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RESEARCH ARTICLE

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

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In this study, 40 biopsy samples collected from cervical cancer patients at the First Affiliated Hospital of Xi'an Jiaotong University, China, were retrospectively assessed using immunohistochemistry for CD4$^+$ and CD8$^+$ tumor-infiltrating lymphocytes (TILs) and were analyzed for the expression of FOXP3, OX40, granzyme B (GrB) and perforin (Prf). The proliferating index of the TILs was determined by assessing Ki67 expression. We determined the prognostic value of low and high numbers of TILs on survival by performing Kaplan–Meier analysis using median values as the cut-off points. Except for the number of CD4$^+$FOXP3$^+$ regulatory T cells (Tregs) and the CD4/CD8 ratio, none of the CD4$^+$, CD8$^+$, OX40$^+$, GrB$^+$ or Prf$^+$ TILs were associated with the overall 5-year survival rate. The 5-year survival rate was significantly lower in patients who had a high percentage of Tregs as compared with the those who had a lower percentage (35.3% versus 88.9%, *P*=0.001), while the 5-year survival rate was significantly higher in patients with a high CD4/CD8 ratio as compared with patients who had a low CD4/CD8 ratio (82.4% versus 44.4%, *P*=0.029). When we considered the deaths and surviving cases as separate groups, we found that both the number of CD4$^+$ T cells and the CD4/CD8 ratio were significantly lower in patients who died as compared with those who survived (26.33±11.80 versus 47.79±38.18, *P*=0.023 and 0.60±0.25 versus 1.17±1.02, *P*=0.019, respectively). In conclusion, decreased proportions of tumor-infiltrating CD4$^+$ T cells with high percentages of Tregs and reversed CD4/CD8 ratios were significantly associated with the clinical outcome of patients with cervical carcinoma.
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Keywords: CD4/CD8 ratio; cervical carcinoma; clinical outcome; tumor-infiltrating lymphocytes

INTRODUCTION

Tumor-infiltrating lymphocytes (TILs) are a part of the tumor surveillance system.[1] They are frequently present in solid human tumors,[2] and their role in different types of cancers has been controversial. The presence of TILs correlates with a better prognosis in several types of cancer, and each T-lymphocyte subset has a unique role in the antitumor response.[3–7] The presence of tumor-infiltrating cytotoxic T lymphocytes (CTLs) has been linked to better patient survival in endometrial, ovarian, pancreatic and colorectal cancers.[8–10] The role of CD4$^+$ T cells is more controversial and is often considered to be a double-edged immunologic sword because CD4$^+$ T cells play a central role in initiating and maintaining anticancer immune responses.[11–13] CD4$^+$ T-cell help is needed during the primary antigen-specific response to imprint CD8$^+$ T cells with the ability to develop into long-lived functional memory cells.[14] In antitumor immunity, CD4$^+$ T cells have also been shown to be important in sustaining the functions of adoptively transferred CD8$^+$ T cells.[15] On the other hand, naturally occurring subsets of CD4$^+$ T cells called regulatory T cells (Tregs) play an essential role in controlling immune responses to self- and non-self-antigens. Tumor-infiltrating Tregs have been reported to be associated with poor prognosis in some cancers because they suppress the proliferation of effector T lymphocytes (i.e., CTL), which prohibits an adequate tumor-specific immune response and enables tumor growth.[16–21] In contrast, tumor-infiltrating Tregs have also been reported to be either associated with a better prognosis or not associated with prognosis in some malignancies such as follicular lymphoma and squamous cell carcinoma.[22–24]

Antitumor immune responses are attributed mainly to cell-mediated immunity. The activation of both CD4$^+$ and CD8$^+$ T lymphocytes is needed for an efficient immune response that destroys

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tumor cells.[11–13,15] In this scenario, the infiltration of the tumor site with high numbers of CD8$^+$ TILs would be desirable. The presence of CD4$^+$ TILs would also be required because CD8$^+$ T cells usually need CD4$^+$ T cells to optimally function. 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.

Recently, the immunogenicity of malignancy has been reappraised according to the type of immune response provoked by the tumor. In our previous study, we observed that highly immunogenic tumor cells in syngeneic mice can induce type I immune responses, whereas poorly immunogenic tumor cells induce type II immune responses.[25] Using an assay to determine the immune microenvironment of cervical cancer, others have shown a type II predominance.[26,27] Cervical cancer is one of the most common malignancies of married women globally and is the result of an uncontrolled persistent infection with a high-risk human papillomavirus (HPV) type, HPV16 and HPV18 in particular, which account for approximately two-thirds of these cancers.[28,29] The immune microenvironment of cervical cancer not only shares the nature of solid tumors in general, but also possesses the properties associated with its etiology. HPV may use different strategies to evade immune recognition. These characteristics make the microenvironment of cervical cancer more complicated.

In the present study, to examine the immune status of cervical carcinoma *in situ* by immunohistochemistry, we evaluated the status of CTLs and Tregs by characterizing their functional phenotypes (using granzyme B (GrB) and FOXP3) as markers of the immune response. This approach may contribute novel information to the field of cervical cancer immunology.

MATERIALS AND METHODS

Samples

Under the approval of the institutional review board and ethics committee, biopsy samples from 40 cervical cancer patients were collected from the First Affiliated Hospital of the Medical College of Xi'an Jiaotong University. All cases had follow-up data for at least 5 years. No cases received radiation or chemotherapy prior to operation but were treated with radiotherapy after diagnosis and biopsy collection. All cases were histopathologically classified according to the International Federation of Obstetrics and Gynecology (FIGO) criteria. The tissue samples were fixed in formalin and embedded in paraffin. All cases were positive for HPV by PCR.[30]

Immunohistochemistry

Immunohistochemistry staining was conducted on 4- to 5-μm paraffin sections using horseradish peroxidase from the Envision Kit or the Catalyzed Signal Amplification Kit (Dako Corporation, Copenhagen, Denmark) for single staining, and alkaline phosphatase was used for double staining, as recommended by the manufacturer. The primary antibodies were mouse monoclonal anti-human anti-CD8 (DakoCytomation, Glostrup, Denmark; 1 : 100 dilution), anti-GrB (Novocastra, Newcastle, UK; 1 : 100), anti-Perforin (Prf; Novocastra; 1 : 20), anti-CD4 (Novocastra; 1 : 30), anti-OX40 (Novocastra; 1 : 50), anti-FOXP3 (Abcam, Cambridge, UK; 1 : 50), and anti-Ki67 (DakoCytomation; 1 : 200). Briefly, the sections were dewaxed in xylene and rehydrated using graded concentrations of ethanol and distilled water. Endogenous peroxidase activity was blocked by submersion of the sections in a 0.5% H_2O_2/methanol solution for 10 min at room temperature (RT). Antigen was retrieved by heating the sections under high temperature and pressure in a stainless steel pressure cooker for 1 min and 30 s in unmasking solution (0.01 M citrate buffer, pH 6.0). After cooling the cooker to RT in running tap water, the sections were placed in TBS-Tween buffer for 5 min, blocked with 3% goat serum for 10 min at 37 °C, and incubated with primary antibody (at RT: CD8, 1 h; GrB, 2 h; Prf, 2 h; OX40, 1 h; Ki67, 1 h; and CD4 overnight at 4 °C). After the sections were rinsed three times with TBS-Tween buffer, they were subsequently incubated with Dako EnVision (or AP 1 : 500 in the case of double staining) for 30 min or processed according to the instructions of the Catalyzed Signal Amplification Kit at RT. After the sections were washed with TBS-Tween buffer, the antigen–antibody reaction was visualized using 3,3′-diaminobenzidine in the case of horseradish peroxidase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium in the case of AP, and the sections were counterstained with hematoxylin or methyl blue, respectively. Tonsil tissue was used as a positive control for all the antibodies. The numbers of labeled TILs were counted by the classical counting method with the light microscope using an ocular grid at ×200 magnification. Labeled cells were counted in the whole tissue section, and the total number of labeled cells was divided by the number of fields to calculate the mean value for each case. Counting was performed twice for each slide.

Table 1 Clinical characteristics of 40 patients with squamous cell carcinoma of the cervix

Characteristics	*No. (%)*
Age	
Median	47
Range	32–70
Clinical stage (FIGO)	
IIB	10 (25.0)
IIIB	30 (75.0)
Lymph node (N) status	Unknown
Distant metastasis (M) status	
M0	38 (95.0)
M1	2 (5.0)
Progression	
No	32 (80.0)
Yes	8 (20.0)
Survival status	
Deceased	15 (37.5)
Survivors	25 (62.5)

Statistical analysis

The non-parametric Wilcoxon signed-rank test was used to compare the mean number of TILs among the groups and subgroups. Follow-up time was calculated as the interval between the date of treatment

Table 2 Descriptive statistics of immunohistochemical variables

Variable[a]	*Mean*	*SD*	*Median*	*Range*
CD8$^+$ TILs	55.89	36.36	48.41	6.88–139.83
Granzyme B$^+$ TILs	13.37	14.45	7.51	0.92–59.03
Perforin$^+$ TILs	15.74	16.64	6.95	0.52–64.34
CD8$^+$FOXP3$^+$ TILs	3.32	4.05	1.89	0.10–19.70
CD8$^+$Ki67$^+$ TILs	0.96	1.22	0.43	0.00–5.44
CD4$^+$ TILs	39.82	32.56	28.58	9.45–168.52
OX40$^+$ TILs	3.66	3.91	2.1	0.00–12.65
CD4$^+$FOXP3$^+$ TILs	11.45	7.51	11.42	0.93–30.39
CD4$^+$Ki67$^+$ TILs	0.95	1.37	0.24	0.00–5.60
CD4$^+$/CD8$^+$ ratio	0.96	0.56	0.69	0.19–4.21

Abbreviation: TIL, tumor-infiltrating lymphocyte.
[a] Number of TILs per field (×400).

and last follow-up or death. Survival rates were analyzed with the Kaplan–Meier method using the log-rank test to assess the difference between survival curves. A P value of less than 0.05 was considered statistically significant. Statistical calculations were performed using SPSS version 14.0 for Windows.

RESULTS

Clinicopathological characteristics of patients

The clinicopathological characteristics of patients are provided in Table 1. The immunohistochemical variables are provided in Table 2. Among the 40 cases studied, five cases were not evaluable after staining, so they were excluded. The remaining 35 cases were evaluated. All data are shown as mean±SD.

Tumor-infiltrating CD8 T lymphocytes

$CD8^+$ T lymphocytes (Figure 1) were found in both the stroma and tumor nest. Independent of whether the samples were from the deceased or surviving groups, the mean number of $CD8^+$ T cells was lower in the stroma as compared with that in the tumor nest (21.06±16.58 versus 34.53±26.98, P=0.039 and 22.57±16.88 versus 33.51±27.79, P=0.050 in the deceased and surviving groups, respectively). Comparing the deceased and surviving groups, we observed no

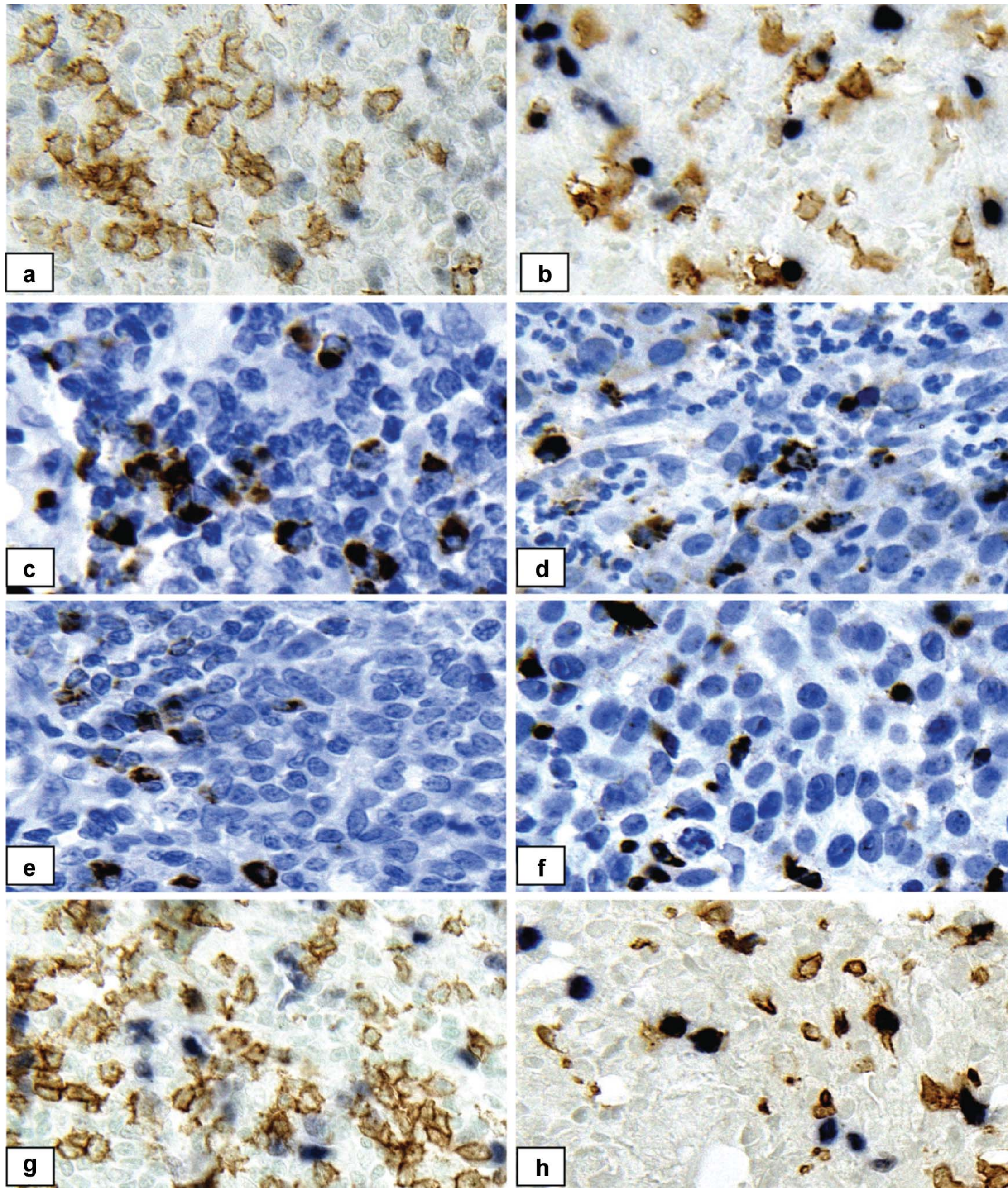

Figure 1 Representative immunohistochemically stained specimens. The left panel shows positive controls (lymph nodes); the right panel shows cervical cancer tissues. (**a**, **b**) Double staining for CD8 (brown) and FOXP3 (pink). (**c**, **d**) Single staining for Granzyme B (brown). (**e**, **f**) Single staining for Perforin (brown). (**g**, **h**) Double staining for CD8 (brown) and Ki67 (pink). Counterstaining: hematoxylin for single immunostaining and methyl green for double immunostaining; magnification: ×400.

significant differences in either the stroma or the tumor nest, and we also observed no significant differences in the overall mean number of $CD8^+$ T cells. When the patients were stratified by FIGO staging, the mean number of $CD8^+$ T cells was higher in the tumor nest as compared with that in the stroma (20.19±14.42 versus 29.92±21.70, P=0.173; and 22.64±17.43 versus 35.22±29.01, P=0.018, in FIGO stage IIB and IIIB, respectively) (Table 3). The functional status of $CD8^+$ T cells was evaluated using the expression of GrB and Prf. No significant differences were observed in the activated CTL either in the stroma or the tumor nest, nor overall in either group (decease or surviving, or FIGO IIB or IIIB groups; data not shown). As expected, the number of GrB^+ and Prf^+ TILs was consistent.

When the survival rate was determined using the Kaplan–Meier method and when the patients were divided into two groups (low and high levels of $CD8^+$ T cells) based on the median value, no significant difference was observed in the 5-year survival rate between the low- and high-level groups (64.7% versus 61.1%, P=0.801) (Figure 2).

Tumor-infiltrating $CD4^+FOXP3^+$ Tregs

The mean number of $CD4^+$ T cells (Figure 3) was almost the same in the stroma and tumor nest in the surviving group (23.79±23.97 versus 24.03±17.68, P=0.950), while the mean number was slightly higher in the stroma than in the tumor nest in the deceased group (15.98±9.73 versus 10.45±4.44, P=0.075). Comparing the deceased and surviving groups, we observed that the overall mean number of $CD4^+$ T cells was significantly lower in the deceased group as compared with the mean number in the surviving group (26.33±11.80 versus 47.79±38.18, P=0.023); the same was observed when we compared the stroma and tumor nest separately (15.98±9.73 versus 23.79±23.97, P=0.039 and 10.45±4.44 versus 24.03±17.68, P=0.023, stroma and tumor nest, respectively). No significant differences were observed in the mean numbers of $CD4^+$ T cells between the tumors-designated FIGO stage IIB and IIIB; no significant differences were observed between the groups or within the groups when the $CD4^+$ T cells were counted overall or separately in the stroma and tumor nest (Table 4). However, although the number of $CD4^+$ TILs was significantly lower in the deceased group as compared with those in the surviving group, we found no statistically significant difference in the 5-year survival rate between the groups with high or low levels of $CD4^+$ TILs (76.5% versus 50.0%, P=0.121) (Figure 4).

To further differentiate the subgroups of $CD4^+$ T cells, we evaluated the Tregs. The number of $CD4^+FOXP3^+$ T cells (Tregs) was significantly higher in the deceased group than in the surviving group in the stroma and tumor nest (47.64±20.41 versus 22.83±18.97, P=0.003 and 68.98±20.76 versus 27.96±21.27, P=0.005, respectively) and also overall (54.47±17.84 versus 23.53±15.59, P=0.002). Interestingly, the mean number of Tregs was significantly higher in the tumor nest than in the stroma in the deceased group (68.98±20.76 versus 47.64±20.41, P=0.023) and in the FIGO stage IIIB group (46.44±28.07 versus 35.16±6.15, P=0.046), while no significant differences were observed between the tumor nest or stroma in the surviving group (27.96±21.27 versus 22.83±18.97, P=0.465) or in the FIGO stage IIB group (33.83±30.94 versus 23.05±18.18, P=0.441) (Table 5).

A significantly lower survival rate was observed in patients with high levels of $CD4^+FOXP3^+$ Tregs as compared with those with low levels of these cells (35.3% versus 88.9%, P=0.001) (Figure 5).

Comparative numbers of $CD4^+$ and $CD8^+$ T lymphocytes and the CD4/CD8 ratio

The overall mean number of $CD4^+$ T cells was significantly lower than the mean number of $CD8^+$ T cells in the deceased group (26.33±11.80 versus 55.60±38.39, P=0.001), but the difference was not significant in the surviving group (47.79±38.18 versus 56.07±36.05, P=0.322). Similarly, there was no significant difference in the number of $CD4^+$ T cells as compared with the number of $CD8^+$ T cells in the stroma (15.98±9.73 versus 21.06±16.58, P=0.221), but it was significantly lower in the tumor nest (10.45±4.44 versus 34.53±26.98, P=0.001) of the deceased group. However, no significant differences were observed in the number of $CD4^+$ T cells as compared with the number of $CD8^+$ T cells either in the stroma or in the tumor nest in the surviving group.

The ratio of $CD4^+/CD8^+$ T cells was significantly lower in the deceased group than in the surviving group (0.60±0.25 versus 1.17±1.02, P=0.019). In comparing the stroma and tumor nest of the two groups, we observed no significant difference in the ratios of $CD4^+/CD8^+$ T cells between the stroma of the deceased and surviving groups (0.99±0.66 versus 1.58±1.43, P=0.075), but the ratio was significantly lower in the tumor nest of the deceased group as compared with that of the surviving group (0.41±0.17 versus 1.16±1.13, P=0.006). In the within-group comparison, we found that the $CD4^+/CD8^+$ T-cell ratio was significantly lower in the tumor nest than in the stroma in the deceased group (0.41±0.17 versus 0.99±0.66, P=0.003), while we found no significant difference in the $CD4^+/CD8^+$ T-cell ratio between the tumor nest and stroma of the surviving group (1.16±1.13 versus 1.58±1.43, P=0.131). In comparing the FIGO stage IIB with IIIB groups, we observed no significant differences in the $CD4^+/CD8^+$ T-cell ratio between the two groups, either

Table 3 Mean numbers of tumor-infiltrating $CD8^+$ T cells[a]

$CD8^+$ TILs	Stroma	Tumor nest	Total
Survivors (22)	22.57±16.88	33.51±27.79	56.07±36.05
Deceased (13)	21.06±16.58[b]*	34.53±26.98	55.60±38.39
FIGO IIB (9)	20.19±14.42	29.92±21.70	50.07±29.34
FIGO IIIB (24)	22.64±17.43[b]*	35.22±29.01	57.91±38.82

Abbreviation: TIL, tumor-infiltrating lymphocyte.
[a] Data shown as mean±SD, *P<0.05.
[b] Stroma versus tumor nest.

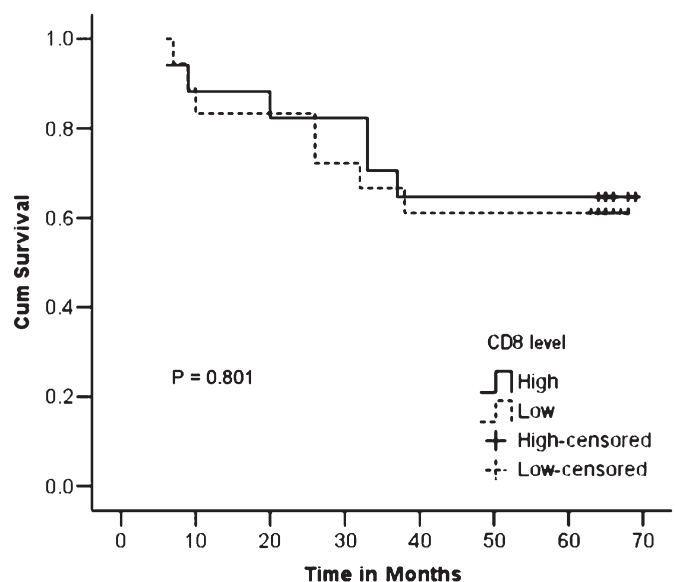

Figure 2 Kaplan–Meier curves showing the 5-year survival rate with respect to the level of $CD8^+$ T cells.

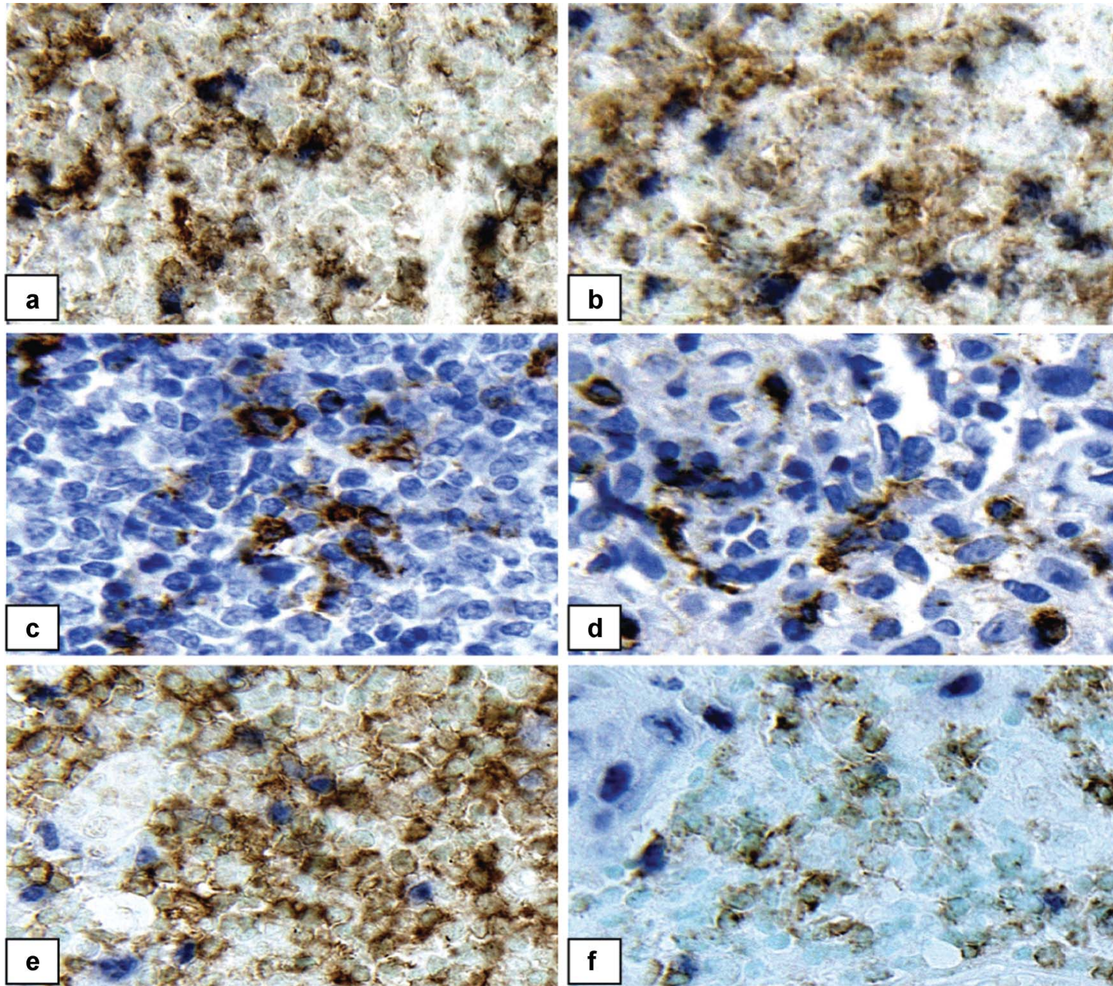

Figure 3 Representative immunohistochemically stained specimens. The left panel shows positive controls (lymph nodes); the right panel shows cervical cancer tissues. (**a**, **b**) Double staining for CD4 (brown) and FOXP3 (pink). (**c**, **d**) Single staining for OX40 (pink). (**e**, **f**) Double staining for CD4 (brown) and Ki67 (pink). Counterstaining: hematoxylin for single immunostaining and methyl green for double immunostaining; magnification, ×400.

overall or separately in the tumor nest and stroma. The ratio of $CD4^+$/ $CD8^+$ T cells was significantly lower in the tumor nest than in the stroma of the FIGO stage IIB group (0.70±0.43 versus 1.68±1.29, P=0.028), while this difference was non-significant in the FIGO stage IIIB group (0.94±1.09 versus 1.25±1.21, P=0.069) (Table 6). A significant difference was observed between the survival rates of patients with high and low $CD4^+$/$CD8^+$ T-cell ratios (82.4% versus 44.4%, P=0.029) (Figure 6).

Table 4 Mean numbers of tumor-infiltrating $CD4^+$ T cells[a]

$CD4^+$ TILs	*Stroma*	*Tumor nest*	*Total*
Survivors (22)	23.79±23.97	24.03±17.68	47.79±38.18
Deceased (13)	15.98±9.73[b]*	10.45±4.44[c]*	26.33±11.80[d]*
FIGO IIB (9)	22.54±11.87	16.34±11.21	38.88±21.45
FIGO IIIB (24)	20.32±22.40	19.90±16.99	40.14±35.97

Abbreviation: TIL, tumor-infiltrating lymphocyte.
[a] Data shown as mean±SD, *P<0.05.
[b] Comparison between stromas.
[c] Comparisons between tumor nests.
[d] Comparison between total.

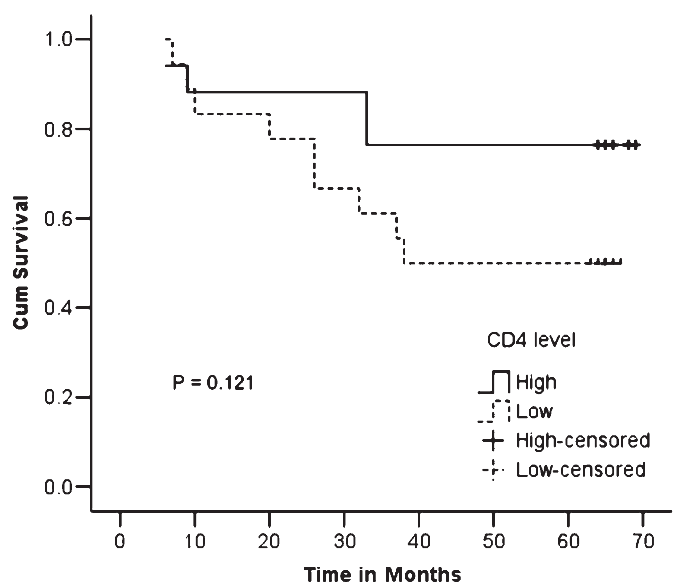

Figure 4 Kaplan–Meier curves showing the 5-year survival rate with respect to the level of $CD4^+$ T cells.

Table 5 Percentage of CD4$^+$FOXP3$^+$ Tregs[a]

CD4$^+$ FOXP3$^+$ %Tregs	Stroma	Tumor Nest	Total
Survivors (22)	22.83±18.97	27.96±21.27	23.53±15.59
Deceased (13)	47.64±20.41[b]*[c]**	68.98±20.76[d]**	54.47±17.84[e]**
FIGO IIB (9)	23.05±18.18	33.83±30.94	26.32±19.77
FIGO IIIB (24)	35.16±6.15[b]*	46.44±28.07	38.03±22.54

Abbreviation: Treg, regulatory T cell.
[a] Data shown as mean±SD, *$P<0.05$, **$P<0.01$.
[b] Stroma versus tumor nest.
[c] Comparison between stromas.
[d] Comparisons between tumor nests.
[e] Comparison between totals.

Table 6 Ratio of CD4$^+$/CD8$^+$ T cells[a]

CD4$^+$/CD8$^+$ TILs	Stroma	Tumor nest	Total
Survivors (22)	1.58±1.43	1.16±1.13	1.17±1.02
Deceased (13)	0.99±0.66[b]*	0.41±0.17[c]**	0.60±0.25[d]*
FIGO IIB (9)	1.68±1.29[b]*	0.70±0.43	1.02±0.44
FIGO IIIB (24)	1.25±1.21	0.94±1.09	0.94±0.92

Abbreviation: TIL, tumor-infiltrating lymphocyte.
[a] Data shown as mean±SD, *$P<0.05$, **$P<0.01$.
[b] Stroma versus tumor nest.
[c] Comparison between tumor nests.
[d] Comparison between totals.

DISCUSSION

In this study, we tried to assess the number and functional status of TILs *in situ* in relation to the 5-year survival of 35 patients with cervical cancer in FIGO stage IIB and IIIB. With regard to the number of CD8$^+$ TILs, we did not find any significant difference between the deceased and surviving groups or between the FIGO stage IIB and IIIB. Similarly, no differences were observed for the number of CD8$^+$FOXP3$^+$ T cells or proliferating CD8$^+$Ki67$^+$ T cells between the deceased and surviving groups and the FIGO stage IIB and IIIB groups (data not shown). Also, we observed no association between the number of CD8$^+$ TILs and survival. This result is in contrast with those from a number of studies that describe CD8$^+$ T lymphocytes as a favorable or non-favorable prognostic factor for survival. Piersma *et al.*[31] observed that the presence of intratumoral CD8$^+$ T cells in cervical cancer was correlated with the lack of pelvic lymph node spread and was, therefore, correlated indirectly with prognosis. A high number of tumor-infiltrating CD8$^+$ T lymphocytes were observed as a favorable prognostic factor for survival in some studies of human cancers, including endometrial cancer,[32] ovarian cancer,[8] colorectal cancer,[33] esophageal cancer[34] and urothelial carcinoma.[35] Other studies on non-small cell lung cancer,[36] anal squamous cell carcinoma[24] and renal cell carcinoma[37] reported that CD8$^+$ T lymphocytes were a negative prognostic factor.

The CD8$^+$ T-cell subpopulation has been considered to be the effector in the antitumor response; however, when the status of activation was explored, most of the cells were underprimed (CD45RO$^+$)[38] or inactivated (in this study, the GrB/CD8 ratio was 7/48), regardless of the effector/target ratio within the tumor tissue (data not shown). It is unlikely that such a weak functional population could eliminate the tumor burden. The similarity in the numbers of CD8 TILs between the two groups suggests that the recruitment of CD8$^+$ T cells to the tumor site occurs under similar conditions. More importantly, the similarity of the two cytotoxic markers (GrB and Prf) reflects that the cytotoxic machinery of the infiltrates was intact, suggesting that the dysfunctional status of the effector cells was due to the influence of the microenvironment[39] rather than to the physical dysfunction of signal transduction in the T cells.[40] Thus, the low expression of the T-cell receptor-ζ chain, the T cell-signaling molecule, observed in CD8$^+$ TILs of cervical cancer patients, may not be associated with chronic viral infection.[39] Interestingly, these results differ from our previous data regarding breast cancer, which revealed a significant discrepancy in the numbers of GrB- and Prf-positive cells.[41] The orchestration of these two molecules is known to be a prerequisite for CD8$^+$ T cells to exert their killing function. This indicates that the status of CTL in different malignant microenvironments requires further exploration.

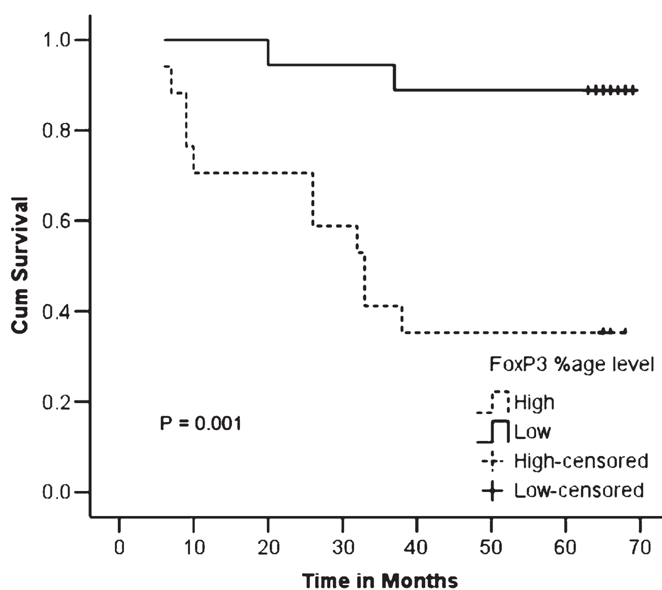

Figure 5 Kaplan–Meier curves showing the 5-year survival rate with respect to the percentage CD4$^+$FOXP3$^+$ T cells.

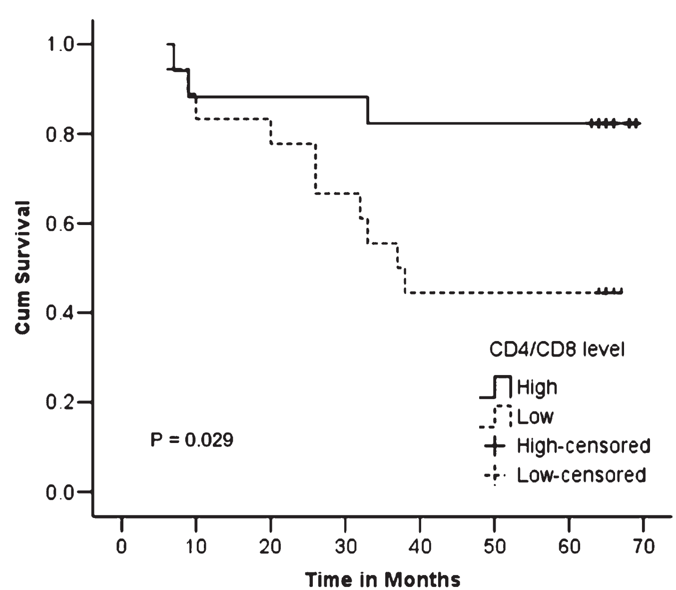

Figure 6 Kaplan–Meier curves showing the 5-year survival rate with respect to the CD4$^+$/CD8$^+$ T-cell ratio.

The main mechanism by which TILs control tumor growth is postulated to be *via* a cytotoxic mechanism. In this scenario, the infiltration of the tumor site with high numbers of activated $CD8^+$ TILs would be desirable. $CD4^+$ TILs would also be required because to function optimally, $CD8^+$ T cells usually require $CD4^+$ T cells (Th1 cells are assumed to play a key role in the antitumor milieu). Therefore, we next examined the number of $CD4^+$ TILs in both the stroma and tumor nest. A significantly lower number of $CD4^+$ TILs was observed in the deceased group than in the surviving group (26.33±11.80 versus 47.79±38.18, P=0.023). Similarly, the number of $CD4^+$ TILs was significantly lower in the deceased group than in the surviving group in both the stroma and tumor nest (15.98±9.73 versus 23.79±23.97, P=0.039 and 10.45±4.44 versus 24.03±17.68, P=0.023, respectively). No differences were observed in the numbers of $CD4^+$ TILs between the FIGO stage IIB and IIIB groups. Although the number of $CD4^+$ TILs was significantly lower in the deceased group than in the surviving group, we observed no statistically significant difference in the 5-year survival rates between the groups with high and low levels of $CD4^+$ TILs (76.5% versus 50.0%, P=0.121). However, when the $CD4^+$ TILs were separately evaluated as helper and Tregs based on their positivity for the FOXP3 marker, we found that the percentage of Tregs was significantly higher in the deceased group than in the surviving group both in the stroma and tumor nest (47.64±20.41 versus 22.83±18.97, P=0.003 and 68.98±20.76 versus 27.96±21.27, P=0.005, respectively) and overall (54.47±17.84 versus 23.53±15.59, P=0.002). In addition, we observed a significantly lower survival rate in patients with high levels of Tregs than in those with low levels (35.3% versus 88.9%, P=0.001).

A low density of $CD3^+$, $CD4^+$ and $CD8^+$ cells is associated with an increased risk of relapse in squamous cell cervical cancer, while a high density of all three cell types is associated with a decreased risk of relapse.[42] High numbers of $CD4^+$ TILs have also been observed as a favorable prognostic marker in other human cancers such as non-small cell lung cancer[36] and head and neck cancer.[43] The characteristics of $CD4^+$ Tregs and the mechanisms leading to their biological effects remain the subject of extensive investigation.[44,45] In principle, Tregs may downregulate the antitumor immune response. Thus, increased Tregs within the primary tumor may predict a poor prognosis. Several studies in mice have shown that Tregs inhibit the antitumor immune response[46–48] and that the depletion of Tregs can enhance effector T-cell antitumor responses.[49,50] HPV-specific Tregs can be detected in both the draining lymph nodes and the tumors of women infected with HPV, and they seem to be another means to downregulate host immune responses. van der Burg *et al.*[51] isolated HPV-specific $CD4^+$ T cells from lymph node biopsies of cervical cancer patients and found that they had the ability to suppress proliferation and cytokine (IFN-γ and IL-2) production by responder T cells. The capacity of HPV-specific $CD4^+$ T cells to exert this suppressive effect depended on their activation by cognate HPV antigen and on close-range interactions with responder T cells. HPV-specific $CD4^+$ Tregs were also retrieved from cervical cancer biopsies, suggesting that they may interfere with the antitumor immune responses on both the induction and effector levels. Notably, the relationship between Tregs and cervical cancer is unknown, but many of these data support a role for Tregs in disease progression.

An earlier study by Sheu *et al.*[52] showed that reversed CD4/CD8 ratios of TILs are correlated with the progression of human cervical carcinoma; the study found that the CD4/CD8 ratios of TILs were reversed in both cervical squamous cell carcinoma and cervical adenocarcinoma. The proportion of $CD4^+$ T cells was significantly lower in tumors from patients with lymph node metastases than in those from patients without lymph node metastases, as was the reversed CD4/CD8 ratio. Similarly, Piersma *et al.*[31] found a significantly stronger $CD8^+$ T-cell tumor infiltration, a higher $CD8^+/CD4^+$ T-cell ratio, and a higher $CD8^+$/Tregs ratio in patients with tumors that failed to metastasize to the tumor-draining lymph node. Loddenkemper *et al.*,[27] in a comparison study of high-grade CIN (CIN III) and cervical carcinoma with colon cancer, skin melanoma and bronchial carcinoma, revealed that HPV-derived lesions have a significantly higher number of infiltrating lymphocytes and $FOXP3^+$ Tregs as compared with three other common tumor types. Similar to the above studies, in our study, we observed a reversed CD4/CD8 ratio in cervical cancer. The overall mean number of $CD4^+$ T cells was significantly lower than the mean number of $CD8^+$ T cells in the deceased group (26.33±11.80 versus 55.60±38.39, P=0.001), but this difference was not significant in the surviving group (47.79±38.18 versus 56.07±36.05, P=0.322); indeed, the ratio of $CD4^+/CD8^+$ T cells was significantly lower in the deceased group than in the surviving group (0.60±0.25 versus 1.17±1.02, P=0.019). The survival rate was significantly higher in patients with high $CD4^+/CD8^+$ T-cell ratios than in patients with low $CD4^+/CD8^+$ T-cell ratios.

The generation of a specific cytotoxic T-cell response is known to depend on sufficient help from activated Th1-type $CD4^+$ T cells.[53,54] The inability of the host to reject a tumor may be due to the insufficient generation of tumor-specific $CD4^+$ T cells.[55,56] The relatively low number of Th1-type $CD4^+$ T cells with reversed CD4/CD8 ratios in the deceased group in our study supports this hypothesis. Unitt *et al.*[57] observed that a high $CD4^+/CD8^+$ T-cell ratio is associated with a reduced risk of tumor recurrence after liver transplantation in hepatocellular carcinoma. Our results obviously reflect the cancerous environment *per se* and demonstrate that the analysis of the functional phenotypes of TILs '*in situ*' may more precisely describe the tumor milieu. However, the mechanism by which Tregs influence the functional maturation of $CD8^+$ T cells remains to be explored because there were no differences in the numbers of activated $CD8^+$ T cells among all of the studied groups.

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