RESEARCH ARTICLE

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An Altered Ratio of CD4+ and CD8+ T Lymphocytes in Cervical Cancer Tissue and Peripheral Blood as a Predictor of Prognostic Outcome: A Clinicopathological Study

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Abstract

Objective: The aim of our study is to compare CD4+ & CD8+ T lymphocytes in the cervical tumor tissue with those in peripheral blood in patients with carcinoma cervix and to access thier association with known prognostic factors. The study also aims to investigate the association between tumor infiltrating lymphocytes (TILs) and the HPV status of the patients. **Methods:** In this prospective study, 42 patients with locally advanced squamous cell carcinoma of cervix were included. Percentages of CD4+ and CD8+ T lymphocytes were obtained using flow cytometry-based method from single-cell suspension prepared from simultaneously collected tumor tissue and peripheral blood samples. DNA extracted from tumor tissue was analysed to detect HPV16 (Human Papillomavirus 16) and HPV18 infection. T lymphocyte subsets in blood and tumor tissue were compared. The association of T lymphocytes with known prognostic factors and HPV status of the tumor was examined. **Result:** Both CD4+ and CD8+ T cells were significantly higher in peripheral blood compared to tumor tissue ($p < 0.001$). The CD4/CD8 ratio was reversed in tumor tissue compared to peripheral blood due to relatively lower reduction of CD8+ cells compared to CD4+ cells in tumor tissue. No significant association of T lymphocyte subpopulation was found with known prognostic parameters of cervical cancer. CD4+ and CD8+ T lymphocyte infiltration was significantly higher in tumor with HPV16 infection ($p < 0.05$). A significant alteration of CD4/CD8 ratio was observed for HPV18 positive tumors ($p < 0.05$). **Conclusion:** Our study demonstrates that both CD4+ and CD8+ T lymphocyte, which are major component of TILs, are significantly lower in tumor tissue compared to peripheral blood in locally advanced cervical cancer. No association was observed between T lymphocyte subpopulations and major prognostic factors of cervical cancer. The enhanced TILs observed in HPV-associated cervical cancer represents a significant alteration with promising therapeutic applications.

Keywords: Tumor infiltrating lymphocytes- HPV- CD4/CD8 ratio

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Introduction

Worldwide, cervical cancer ranks as the 4th most common cancer in females and the 8th most common cancer in both sexes, according to Globocan 2022 data. In India, cervical cancer has an annual incidence of 127,526, ranking as the 2nd most common cancer in females and the 3rd most common cancer in both the sexes, with a mortality of 79,906 [1]. Infection with the carcinogenic strains of HPV (Human Papillomavirus) is a common risk factor, especially in the Indian subcontinent, in the development of cervical cancer [2]. If the innate immune system is unable to prevent an incipient infection from HPV, local T-cell responses, including those mediated by lesion-infiltrating cytotoxic and helper T-cells come to play. Similarly, when (aberrant) cancer cells find niche in the human body, the body's adaptive immune system tries to clear these abnormal cells.

The elimination phase of an anti-tumour immune response in the body is mounted by cell-mediated immunity using cytotoxic and helper T cells after interaction with antigen presenting cells (APC's) that recognize tumour antigens through major histocompatibility complex (MHC). T cells differentiate into type 1 or type 2 helper T cells (Th1 and Th2), respectively, with the phenotype CD4+ and effector memory T cells expressing the phenotype CD8+, CD45RO+, CCR7– (negative for CC chemokine receptor 7), CD62L– (negative for CD62 ligand), perforin+, granulysin+, granzyme B+. Stimulation with tumour antigen induces these cells to exert an immediate effector function by releasing cytotoxic mediators [3]. However insufficient elimination enables

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tumour cells to develop tumour evasive mechanism and further on, immune escape mechanisms lead to cancer progression.

A deep understanding of the immunological microenvironment in tumor is pivotal in the current age of immuno-oncology, not just for therapeutic goals, but a possible role in the pre-treatment prognostication as well as in the post-treatment surveillance of various cancers. Needless to say, studying the cell-mediated immunity against tumor antigens is of utmost importance.

A myriad of studies on the role of cytotoxic T cells in the tumor microenvironment have been conducted, such as, in ovarian cancer, endometrial cancer and colon cancer which have concluded that intra-tumoral CD8+ cells is correlated to improved prognosis [4-6]. Jérôme Galon, et al. studied tumor infiltrating lymphocytes (TILs) and conducted genomic and in-situ immunostaining analyses and found that the type, density, and location of immune cells in colorectal cancers had a prognostic value that was superior to and independent of those of the UICC-TNM classification [7].

It is pertinent to find the role of the tumor microenvironment in cervical cancer, which has a heavy burden worldwide, and study results from an Indian population with diverse ethnicity, will have substantial clinical implications. The present study aims to compare the subsets of T lymphocyte i.e. CD4+ & CD8+ T lymphocytes and its ratio in the cervical tumor tissue with that of peripheral blood in patients with carcinoma cervix; to find out its association with known prognostic factors of cervical cancer. We have also attempted to assess the HPV DNA PCR status of cervical cancer patients and to find out its association with the TIL, if any.

Materials and Methods

We obtained approval for the study from the IPGME&R Research Oversight Committee (Institutional Ethics Committee for research involving human participants), with Memo Number: IPGME&R/IEC/2022/409 dated 15.09.2022. The study was conducted from October 2022 to September 2024 in a tertiary care hospital of eastern India.

Patient selection and sample collection

Written informed consents were obtained from all participating patients before the study. All consenting patients between age of 18-75 years, suffering from nonmetastatic cervical cancer with non-keratinizing or keratinizing squamous cell carcinoma histology of any size, and of any stage are being included.

Patients with positive serology for HIV, Hepatitis B, Hepatitis C, suffering from major systemic diseases or those receiving immunosuppressive therapy and those with performance status of Eastern Cooperative Group (ECOG) score 3 or more are excluded from the study.

A total of 42 patients were included during the period of the study. History and physical examination findings were recorded for each patient. All patients had undergone pre-treatment investigations as per the standard guideline. Relevant findings were noted from pre-treatment investigations. Primary tumor volume was determined from MRI pelvis findings.

A fresh tumor tissue is obtained from primary tumor and immediately sent to designated molecular laboratory in 0.9% normal saline solution. At the same setting, 3 ml of peripheral blood sample is also collected in EDTA tube.

Blood Cell Preparation

100 µl of whole blood wad collected in the 2 ml tube and stained with florescent labeled antibodies (CD4 PE-CY7 BD 557852, CD8 APC-Cy7 BD 557834) for 30 minutes. The RBC was lysed using BD FACS (Fluorescence Activated Cell Sorter) lysing solution (BD 349202) for 15 minutes and washed twice with PBS. The pellet was then suspended with 400 µl PBS and acquired in BD FACS Verse.

Single cell preparation from tumor tissue

To obtain tumor infiltrating lymphocytes from the biopsy tissue, the tissue was excised, chopped finely using tweezers and scissors. The fragments were then washed in PBS by centrifugation at 1400 rpm for 10 minutes at 4˚ C. Following this step, pellet (tissue) was digested with 5mg/ml collagenase type IV (stem cell technologies, Vancouver, BC) for 70 minutes. Following digestion, 3 ml culture media was added to the pellet and filtered through 70 µm cells strainers (Corning) and homogenized using plunger end of a syringe (one fragment at a time to ensure complete homogenization), this filtered solution was centrifuged at 1400 rpm for 5 minutes at 4˚ C. After the second wash the pellet was washed in PBS by centrifugation at 1400 rpm for 5 minutes at 4˚ C. The pellet was then suspended in 500 µl of complete media and then stained with CD4 and CD8 antibodies.

Flow cytometry based analysis of immune cell subsets

The percentages of CD4 and CD8 positive cell was then obtained using the BD FACS Verse (Flow Cytometer Equipment) from the single cell suspension prepared from peripheral blood and tumor tissue.

Detection of HPV DNA from tumor tissue

DNA Isolation from Tissue: Performed via Tissue Direct DNA Isolation Kit (Omega Biotech- TQ2310-01). The extracted DNA was then quantified using Nanodrop (Low Volume Spectrophotometer, Thermo Fisher Scientific).

Polymerase Chain Reaction for Gene of interest amplification: Depending upon the gene (HPV16 &18), forward and reverse primers were designed. The primer sequences used are as follows:

HPV-16 forward: AAGGCCAACTAAATGTCAC
HPV-16 reverse: CTGCTTTTATACAACCGG
HPV-18 forward: ACCTTAATGAAAAACCACGA
HPV-18 reverse: CGTCGTTTAGAGTCGTTCCTG

Statistical Analysis Plan

Analysis was performed using Statistical Package for Social Science (SPSS) software (Version 22.0, SPSS, Inc., Chicago, IL, USA). Continuous data are presented as the median (range), and categorical data are presented

as proportions. Paired t-test is used to compare means of two dependent groups. Subjects will be categorised into two prognostically different groups based on group staging, primary tumor stage, primary tumor volume and presence of lymph node metastasis. Unpaired t-test is used to compare the means of two prognostically different groups. Unpaired t-test will also be used to compare HPV positive and negative cohort of patients.

Results

A total of 42 patients were enrolled in the study. The mean age of study population is 53.76 years with minimum age being 33 years and maximum being 73 years. Frequency distribution of clinic-pathological variables is given as per Table 1. All patients were married with history of multiple pregnancies. Majority of the patients (83%) were post-menopausal women. Vaginal bleeding was the primary presenting symptom in majority of patients. A history of tobacco product addiction was reported by 26% of patients.

Mean percentage value of CD4 T Lymphocytes, CD8 T Lymphocytes and value of CD4/CD8 ratio in peripheral blood and tumor tissue given as per the Table 2. To assess the normality of the parameters mentioned in Table 2, Kolmogorov-Smirnov test of normality was performed. CD4 T Lymphocyte and CD8 T Lymphocyte percentage in both peripheral blood and cervical tissue were found to have normally distributed. However, CD4/CD8 ratio in both peripheral blood and tumor tissue had skewed distribution. We have compared the means of the values by using paired t-test for normally distributed parameters and by Wilcoxon Signed-Rank test for non-parametric variables. The results were depicted in Table 2. Both CD4+ and CD8+ T lymphocytes percentages were found

Table 1. Clinicopathological Characteristics of Study Population

Clinicopathological Features	Classification	Number (42)	Percent (%)
Menstrual status	Pre-menopausal	7	17
	Post-menopausal	35	83
Marital Status	Married	42	100
Number of Pregnancies	< 4	18	43
	> = 4	24	57
Tobacco history	H/o tobacco addiction	11	26
	No h/o tobacco addiction	31	74
Performance Status	ECOG 1	28	66
	ECOG 2	14	34
Symptoms	Bleeding PV	35	83
	Combination of Symptom	20	47
Figo Stage	IA & IB	0	0
	IIA & IIB	19	45
	IIIA & IIIB	23	55
	IVA	0	0
LN Involvement	Present	17	40
	Absent	25	60
Primary Tumor Volume	< 100 cc	26	62
	> 100 cc	16	38
Keratinisation	Keratinizing squamous cell carcinoma	26	62
	Non-Keratinizing squamous cell carcinoma	16	38
Tumor Differentiation	Well Differentiated	2	5
	Moderately Differentiated	36	85
	Poorly Differentiated	4	10
HPV Infection	HPV 16 positive	17	40
	HPV 18 positive	8	19
	Co-Infection positive	2	5
	HPV Negative	19	45

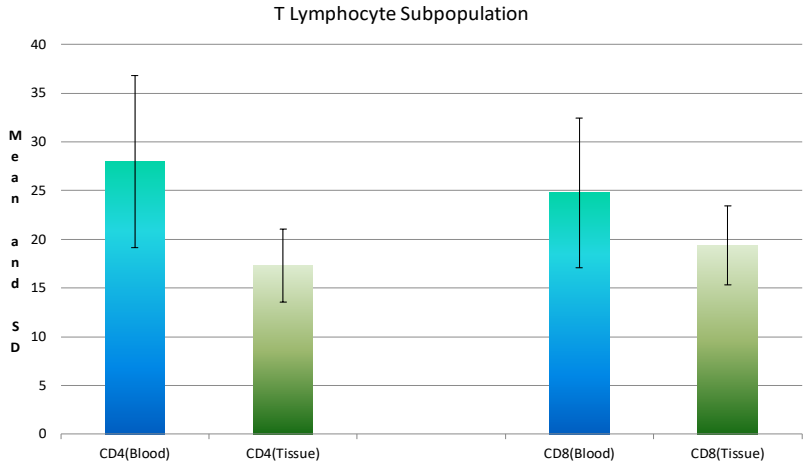

Figure 1. Comparison of CD4 & CD8 T Cells (Percentage) in Peripheral Blood and Tumor Tissue. Both CD4+ and CD8+ T lymphocytes percentages were found to be higher in peripheral blood compared to that of tumor tissue ($p < 0.001$).

Table 2. Comparison of T Lymphocyte Subsets in Peripheral Blood and Tumor Tissue

T Lymphocyte Subpopulation	Peripheral Blood (n=42)	Cervical Cancer Tissue (n=42)	p value
CD4 T Lymphocyte	27.95 ± 8.82 %	17.33 ± 3.73 %	< 0.001*
CD8 T Lymphocyte	24.77 ± 7.65 %	19.36 ± 4.04 %	< 0.001*
CD4/CD8 Ratio	1.13 ± 0.21	0.89 ± 0.07	< 0.001λ

Values are in number ± standard deviation; CD4+ and CD8+ cells are in percentage; '*' paired t-test; 'λ' Wilcoxon Signed-Rank test

Table 3. Comparison of T Lymphocyte Subpopulations in Two Prognostically Different Groups

	Peripheral Blood (n=42)			Tumor Tissue (n=42)		
FIGO stage	Stage II (n=19)	Stage III (n=23)	p value	Stage II (n=19)	Stage III (n=23)	p value
CD4+	27.08±8.65	28.68±9.08	0.564	16.51±3.64	18.01±3.75	0.199
CD8+	23.62±7.55	25.72±7.78	0.383	18.67±3.84	19.92±4.20	0.323
CD4+/CD8+	1.15±0.0.13	1.12±0.26	0.71	0.87±0.08	0.90±0.05	0.265
Primary Stage	Stage T_2 (n=29)	Stage T_3 (n=13)	p value	Stage T_2 (n=29)	Stage T_3 (n=13)	p value
CD4+	28.05±8.28	27.75±10.27	0.918	17.50±3.65	16.95±4.05	0.661
CD8+	25.11±7.68	24.02±7.84	0.676	19.61±3.72	18.80±4.81	0.557
CD4+/CD8+	1.12±0.11	1.16±0.34	0.536	0.88±0.07	0.89±0.06	0.564
LN involvement	No LN involvement (n=25)	LN involvement present (n=17)	p value	No LN involvement (n=25)	LN involvement present (n=17)	p value
CD4+	27.37±8.57	28.82±9.36	0.607	16.71±3.92	18.24±3.35	0.197
CD8+	24.36±7.74	25.37±7.72	0.678	18.76±4.25	20.25±3.67	0.245
CD4+/CD8+	1.12±0.0.12	1.15±0.30	0.729	0.88±0.08	0.89±0.06	0.727
Primary tumor volume	Less than 100 cc (n=26)	More than 100 cc (n=16)	p value	Less than 100 cc (n=26)	More than 100 cc (n=16)	p value
CD4+	28.59±9.92	27.00±6.85	0.586	17.25±4.10	17.46±3.18	0.859
CD8+	24.72±8.29	24.84±6.75	0.962	19.47±4.41	19.19±3.50	0.831
CD4+/CD8+	1.16±0.0.26	1.09±0.05	0.255	0.88±0.08	0.90±0.04	0.357

Values are in number ± standard deviation; CD4+ and CD8+ cells are in percentage. Unpaired t-test was performed to compare the values.

to be higher in peripheral blood compared to that of tumor tissue ($p < 0.001$) (Figure 1).

Overall stage, T stage, LN involvement and primary tumor volume are considered as important prognostic factor for carcinoma cervix. To find out correlation between these prognostic groups and the T lymphocyte percentage and CD4/CD8 ratio in blood and tumor tissue, Pearson's correlation for parametric values and Spearman correlation study were performed.

FIGO group stage, T stage, lymph node metastasis and primary tumor volume are established prognostic factor in cervical cancer. Based on these prognostic factors, independent t-test was performed between to two prognostically different groups to see any difference in T lymphocyte subpopulation or ratio (Table 3). We found no statistically significant difference in T lymphocyte subpopulations between two prognostically distinct groups both in tumor tissue and in peripheral blood.

Out of 42 patients, 23 patients (55%) tested positive for HPV, while 19 patients (45%) were negative. HPV 16 was detected in 17 patients, whereas HPV 18 was positive in 8 patients. Two patients had co-infection of both serotypes of HPV. Independent t-test was performed to understand any differences of T lymphocyte subpopulations between positive and negative subjects.

Comparison was made of T lymphocyte subpopulations in HPV 16 positive versus negative cases (Table 4). HPV 16 infection was significantly associated with CD4+ and CD8+ T lymphocyte infiltration in tumor tissue ($p < 0.05$). Comparison was also made of T lymphocyte

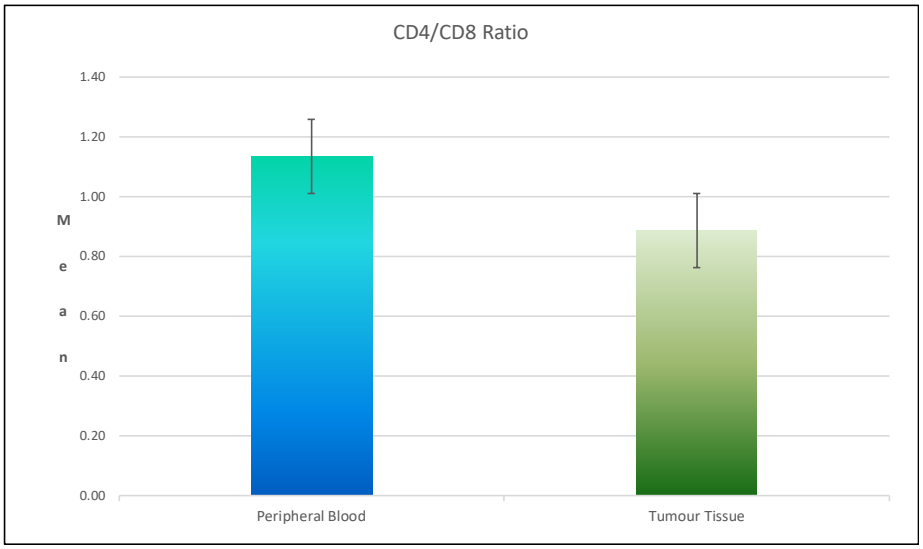

Figure 2. Comparison of CD4/CD8 Ratio in Peripheral Blood and Tumor Tissue. The mean of CD4/CD8 ratio in peripheral blood (1.13) is reversed in tumor tissue (0.89).

Table 4. Comparison of T Lymphocyte Subpopulations in HPV 16 Positive Versus Negative Cases. HPV 16 infection was significantly associated with CD4+ and CD8+ T lymphocyte infiltration in tumor tissue ($p < 0.05$)

	Peripheral blood (n=42)			Tumor tissue (n=42)		
	HPV 16 positive (n=17)	HPV 16 negative (n=25)	p value	HPV 16 Positive (n=17)	HPV 16 negative (n=25)	p value
CD4+	30.52±6.85	26.22±9.68	0.122	19.10±3.06	16.13±3.72	0.010*
CD8+	27.11±5.07	23.18±8.74	0.103	21.03±3.24	18.22±4.20	0.025*
CD4+/CD8+	1.14±0.0.30	1.13±0.12	0.914	0.89±0.06	0.88±0.08	0.509

Values are in number ± standard deviation; CD4+ and CD8+ cells are in percentage. Unpaired t-test was performed to compare the values. '*' $p < 0.05$

Table 5. Comparison of T Lymphocyte Subpopulations in HPV 18 Positive Versus Negative Cases. A significant alteration of CD4/CD8 ratio was observed in both peripheral blood and tumor tissue.

	Peripheral blood			Tumor tissue		
	HPV 18 positive (n=8)	HPV 18 negative (n=34)	"P"	HPV 18 Positive (n=8)	HPV 18 negative (n=34)	"P"
CD4+	28.23±12.50	27.89±7.97	0.925	15.57±4.40	17.74±3.51	0.140
CD8+	22.32±8.98	25.35±7.34	0.321	18.79±4.73	19.49±3.93	0.666
CD4+/CD8+	1.27±0.0.44	1.10±0.08	0.033*	0.83±0.12	0.90±0.04	0.008*

Values are in number ± standard deviation; CD4+ and CD8+ cells are in percentage. Unpaired t-test was performed to compare the values. '*' $p < 0.05$

subpopulations in HPV 18 positive versus negative cases (Table 5). In both peripheral blood and tumor tissue, HPV 18-positive cases exhibited a significantly altered CD4/CD8 ratio compared to HPV 18-negative cases ($p < 0.05$).

Discussion

We performed this descriptive study to characterize T lymphocyte subpopulations, particularly CD4+ and CD8+ cells in cervical cancer tissue and to compare with that in peripheral blood. Our analysis focused on CD4+ and CD8+ T lymphocyte subpopulations, as these are the predominant cell types in TILs. Result shows that there is substantial infiltration of T lymphocytes in the tumor tissue (Table 2). Flowcytometry method was employed to identify percentage of CD4+ and CD8+ cells from a single cell suspension. Similar method was used by Yutuan et al. to identify subsets of T lymphocytes, granulocytes and B lymphocytes [8]. While, some studies, particularly retrospective one, have used immunohistochemistry method to identify TILs from paraffin embedded tumor tissues using specific antibodies [9, 10]. In our study cohort, percentage of CD8+ T cells was higher than that of CD4+ T cells in the tumor tissue with resultant CD4/CD8 ratio being less than 1. This finding corroborates result reported by Sheu et al. where flowcytometry method was used to identify TILs [11].

Blood sample was also drawn concurrently during biopsy sample collection. Percentage of T lymphocyte subpopulations was obtained from single cell suspension obtained from peripheral blood. We have compared T lymphocyte subsets in peripheral blood and tumor tissue (Table 2). Both CD4+ and CD8+ T lymphocytes percentages were found to be higher in peripheral blood compared to that of tumor tissue ($p < 0.001$) (Figure 1). In our previous study involving 20 patients, CD4+ count was significantly higher in peripheral blood compared to tumor tissue. However, no statistically significant difference was observed in CD8+ counts between peripheral blood and tumor tissue [12]. With respect to CD4+ cell count, similar finding was reported by Yutuan et al. However, CD8+ cells were found to be increased in tumor tissue compared to peripheral blood [8]. Our study shows that there is significant alteration of T lymphocyte subpopulation percentage in tumor tissue compared to that in peripheral blood. CD8+ cells are primarily responsible for killing cancerous cells in an MHC class I – matched manner, while CD4+ cells mainly provide help to CD8+ cells [13].

In our study cohort, CD4/CD8 ratio is significantly lower in tumour tissue compared to peripheral blood ($p < 0.001$) (Table 2). The mean of CD4/CD8 ratio in peripheral blood (1.13) is reversed in tumor tissue (0.89) (Figure 2). This is in consistence with the findings of other investigations [8, 10, 12]. Both CD4+ and CD8+ T cells are reduced in tumour tissue, however relatively lower reduction of CD8+ cells compared to the CD4+ cells (Figure 1) leads to reversal of CD4/CD8 ratio in tumour tissue.

Stage is considered most important prognostic factor in carcinoma cervix. Other important prognostic factors include primary tumor stage (T stage), presence of lymph node involvement and bulky primary disease [14]. Based on each of these prognostic factors, patients were categorised in two different groups. We found no statistically significant difference in T lymphocyte subpopulations between two prognostically distinct groups both in tumor tissue and in peripheral blood (Table 3). In a study involving 59 patients with early-stage cervical carcinoma treated with radical hysterectomy, Piersma et al. analysed association of TILs with the prognostic factors such as lymph node metastasis, tumor size, vasoinvasion and depth of infiltration of the tumor [15].

They reported significantly higher CD8+ cell counts in patients with lymph node metastasis compared to patients without. Notable distinctions of our study encompass the reliance of imaging studies for the identification of lymph node metastasis and the application of flowcytometry method for characterisation of TILs. Other investigators have reported similar findings, albeit in the context of early-stage disease [8]. Ohno et al. recently reported similar study involving locally advanced cases of cervical carcinoma patients treated with definitive chemoradiotherapy [16]. Their study investigated correlation between TILs and prognostic factors such as age, clinical stage, tumor size, pelvic lymph node metastasis, para-aorta lymph node metastasis, parametrial invasion, and vaginal wall invasion. Weak infiltration of CD8+ lymphocytes was found to be associated with pelvic lymph node metastasis. No other factors were found to be associated with immune cell infiltration [16]. Garg et al. investigated the association with T lymphocyte subsets in tumor and peripheral blood from patients with locally advanced cervical cancer, using methods similar to those in our study [17]. No significant difference of T lymphocyte subsets was reported in peripheral blood and tumor tissue in relation to FIGO staging and lymph node metastasis; however, an association with primary tumor volume was found.

Cervical cancer is caused by high risk types of HPV, particularly HPV 16 and HPV 18, which account for approximately two third of all cervical carcinomas [18, 19]. In our study population, HPV (HPV 16 & 18) association was found in 55% cases. HPV 16 infection was significantly associated with CD4+ and CD8+ T lymphocyte infiltration in tumor tissue ($p < 0.05$) (Table 4). For patients with HPV 18 infection, a significant alteration of CD4/CD8 ratio was observed in both peripheral blood and tumor tissue ($p < 0.05$) (Table 5). The HPV early 6 (E6) and early 7 (E7) gene encoded proteins are two well-known oncoproteins involved in the pathogenesis of cervical cancer, and defective T cell immunity against HPV has been considered an important microenvironment factor influencing tumor biological characteristics [11, 20]. Significant alteration of TILs in tumor tissue of HPV associated cervical cancer may explain impaired or defective immune response against viral oncoproteins leading to development of invasive malignancy. Stimulation of impaired immune mechanism through immunotherapy holds potential therapeutic implications in the management of cervical cancer. Recently, the therapeutic value of adoptive transfer of TILs in HPV-associated epithelial cancers including cervical cancer has been reported, and new immunotherapies such as Pembrolizumab, a humanized anti-PD-1 antibody, was approved for patients with recurrent or metastatic cervical cancer in 2018 [21, 22].

The modest sample size constitutes a limitation of the current investigation. A further limitation is the lack of separate assessment of infiltrating T lymphocytes in tumor nest and stroma of the tumor. A lack of prospective follow-up limits our ability to determine the prognostic implications of altered CD4/CD8 ratio of TILs. The study also has certain strengths. We included patients with locally advanced cervical carcinoma, the predominant stage of presentation in our country, enhancing the study's clinical relevance. We have also explored association of HPV infection which is attributable to majority of cervical cancer cases.

In conclusion, our study demonstrates that both CD4+ and CD8+ T lymphocyte, major component of TILs, are significantly decreased in tumor tissue compared to peripheral blood of locally advanced cervical cancer. No association is observed between T lymphocyte subpopulations and major prognostic factors of cervical cancer in our study. The enhanced tumor infiltration by T lymphocytes (TILs) observed in HPV-associated cervical cancer represents a significant alteration with promising therapeutic applications.

Author Contribution Statement

Study was conceptualised and designed by Prof Diptimay Das. Material preparation and data collection were performed by Dr. Arpan Adhikary, Dr. Miranda Thoudam and Dr. Bidhan Chandra Chakraborty. The data was analysed by Dr. Suparna Kanti Pal. The first draft of the manuscript was written by Dr. Harris Mahammad Sepai and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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General

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Data Availability

Research data is accessible upon reasonable request to the correspondence author.

Funding Statement

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Approval

The research project was approved by the Research Advisory Committee of IPGME&R, Kolkata (Memo no. IPGME&R/RAC/340 dated 04/08/2022)

Ethical Declaration

The study was approved by IPGME&R Research Oversight Committee (Institutional Ethics Committee) (Memo no. IPGME&R/IEC/2022/409 dated 15.09.2022). Written informed consent to participate in this study was

provided by the participants. All the methods were carried out following the guidelines of the Declaration of Helsinki.

Conflict of Interest

The authors declare that there is no conflict of interests.

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