Increase of programmed death-1-expressing intratumoral CD8 T cells predicts a poor prognosis for nasopharyngeal carcinoma

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Intratumoral cytotoxic T lymphocytes are critical for controlling tumor recurrence, and programmed death-1 (PD-1) is a recognized marker of T-cell dysfunction. We analyzed this marker and its binding ligands in nasopharyngeal tumor tissue and non-cancerous nasopharyngeal control tissue to retrospectively evaluate the correlation between its expression and the post-treatment outcome of nasopharyngeal carcinoma patients. Using double immunofluorescence staining, we found that the expression of PD-1 in CD8 T cells in tumor tissue was significantly higher than in control tissue (mean: 28.4 *vs* 3.9%, $P<0.0001$). Although the expression rate of PD-1 in intratumoral CD8 cells was not associated with the other clinicopathological parameters examined, the higher expression rate in this subset of T cells significantly correlated with a poorer prognosis of overall survival, disease-free survival, and locoregional recurrence-free survival of the cancer patients ($P=0.05$, 0.007, and 0.004, respectively). Multivariate analysis confirmed it as an independent risk factor for death, treatment failure, and local recurrence of nasopharyngeal carcinoma. On the other hand, the expression of PD-1 in CD4 T cells and of its ligands in epithelial and stromal cells was not significantly different between tumor and control tissue, and its expression was not associated with clinical outcome of the cancer patients. We propose that PD-1 expression in CD8 cells reflects the selective suppression of cytotoxic lymphocytes in the tumor microenvironment and predicts recurrence of nasopharyngeal carcinoma after conventional therapies.
Modern Pathology (2010) **23,** 1393–1403; doi:10.1038/modpathol.2010.130; published online 23 July 2010

Keywords: immunofluorescence; intratumoral lymphocyte; nasopharyngeal carcinoma; prognosis; programmed death-1

Nasopharyngeal carcinoma is distinguished from other head-and-neck epithelial cancers by its epidemiological and pathological features and its clinical presentation, including the unique distribution of its endemic areas (southern China, southeast Asia, the Arctic, and North Africa), close association with Epstein–Barr virus infection, poor differentiation of tumor cells, and high susceptibility to radiotherapy and chemotherapy.[1] Although radiotherapy alone or combined with chemotherapy is relatively successful for curing the cancer, a substantial proportion of patients do not benefit from these conventional treatments, but show locoregional or distant recurrence.[1,2] To develop more effective therapies for refractory nasopharyngeal carcinoma, we must be able to identify early those patients who urgently require advanced therapies.[2,3]

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Received 12 February 2010; revised 22 June 2010; accepted 23 June 2010; published online 23 July 2010

Recent studies have underlined that effectiveness of radiotherapy and chemotherapy can be influenced by anti-tumor immune responses.[4,5] After conventional cancer treatments that massively destroy tumor cells, activated immune cells such as cytotoxic tumor-infiltrating lymphocytes (TILs) are important for eliminating residual cancer cells and reducing their recurrence. Nevertheless, multiple immunosuppressive mechanisms in tumor microenvironments can inactivate TILs, which may increase the risk of tumor recurrence after radiotherapy and chemotherapy.[4,6] A marker indicating TIL dysfunction and predicting a poor outcome after conventional treatment would help identify patients refractory nasopharyngeal carcinoma.

Programmed death-1 (PD-1) is a surface receptor that is expressed by lymphocytes and inhibits their proliferation and effector functions after it binds with PD-1 ligands such as B7-H1 (also known as PD-L1) and B7-DC (also known as PD-L2).[7,8] PD-1 is a significant marker for T lymphocytes malfunctioning in response to viral infection.[9–12] Recently, elevated PD-1 expression in intratumoral lymphocytes and its association with the functional impairment of TILs have also been reported in melanoma, hepatocellular carcinoma, and Hodgkin lymphoma.[13–15] However, reports on the prognostic value of PD-1 expression for cancer therapy, analyzed in only a few studies, are inconsistent. Increased PD-1 expression in intratumoral immune cells correlates with a poor clinical outcome in renal cell carcinoma and classical Hodgkin lymphoma, but is associated with improved overall survival in follicular lymphoma;[16–18] however, it is not significantly associated with the prognosis of pancreatic cancer.[19] These inconsistent results may be attributable to two possibilities that have not been clearly addressed. First, PD-1 expression in different subsets of tumor-infiltrating immune cells may have differential effects on clinical outcome. Second, the functional suppression of PD-1-expressing TILs and its prognostic relevance may depend on whether the ligands of PD-1 are present in the tumor tissue. Therefore, we examined the expression of PD-1 and its ligands in biopsy specimens of nasopharyngeal carcinoma and non-cancerous nasopharyngeal control tissue to determine their correlation with clinical outcome after therapies.

Materials and methods

Patients and Biopsy Specimens

Biopsy specimens and clinical data were collected from the Surgical Pathology Laboratory of National Cheng Kung University Hospital. The study protocol, specimen usage, and data retrieval were approved by the Institutional Human Experiment and Ethics Committee of National Cheng Kung University Hospital (approval number ER-95-107). We retrospectively reviewed the charts of nasopharyngeal carcinoma patients whose pre-treatment tumor biopsies, clinicopathological data, and follow-up records were available, who were free of distant metastasis at diagnosis, and who had completed curative radiotherapy with or without chemotherapy at National Cheng Kung University Hospital during 2003 and 2004. We obtained formalin-fixed, paraffin-embedded tissue blocks from 46 primary tumor biopsies, and the control specimens were non-cancerous nasopharyngeal tissue samples from 22 age-matched patients diagnosed with lymphoid hyperplasia. From a second group of patients, we also obtained snap-frozen tissue specimens from 28 primary tumor biopsies and 29 control nasopharyngeal tissue specimens of lymphoid hyperplasia. Histologically, all the tumors were classified as World Health Organization (WHO) type 2 or 3, showing a poorly differentiated or undifferentiated phenotype and containing a large number of infiltrating lymphocytes. The age of the cancer patients ranged from 28 to 77, with a median of 51. Among the 74 cancer patients studied (46 with paraffined specimens and 28 with frozen specimens), 23 patients had locoregional relapse or distant metastasis and a total of 14 patients died during the follow-up.

Immunofluorescence Staining

As the antibodies used in this study were suitable for immunostaining on different tissue sections, immunofluorescence co-staining of PD-1 and T-cell markers (CD8 and CD4) was performed using paraffined tissue sections, and co-staining of the PD-1 ligands (B7-H1 and B7-DC) and an epithelial cell adhesion molecule (EpCAM) was performed using frozen tissue sections. Paraffined sections (3-μm thick) of tumor and control tissues were laid on silanized slides (DakoCytomation, Carpinteria, CA, USA), deparaffinized, rehydrated in phosphate-buffered saline (PBS), and then autoclaved in ethylene diamine tetraacetic acid buffer (pH 8.0) for 10 min to retrieve the antigens. The slides were co-incubated with a goat polyclonal antibody that recognizes human PD-1 (diluted 1:15) (R&D Systems, Minneapolis, MN, USA) and mouse monoclonal antibodies against human CD8 (clone C8/144B, ready-to-use; DakoCytomation) or human CD4 (clone 4B12, 1:30 dilution; Novocastra Laboratories, Newcastle upon Tyne, UK) at room temperature for 1 h. After they had been washed three times with PBS, the slides were co-incubated with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat antibody (DakoCytomation) and a tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse antibody (DakoCytomation) in the dark at room temperature for 1 h. In the final step, the slides were washed with PBS, mounted with

a mounting medium (Vector Laboratories, Burlingame, CA, USA), and then examined under a fluorescence microscope (Olympus, Tokyo, Japan) by a pathologist blinded to the clinical information of the patients. Cells that stained positive for PD-1 were green (FITC), and those that stained positive for CD4 and CD8 were red (TRITC). To analyze the expression rates of PD-1 in CD8 and CD4 cells, randomly selected micrographs of the double-staining results were digitally captured and merged, and 800 cells from each sample were manually counted to quantify the percentage of PD-1-positive (PD-1$^+$) CD8 and CD4 cells.

To detect PD-1 ligands and EpCAM, cryostat sections (5-μm thick) of tumor and control tissue were fixed in acetone, rehydrated in PBS for 5 min, and then co-incubated in the dark at room temperature for 1 h with an FITC-conjugated monoclonal antibody that recognizes human EpCAM (1:10 dilution) (clone HEA-125; Miltenyi Biotec, Bergisch Gladbach, Germany) and with phycoerythrin-conjugated monoclonal antibodies against human B7-H1 (clone MIH1, ready-to-use; eBioscience, San Diego, CA, USA) or human B7-DC (clone MIH18, ready-to-use; eBioscience). After they had been washed three times with PBS, the sections were then co-incubated in the dark at room temperature for 30 min with a biotinylated rabbit anti-phycoerythrin antibody (1:100 dilution) (eBioscience) and an Alexa488-conjugated rabbit anti-FITC antibody (1:100 dilution) (Invitrogen, Carlsbad, CA, USA). After the specimens had been washed three more times with PBS, the sections were reacted with Alexa594-conjugated streptavidin (1:500 dilution) (Invitrogen) in the dark at room temperature for 30 min. Finally, the sections were washed again, mounted, and examined under a fluorescence microscope. Cells that stained positive for EpCAM were green (FITC plus Alexa488), and those that stained positive for B7-H1 and B7-DC were red (phycoerythrin plus Alexa594). Micrographs of the double-staining results were digitally captured and merged to evaluate whether the ligands of PD-1 were expressed by EpCAM-positive (EpCAM$^+$) epithelial cells or EpCAM-negative (EpCAM$^-$) stromal cells.

Statistical Analysis

Student's *t*-test was used to evaluate the differences between PD-1$^+$ rates in CD8 and CD4 T cells. On the basis of the positive rates of PD-1 in the two types of intratumoral T cells, cancer patients were divided into high PD-1$^+$-CD8 (>median expression rate: 27.8%) and low PD-1$^+$-CD8 (<median expression rate: 27.8%) groups, or into high PD-1$^+$-CD4 (>median expression rate: 14.5%) and low PD-1$^+$-CD4 (<median expression rate: 14.5%) groups. Alternatively, based on the cell types expressing B7-H1 in tumor tissue, the cancer patients were grouped into B7-H1$^+$ in EpCAM$^+$ tumor cells *vs* B7-H1$^+$ in EpCAM$^-$ stromal cells. Student's *t*-test was also used to examine whether the different expression patterns of PD-1 or B7-H1 were associated with age. A χ^2 test was used to assess whether expression patterns of PD-1 or B7-H1 correlated with other clinicopathological parameters such as gender and clinical tumor-node-metastasis (TNM) staging. Overall survival, disease-free survival, locoregional relapse-free survival, and distant metastasis-free survival were measured from the initial diagnosis until death or specific events; follow-up data of surviving patients were assessed on the last contact date. Kaplan–Meier analysis was performed to estimate the survival distribution, and a log-rank test was used to evaluate the correlation between expression patterns of PD-1 or B7-H1 with the post-treatment survival. Multivariate Cox proportional hazard regression modeling was used to test the influence of covariates (PD-1$^+$-CD8 rate, PD-1$^+$-CD4 rate, gender, age, histological type, tumor stage, nodal status, and clinical staging) on locoregional relapse-free, disease-free, and overall survival. *P*-values are two sided. All analyses were performed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA).

Results

PD-1 Expression in CD8 and CD4 Cells in Nasopharyngeal Carcinoma and Control Tissue

As both CD8 and CD4 cells can be involved in controlling tumor growth, we used immunofluorescence co-staining to simultaneously detect the expression of PD-1 as well as CD8 and CD4 in paraffined tissue sections. Substantially different patterns of PD-1 were detected in nasopharyngeal tumor and control tissue (Figure 1). PD-1 was co-stained with CD4, but not CD8 cells in the control specimens diagnosed as lymphoid hyperplasia (Figures 1a and b). A similar staining pattern was also reported in tonsils,[16] which suggests that PD-1 is expressed predominantly by CD4 but not CD8 cells during an active immune response in lymphoid tissue. In contrast, PD-1 was markedly detected in not only CD4, but also CD8 cells in tumor tissue (Figures 1a and b), which indicated preferential increase of PD-1 expression in the intratumoral CD8 cells. The PD-1$^+$ percentage of CD8 cells was significantly higher in tumor tissue than in control tissue (mean: 28.4 *vs* 3.9%, 95% confidence interval (CI): 23.93–32.79% *vs* 1.57–6.25%, $P<0.0001$; Figure 1c); however, PD-1 expression rates in CD4 cells were not significantly different between tumor and control tissue (mean: 15.6 *vs* 20.5%, 95% CI: 12.91–18.20% *vs* 14.56–26.35%; Figure 1d). The PD-1 expression rates of CD8 and CD4 cells were not associated (data not shown).

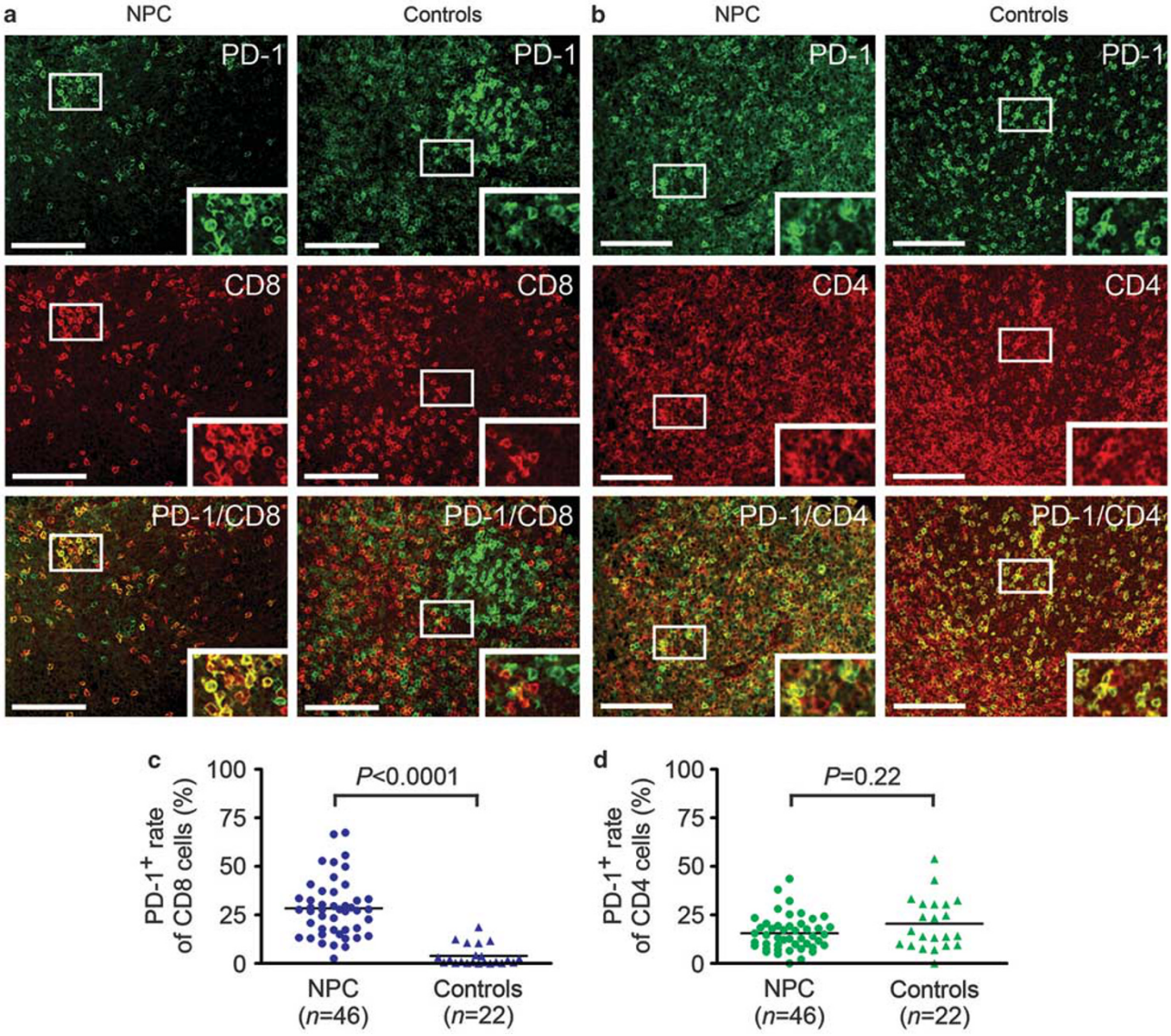

Figure 1 PD-1 expression in CD4 and CD8 T cells in nasopharyngeal carcinoma tumor (NPC) and control nasopharyngeal tissue. (**a**) Representative results of immunofluorescence staining of PD-1 and CD8 in NPC and control tissue. (**b**) Representative results of immunofluorescence staining of PD-1 and CD4 in NPC and control tissue. PD-1-positive (PD-1$^+$) cells are stained green; CD8 and CD4 cells are stained red. PD-1$^+$-CD8 and PD-1$^+$-CD4 cells in the merged images (third row) are yellow. Scale bar, 100 μm. (**c**) PD-1$^+$-CD8 rates in NPC and control tissue. (**d**) PD-1$^+$-CD4 rates in NPC and control tissue. The expression rates in 46 NPC and 22 control samples are plotted; each dot represents one specimen. Black bars = mean values.

Association of PD-1 Expression in Intratumoral CD4 and CD8 Cells with Clinicopathological Parameters and Post-treatment Outcome of Nasopharyngeal Carcinoma

To analyze whether PD-1 expression correlates with clinicopathological parameters or the prognosis of nasopharyngeal carcinoma, we divided the cancer patients according to their PD-1-positive percentage of intratumoral CD8 or CD4 cells: high PD-1$^+$-CD8 group (>median expression rate: 27.8%; 23 cases) *vs* low PD-1$^+$-CD8 group (<median expression rate: 27.8%; 23 cases), and high PD-1$^+$-CD4 group (>median expression rate: 14.5%; 23 cases) *vs* low PD-1$^+$-CD4 group (<median expression rate: 14.5%; 23 cases). PD-1 expression rates and the clinicopathological parameters of age, gender, tumor stage, nodal involvement, and clinical TNM staging at diagnosis were not correlated (Table 1). Notably, a Kaplan–Meier survival analysis showed that the patients in the high PD-1$^+$-CD8 group had poorer post-treatment outcomes than did the patients in the low PD-1$^+$-CD8 group. The prognoses for overall survival ($P = 0.05$; Figure 2a) and disease-free survival ($P = 0.007$; Figure 2b) were significantly worse in the high PD-1$^+$-CD8 group. An analysis of the types of treatment failure showed that a high PD-1$^+$-CD8 rate strongly correlated with a locoregional relapse

Table 1 Correlation between clinicopathological parameters and expression of PD-1 or B7-H1 in biopsies of nasopharyngeal carcinoma

Parameter	*PD-1 expression rate of CD8 TILs*			*PD-1 expression rate of CD4 TILs*			*B7-H1 expression*		
	Low PD-1-CD8 (<27.8%) (n=23)	*High PD-1-CD8 (>27.8%) (n=23)*	P-*value*	*Low PD-1-CD4 (<14.5%) (n=23)*	*High PD-1-CD4 (>14.5%) (n=23)*	P-*value*	*B7-H1⁺ in EpCAM⁻ cells (n=10)*	*B7-H1⁺ in EpCAM⁺ cells (n=18)*	P-*value*
Age (mean, 95% CI)	50.8, 46.7–54.9	49.7, 44.0–55.5	0.76[a]	51.7, 47.0–56.3	48.9, 43.7–54.1	0.42[a]	50.9, 40.6–61.2	52.3, 46.5–58.1	0.78[a]
Gender									
Male	19 (82.6%)	20 (87.0%)	0.68[b]	21 (91.3%)	18 (78.3%)	0.22[b]	7 (70.0%)	11 (61.1%)	0.70[b]
Female	4 (17.4%)	3 (13.0%)		2 (8.7%)	5 (21.7%)		3 (30.0%)	7 (38.9%)	
Tumor stage									
T1	6 (26.1%)	5 (21.7%)	0.60[b]	6 (26.1%)	5 (21.7%)	0.81[b]	4 (40.0%)	6 (33.3%)	0.72[b]
T2	6 (26.1%)	7 (30.4%)		5 (21.7%)	8 (34.8%)		1 (10.0%)	2 (11.1%)	
T3	5 (21.7%)	8 (34.8%)		8 (34.8%)	5 (21.7%)		4 (40.0%)	5 (27.8%)	
T4	6 (26.1%)	3 (13.0%)		4 (17.4%)	5 (21.7%)		1 (10.0%)	5 (27.8%)	
Nodal status									
N0	3 (13.0%)	2 (8.7%)	0.89[b]	3 (13.0%)	2 (8.7%)	0.64[b]	3 (30.0%)	4 (22.2%)	0.59[b]
N1	6 (26.1%)	6 (26.1%)		7 (30.4%)	5 (21.7%)		4 (40.0%)	6 (33.3%)	
N2	8 (34.8%)	10 (43.5%)		7 (30.4%)	11 (47.8%)		3 (30.0%)	5 (27.8%)	
N3	6 (26.1%)	5 (21.7%)		6 (26.1%)	5 (21.7%)		0 (0%)	3 (16.7%)	
TNM staging									
1	1 (4.4%)	0 (0%)	0.36[b]	1 (4.4%)	0 (0%)	0.85[b]	1 (10.0%)	2 (11.1%)	0.17[b]
2	4 (17.4%)	4 (17.4%)		5 (21.7%)	3 (13.0%)		3 (30.0%)	3 (16.7%)	
3	7 (30.4%)	12 (52.2%)		9 (39.1%)	10 (43.5%)		5 (50.0%)	4 (22.2%)	
4	11 (47.8%)	7 (30.4%)		8 (34.8%)	10 (43.5%)		1 (10.0%)	9 (50.0%)	

[a]*P*-values were obtained from Student's *t*-test.
[b]*P*-values were obtained from χ^2 tests.

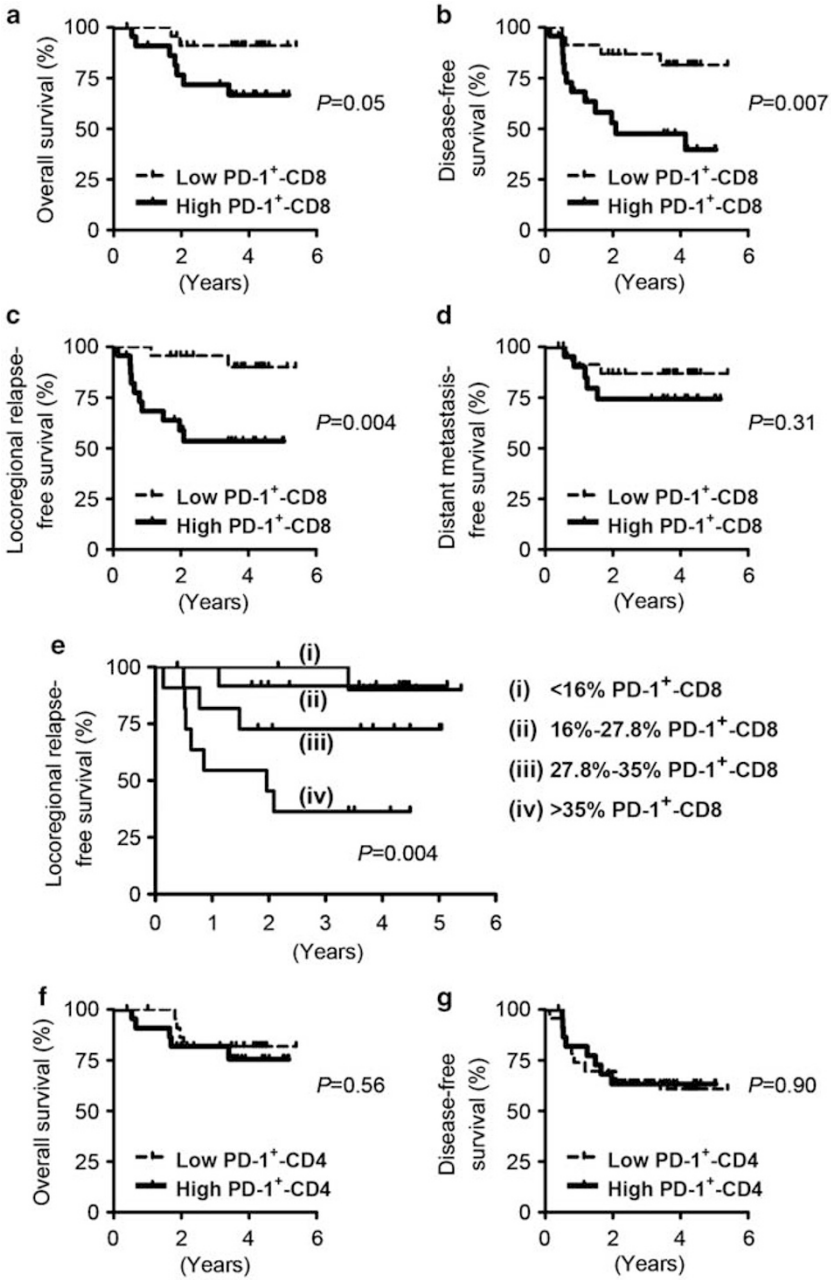

Figure 2 Kaplan–Meier analysis of the correlation between PD-1 expression in intratumoral CD4 and CD8 cells and post-treatment survival of nasopharyngeal carcinoma patients. (**a**) Correlation between PD-1$^+$-CD8 expression and overall survival. (**b**) Correlation between PD-1$^+$-CD8 expression and disease-free survival. (**c**) Correlation between PD-1$^+$-CD8 expression and locoregional recurrence-free survival. (**d**) Correlation between PD-1$^+$-CD8 expression and distant metastasis-free survival. (**e**) Correlation between PD-1$^+$-CD8 expression and locoregional recurrence-free survival (four groups). (**f**) Correlation between PD-1$^+$-CD4 expression and overall survival. (**g**) Correlation between PD-1$^+$-CD4 expression and disease-free survival.

of nasopharyngeal carcinoma ($P = 0.004$; Figure 2c). Although the high PD-1$^+$-CD8 group also had more distant metastasis than did the low PD-1$^+$-CD8 group, the difference was not significant (Figure 2d). We also stratified the cancer patients into four groups based on their PD-1$^+$-CD8 percentage, and still observed a pronounced trend that the incidence of locoregional recurrence increased as the PD-1 expression rate of CD8 cells rose; the group with the highest PD-1$^+$ rate showed the highest incidence of recurrence ($P = 0.004$; Figure 2e). However, the PD-1$^+$-CD4 expression rate was not correlated with the overall or disease-free survival of the cancer patients (Figures 2f and g). In both univariate and multivariate analyses, a high PD-1$^+$-CD8 rate, but not a high PD-1$^+$-CD4 rate, significantly predicted a poor prognosis after therapy, even though the TNM staging did not reliably predict clinical outcome in this study (Tables 2 and 3). Multivariate analysis showed that, compared with the low PD-1$^+$-CD8

Table 2 Univariate analysis to assess the association of clinicopathological parameters with prognosis of nasopharyngeal carcinoma

Parameter	*Prognostic factor*	*Univariate analysis (P-value[a])*		
	Case number/total, percentage	*LRFS*	*DFS*	*OS*
PD-1$^+$ rate of CD8 T cells	High PD-1-CD8 (>27.8%) 23/46, 50%	**0.004**	**0.007**	**0.050**
PD-1$^+$ rate of CD4 T cells	High PD-1-CD4 (>14.5%) 23/46, 50%	0.989	0.900	0.560
Gender	Male (*vs* female) 39/46, 84.8%	0.946	0.653	0.933
Age	>50 years 19/46, 41.3%	0.334	0.883	0.758
Histological type	WHO type 3 (*vs* WHO type 2) 35/46, 76.1%	0.726	0.502	0.348
Tumor stage	T3/4 (*vs* T1/2) 22/46, 47.8%	0.474	0.351	0.546
Nodal status	N2/3 (*vs* N0/1) 29/46, 63.0%	0.671	0.939	0.951
TNM staging	Stage 3/4 (*vs* stage 1/2) 37/46, 80.4%	0.86	0.314	0.531

LRFS, locoregional relapse-free survival; DFS, disease-free survival; OS, overall survival.
[a]*P*-values were obtained from log-rank test. Bold values signify *P*-value ≤ 0.05.

group, the high PD-1$^+$-CD8 group had a 6.5 times higher risk of locoregional recurrence (95% CI: 1.77–24.01, $P=0.005$), a 6.5 times higher risk of treatment failure (95% CI: 1.48–28.22, $P=0.013$), and a 9.5 times higher risk of death (95% CI: 1.55–58.26, $P=0.015$), which suggested that PD-1 expression in intratumoral CD8 cells may have an independent effect on the post-treatment outcome of nasopharyngeal carcinoma.

PD-1 Ligand Expression in Nasopharyngeal Carcinoma and Control Tissue

Other studies have reported that the expression of PD-1 ligands, especially of B7-H1, is higher in tumor tissue than in control tissue, and that it is associated with a poor prognosis.[20–23] Therefore, we also used immunofluorescence co-staining of individual PD-1 ligands (B7-H1 and B7-DC) and an epithelial marker EpCAM to check the cell types expressing the ligands in the tumor and control tissue. As staining antibodies for these markers were only suitable for frozen sections, we used tissue sections of snap-frozen specimens from a separate group of patients. B7-DC was not detected in any of the specimens (data not shown), but B7-H1 was positive in 28 (100%) tumor samples and in 23 of 29 (79%) controls. Both the tumor and control samples showed two distinct staining patterns of B7-H1 (Figures 3a and b). In 18 of 28 (64%) tumor specimens, B7-H1 was detected predominantly in EpCAM$^+$ epithelial cells, but in the remaining 10, B7-H1 was detected exclusively in EpCAM$^-$ stromal cells (Figures 3a and 4a). Of the 23 B7-H1$^+$ control samples, 8 cases showed B7-H1 staining in EpCAM$^+$ epithelial cells and 15 in EpCAM$^-$ cells (Figures 3b and 4a).

Association of B7-H1 Expression with Clinicopathological Parameters and Post-treatment Outcome of Nasopharyngeal Carcinoma

As B7-H1 was expressed at similar levels in all nasopharyngeal carcinoma biopsy samples, we divided the cancer patients into two groups: B7-H1$^+$ in EpCAM$^+$ epithelial cells *vs* B7-H1$^+$ in EpCAM$^-$ stromal cells. The clinicopathological parameters of age, gender, tumor stage, nodal status, and TNM staging were not significantly different between the groups (Table 1), nor were there significant differences in post-treatment overall survival or disease-free survival (Figures 4b and c).

Discussion

In summary, we found that the expression of PD-1 in CD8 T cells was significantly higher in nasopharyngeal tumor tissue than in non-cancerous nasopharyngeal tissue. In addition, we found that the high expression rate of PD-1 in intratumoral CD8 cells predicted a poor clinical outcome in terms of mortality, treatment failure, and locoregional recurrence. However, the expression of PD-1 in CD4 T cells and of B7-H1 in epithelial and stromal cells was not significantly different between tumor and non-cancerous control tissue, and its expression was not associated with clinical outcome of the cancer patients.

During a chronic viral infection, persistent stimulation with viral antigens exhausts virus-specific T cells: they are rendered phenotypically activated, but functionally impaired.[7,8] PD-1 has been recognized as a remarkable marker for the exhausted T cells.[7,9,10,12] Consistent with the idea that continuous exposure to tumor antigens may also facilitate the exhaustion of infiltrating lymphocytes in tumor

Table 3 Multivariate analysis to estimate the association of clinicopathological parameters with prognosis of nasopharyngeal carcinoma

Parameter	*Risk factor*	*LRFS*		*DFS*		*OS*	
	Case number/total, percentage	*Hazard ratio (95% CI)*	P-*value*[a]	*Hazard ratio (95% CI)*	P-*value*[a]	*Hazard ratio (95% CI)*	P-*value*[a]
PD-1$^+$ rate of CD8 T cells	High PD-1-CD8 (>27.8%) 23/46, 50%	6.512 (1.767–24.005)	**0.005**	6.466 (1.481–28.220)	**0.013**	9.509 (1.552–58.261)	**0.015**
PD-1$^+$ rate of CD4 T cells	High PD-1-CD4 (>14.5%) 23/46, 50%	1.410 (0.404–4.919)	0.590	1.535 (0.374–6.294)	0.552	2.825 (0.482–16.552)	0.250
Gender	Male (*vs* female) 39/46, 84.8%	1.225 (0.222–6.754)	0.816	2.381 (0.435–13.040)	0.317	1.797 (0.161–20.009)	0.633
Age	>50 years 19/46, 41.3%	0.882 (0.240–3.247)	0.851	0.998 (0.258–3.866)	0.998	0.944 (0.209–4.272)	0.940
Histological type	WHO type 3 (*vs* WHO type 2) 35/46, 76.1%	3.367 (0.662–17.123)	0.143	3.780 (0.533–26.807)	0.183	7.387 (0.495–110.259)	0.147
Tumor stage	T3/4 (*vs* T1/2) 22/46, 47.8%	2.112 (0.452–9.877)	0.342	1.749 (0.347–8.825)	0.498	2.814 (0.374–21.163)	0.315
Nodal status	N2/3 (*vs* N0/1) 29/46, 63.0%	1.007 (0.219–4.632)	0.993	0.895 (0.175–4.580)	0.894	1.222 (0.181–8.241)	0.837
TNM staging	Stage 3/4 (*vs* stage 1/2) 37/46, 80.4%	0.802 (0.066–9.767)	0.863	2.360 (0.117–47.605)	0.575	0.753 (0.021–26.396)	0.876

LRFS, locoregional relapse-free survival; DFS, disease-free survival; OS, overall survival.
[a]Bold values signify *P*-value <0.05.

tissue, recent studies have reported an association between PD-1 expression and the functional impairment of TILs in some cancers.[13–15] Nasopharyngeal carcinoma tumors, especially those of WHO type 2 and type 3 in the endemic areas, are characterized by a prevailing Epstein–Barr virus infection in cancerous cells with intensive lymphocytic infiltration and, therefore, represent a tissue microenvironment for both tumor development and chronic viral infection.[1] PD-1 expression in the TILs suggests that T-cell exhaustion may also occur in this cancer, which is supported by previous findings that the cytotoxic activity and cytokine production of T lymphocytes in nasopharyngeal carcinoma tumors are defective even if some of the TILs exhibit activated phenotypes.[24,25] Additional studies are required to determine whether the PD-1-expressing TILs are specific to antigens of the tumor cells or of Epstein–Barr virus, and whether they are functionally exhausted.

Impairment of anti-tumor immune responses prevents the body from clearing residual tumor cells after radiotherapy or chemotherapy, which increases the risk of tumor recurrence after treatment.[4,5] Multiple immunosuppressive mechanisms in nasopharyngeal carcinoma have been proposed: interleukin-10,[26] transforming growth factor β1,[27] Fas ligand,[28] regulatory T cells,[29] and galectin-9.[30] As most of these mechanisms converge to inhibit effector T cells, we hypothesized that a marker summarizing the suppressive status of TILs would help identify nasopharyngeal cancer patients with a high risk of treatment failure after standard radiotherapy or chemotherapy. PD-1 is a candidate marker because it is not only a specific receptor that suppresses T cells, but it is also an indicator of the severity of T-cell dysfunction.[31,32] PD-1 expression in primary nasopharyngeal carcinoma tumors was higher in CD8 T cells, the major cytotoxic effector cells that eliminate tumor cells, than in CD4 T cells. This finding suggests that CD8 cells are selectively exhausted in the tumor microenvironment, which may explain the significant association of the high PD-1$^+$-CD8 group with frequent locoregional recurrence. However, its weak association with distant metastasis implies that the local immunosuppression status at primary tumor sites is not necessarily linked to the status at metastatic sites, but we did not use metastatic tumor tissue to test this hypothesis.

Our finding of a preferential increase of PD-1$^+$-CD8 cells in nasopharyngeal carcinoma tumors differs from the findings of studies that reported elevated PD-1 expression in both CD4 and CD8 cells in melanoma, Hodgkin lymphoma, and cervical carcinoma tissue.[13,15,33] The high PD-1$^+$-CD8 rate was significantly correlated with a poor prognosis in our study. In contrast, for follicular lymphoma, in which PD-1 is highly expressed by CD4 cells, higher PD-1 expression in TILs predicts improved overall survival.[16] We hypothesize that PD-1 expression in

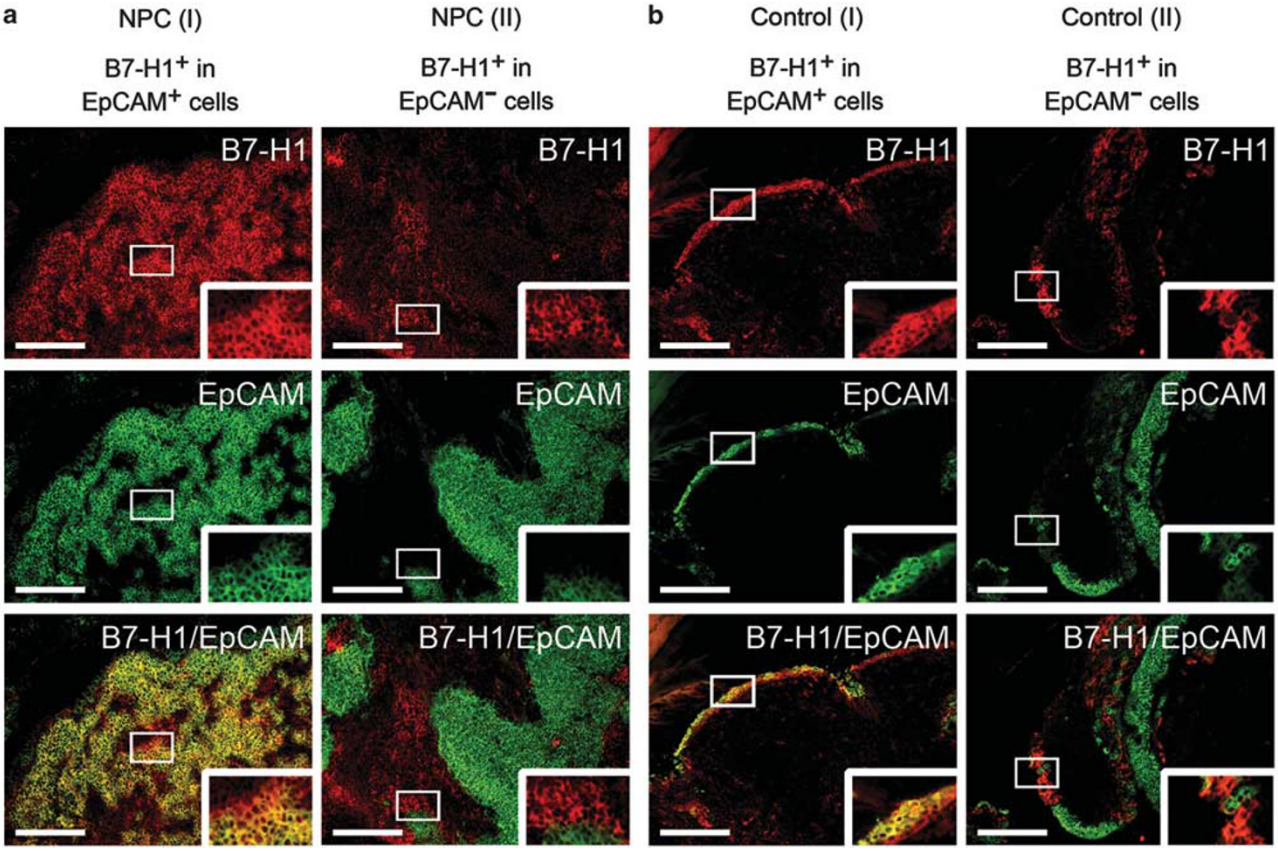

Figure 3 Immunofluorescence detection of B7-H1 and EpCAM in nasopharyngeal carcinoma tumor (NPC) and control tissue. (**a**) Representative staining patterns of B7-H1 and EpCAM in two tumor samples. (**b**) Representative staining patterns of B7-H1 and EpCAM in two control samples. B7-H1$^+$ staining is red; EpCAM$^+$ staining is green. B7-H1$^+$ and EpCAM$^+$ co-staining in the merged images (third row) is yellow. Scale bar, 200 μm.

different types of intratumoral lymphocytes is likely to differentially influence clinical outcome, which may account for the variable prognostic impact of PD-1 in studies that did not discriminate between the types of PD-1$^+$ TILs.[16–19] Therefore, to evaluate the clinical relevance of PD-1, the types of PD-1-expressing cells should be taken into consideration. Interestingly, a poor prognosis for nasopharyngeal carcinoma has been predicted by a large number of CD8 cells expressing granzyme B, a marker of activated cytotoxic T cells.[34] It remains to be clarified whether those granzyme B-positive TILs express PD-1 and are functionally suppressed.

Our findings are different from the previous reports that B7-H1 expression is upregulated in many kinds of tumors and predicts a poor clinical outcome.[20–23] Although we were unable to obtain both parafinned and frozen specimens from the same patients and examine PD-1 and its ligands in the same tissue samples, we found that B7-H1 was expressed generally in nasopharyngeal carcinoma and control tissue, and either EpCAM$^+$ epithelial tumor cells or EpCAM$^-$ stromal cells are the source of this PD-1 ligand. B7-H1 expression in the non-cancerous control tissues is consistent with a previous report that B7-H1 is induced in infected or inflamed nasal tissue.[35] Thus, unlike in many other cancers, B7-H1 expression is not specific to malignant tissue in the nasopharynx. Abundant B7-H1 expression in all the tested tumor biopsy tissue samples suggests that this ligand is probably not a limiting factor for PD-1-mediated suppression of TILs in nasopharyngeal carcinoma. Our finding that B7-H1 was not associated with the clinical prognosis of the cancer supports this notion. Therefore, it is possible that in nasopharyngeal carcinoma tumors, the cells that express PD-1, rather than those that express its ligands, are critical for determining the extent of TIL dysfunction and for affecting the clinical outcome.

We conclude that PD-1$^+$-CD8 cells are a prognostic marker of nasopharyngeal carcinoma. Our findings have two major clinical implications. First, because cancer patients with low PD-1$^+$-CD8 expression respond well to conventional therapies, efforts to reduce the damage and side effects of standard treatments should benefit them the most. Second, patients with high PD-1$^+$-CD8 expression should be carefully monitored after treatments and considered for advanced therapies.[2] The inhibitory effects of PD-1 and B7-H1 can be overcome by certain immunoactivators,[36,37] and blocking PD-1

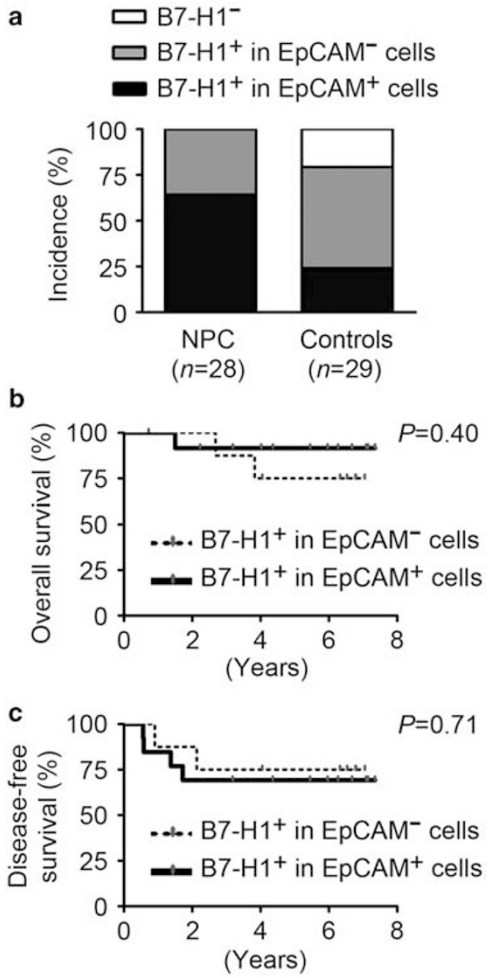

Figure 4 B7-H1 expression in nasopharyngeal carcinoma tumor (NPC) and control tissue, and its clinical relevance to post-treatment survival of cancer patients. (**a**) Distribution of the B7-H1 staining patterns in 28 NPC and 29 control tissue samples. (**b**) Correlation between B7-H1 staining patterns and overall survival. (**c**) Correlation between B7-H1 staining patterns and disease-free survival.

and B7-H1 restores the anti-tumor activity of TILs in some cancers,[14,15,22] which indicates that local immunosuppression in tumors can be reversed. Therefore, we hypothesize that alleviating the strong local immunosuppression of nasopharyngeal carcinoma by counteracting PD-1 and its ligands will not only improve the outcomes of Epstein–Barr virus-targeted cancer immunotherapy, but also improve the efficacy of conventional nasopharyngeal carcinoma therapy.

Acknowledgements

We thank Fan-Chen Tseng and Jeffrey Chang for advice about statistical analysis, and Chaio-Wei Chen and Mei-Ling Tsai for technical assistance. This study was supported by the Taiwan National Health Research Institutes (grant numbers CL-097-PP-15, CL-098-PP-15, and ID-099-PP-16) and the Comprehensive Cancer Center in Southern Taiwan (DOH99-TD-C-111-003).

Disclosure/conflict of interest

The authors declare no conflict of interest.

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