4/CD8 T-cell ratio in lymph node fine needle aspiration helpful for diagnosing Hodgkin lymphoma? A study of 85 lymph node FNAs with increased CD4/CD8 ratio. *Cyto J*, **2**, 14.

Ho WY, Yee C, Greenberg PD (2002). Adoptive therapy with CD8(+) T cells: it may get by with a little help from its friends. *J Clin Invest*, **110**, 1415–17.

Hou F, Li Z, Ma D, et al (2012). Distribution of Th17 cells and Foxp3-expressing T cells in tumor-infiltrating lymphocytes in patients with uterine cervical cancer. *Clin Chim Acta Int J Clin Chem*, **413**, 1848–54.

Imlach S, McBreen S, Shirafuji T, et al (2001). Activated peripheral CD8 lymphocytes express CD4 in vivo and are targets for infection by human immunodeficiency virus type 1. *J Virol*, **75**, 11555–4.

Itoh K, Hayakawa K, Salmeron MA, et al (1991). Alteration in interactions between tumor-infiltrating lymphocytes and tumor cells in human melanomas after chemotherapy or immunotherapy. *Cancer Immunol Immunother*, **33**, 238–46.

Jiang W, Kang L, Lu H-Z, et al (2004). Normal values for CD4 and CD8 lymphocyte subsets in healthy Chinese adults from Shanghai. *Clin Diagn Lab Immunol*, **11**, 811–13.

Johnson M (2015). FITC/Fluorescein Mater Methods [Internet]. Available from: /method/FITC-Fluorescein.html [cited 2017 Dec 3]

Katz SC, Bamboat ZM, Maker AV, et al (2013). Regulatory T cell infiltration predicts outcome following resection of colorectal cancer liver metastases. *Ann Surg Oncol*, **20**, 946–55.

Kenny E, Mason D, Pombo A, Ramírez F (2000). Phenotypic analysis of peripheral CD4+ CD8+ T cells in the rat. *Immunology*, **101**, 178–84.

Kim HR, Ha S-J, Hong MH, et al (2016). PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. *Sci Rep*, **6**, 36956.

Lee N-R, Song E-K, Jang KY, et al (2008). Prognostic impact of tumor infiltrating FOXP3 positive regulatory T cells in diffuse large B-cell lymphoma at diagnosis. *Leuk Lymphoma*, **49**, 247–56.

Li J, Tang Y, Huang L, et al (2016). A high number of stromal tumor-infiltrating lymphocytes is a favorable independent prognostic factor in M0 (stages I-III) esophageal squamous cell carcinoma. *Dis Esophagus Off J Int Soc Dis Esophagus*, https://doi.org/10.1111/dote.12518

Liakou CI, Narayanan S, Ng Tang D, Logothetis CJ, Sharma P (2007). Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human bladder cancer. *Cancer Immun*, **7**, 10.

Mishra AK, Kadoishi T, Wang X, et al (2016). Squamous cell carcinomas escape immune surveillance via inducing chronic activation and exhaustion of CD8+ T Cells co-expressing PD-1 and LAG-3 inhibitory receptors. *Oncotarget*, https://doi.org/10.18632/oncotarget.13228

Monnier-Benoit S, Mauny F, Riethmuller D, et al (2006). Immunohistochemical analysis of CD4+ and CD8+ T-cell subsets in high risk human papillomavirus-associated pre-malignant and malignant lesions of the uterine cervix. *Gynecol Oncol*, **102**, 22–31.

Nardone V, Botta C, Caraglia M, et al (2016). Tumor infiltrating T lymphocytes expressing FoxP3, CCR7 or PD-1 predict the outcome of prostate cancer patients subjected to salvage radiotherapy after biochemical relapse. *Cancer Biol Ther*, **17**, 1213–20.

Pardoll DM, Topalian SL (1998). The role of CD4+ T cell responses in antitumor immunity. *Curr Opin Immunol*, **10**, 588–94.

Patel S, ChiplunkarS (2009). Host immune responses to cervical cancer. *Curr Opin Obstet Gynecol*, **21**, 54–59.

Picot J, Guerin CL, Le Van Kim C, Boulanger CM (2012). Flow cytometry: retrospective, fundamentals and recent instrumentation. *Cytotechnology*, **64**, 109–30.

Piersma SJ, Jordanova ES, Poelgeest MIE,et al (2007). High number of intraepithelial CD8+ tumor-infiltrating lymphocytes is associated with the absence of lymph node metastases in patients with large early-stage cervical cancer. *Cancer Res*, **67**, 354–61.

Prall F, Dührkop T, Weirich V, et al (2004). Prognostic role of CD8+ tumor-infiltrating lymphocytes in stage III colorectal cancer with and without microsatellite instability. *Hum Pathol*, **35**, 808–16.

Radvanyi LG (2015). Tumor-infiltrating lymphocyte therapy: addressing prevailing questions. *Cancer J Sudbury Mass*, **21**, 450–64.

Reichert T, DeBruyère M, Deneys V, et al (1991). Lymphocyte subset reference ranges in adult Caucasians. *Clin Immunol Immunopathol*, **60**, 190–208.

Ruffini E, Asioli S, Filosso PL, et al (2009). Clinical significance of tumor-infiltrating lymphocytes in lung neoplasms. *Ann Thorac Surg*, **87**, 365-71.

Saranchova I, Han J, Huang H, et al (2016). Discovery of a metastatic immune escape mechanism initiated by the loss

of expression of the tumour biomarker interleukin-33. *Sci Rep*, **6**, 30555.

Sato E, Olson SH, Ahn J, et al (2005). Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A*, **102**, 18538–43.

Scotch M, Parmanto B, Gadd CS, Sharma RK (2006). Exploring the role of GIS during community health assessment problem solving: experiences of public health professionals. *Int J Health Geogr*, **5**, 39.

Semeraro M, Adam J, Stoll G, et al (2016). The ratio of CD8(+)/FOXP3 T lymphocytes infiltrating breast tissues predicts the relapse of ductal carcinoma in situ. *Oncoimmunology*, **5**, e1218106.

Shah W, Yan X, Jing L, et al (2011). A reversed CD4/CD8 ratio of tumor-infiltrating lymphocytes and a high percentage of CD4+FOXP3+ regulatory T cells are significantly associated with clinical outcome in squamous cell carcinoma of the cervix. *Cell Mol Immunol*, **8**, 59–66.

Sheu BC, Hsu SM, Ho HN, et al (1999). Reversed CD4/CD8 ratios of tumor-infiltrating lymphocytes are correlated with the progression of human cervical carcinoma. *Cancer*, **86**, 1537–43.

Sim GC, Chacon J, Haymaker C, et al (2014). Tumor-infiltrating lymphocyte therapy for melanoma: rationale and issues for further clinical development. *Bio Drugs Clin Immunother Biopharm Gene Ther*, **28**, 421–37.

Toes RE, Ossendorp F, Offringa R, Melief CJ (1999). CD4 T cells and their role in antitumor immune responses. *J Exp Med*, **189**, 753–6.

Turk JL (1994). Paul Ehrlich-the dawn of immunology. *J R Soc Med*, **87**, 314–15.

Umansky V, Schirrmacher V, Rocha M (1996). New insights into tumor-host interactions in lymphoma metastasis. *J Mol Med Berl Ger*, **74**, 353–63.

Uppal SS, Verma S, Dhot PS (2003). Normal values of CD4 and CD8 lymphocyte subsets in healthy indian adults and the effects of sex, age, ethnicity, and smoking. *Cytometry B Clin Cytom*, **52**, 32–36.

Wu R, Forget M-A, Chacon J, et al (2012). Adoptive T-cell therapy using autologous tumor-infiltrating lymphocytes for metastatic melanoma: current status and future outlook. *Cancer J Sudbury Mass*, **18**, 160–75.

Ye Z, Tang C, Xu S, et al (2007). Type 1 CD8+ T cells are superior to type 2 CD8+ T cells in tumor immunotherapy due to their efficient cytotoxicity, prolonged survival and type 1 immune modulation. *Cell Mol Immunol*, **4**, 277–85.

Yoshioka T, Miyamoto M, Cho Y, et al (2008). Infiltrating regulatory T cell numbers is not a factor to predict patient's survival in oesophageal squamous cell carcinoma. *Br J Cancer*, **98**, 1258–63.

Yu P, Fu Y-X (2006). Tumor-infiltrating T lymphocytes: friends or foes? Lab. *Investig J Tech Methods Pathol*, **86**, 231–45.