Association between circulating CD39+CD8+ T cells pre-chemoradiotherapy and prognosis in patients with nasopharyngeal carcinoma

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Abstract

Background: The mortality rate among patients with nasopharyngeal carcinoma (NPC) has improved significantly with the advent of chemoradiotherapy strategies. However, distant metastasis remains problematic. Tumor-specific reactivity in cancer patients has been detected exclusively in CD39+ T cells, particularly in CD39+CD103+ T cells. Circulating cancer-specific T cells are important for protecting against metastasis. This study aimed to evaluate the predictive value of circulating CD39+CD8+ T cells for metastasis in patients with NPC.

Methods: We performed a cross-sectional, longitudinal study of 55 patients with newly diagnosed NPC of stage III–IVa. All patients were initially treated with standard combined chemoradiotherapy. Blood samples were obtained from 24 patients before and at 1 month and 6 months after treatment. T cell expression of CD39 and CD103, together with the markers of T cell exhaustion programmed death-1 (PD-1)/T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3) and markers of cell differentiation CD27/CC-chemokine receptor 7/CD45RA, was examined by flow cytometry. The Wilcoxon rank-sum test analysis was used to analyze the differences between two groups. Kaplan-Meier analysis was used for analysis of progression-free survival (PFS).

Results: The expression of circulating CD39+CD8+ and CD39+CD103+ CD8+ T cells was significantly higher in patients without distant metastasis (CD39+CD8+: 6.52% [1.24%, 12.58%] *vs.* 2.41% [0.58%, 5.31%], $Z=-2.073$, $P=0.038$ and CD39+CD103+ CD8+: 0.72% [0.26%, 2.05%] *vs.* 0.26% [0.12%, 0.64%], $Z=-2.313$, $P=0.021$). Most CD39+ T cells did not express PD-1 or Tim-3. Patients with high expression of CD39+CD103+CD8+ T cells had better PFS than patients with low expression (log rank value = 4.854, $P=0.028$). CD39+CD8+ T cells were significantly elevated at 1-month post-treatment (10.02% [0.98%, 17.42%] *vs.* 5.91% [0.61%, 10.23%], $Z=-2.943$, $P=0.003$). The percentage of advanced differentiated CD8+ T cells also increased at 1-month post-treatment compared with pre-treatment (33.10% [21.60%, 43.05%] *vs.* 21.00% [11.65%, 43.00%], $Z=-2.155$, $P=0.031$). There was a significant correlation between elevated CD39+CD8+ T cells and increased effector memory T cells (intermediate stage: $r=0.469$, $P=0.031$; advanced stage: $r=0.508$, $P=0.019$).

Conclusions: CD39+CD8+ circulating T cells have preserved effector function, contributing to an improved prognosis and a reduced risk of metastasis among NPC patients. These cells may thus be a useful predictive marker for a better prognosis in patients with NPC.

Keywords: Nasopharyngeal carcinoma; CD39; T cell; Prognosis

Introduction

Nasopharyngeal carcinoma (NPC) remains a significant clinical problem, particularly in relation to distant metastasis, especially in China.[1,2] The current standard treatment for NPC in China is chemoradiotherapy.[3] Although early diagnosis and treatment are associated with promising outcomes, the potential for distant metastasis in patients with late-stage NPC (ie, stages III and IV) remains a significant problem.[4]

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The prognosis of cancer is largely influenced by the cytotoxic T cell response: a robust and appropriate T cell response is beneficial to the host, while a weak or inappropriate response can be ineffective or may even have a detrimental effect.[5] Cancer patients are characterized by impaired anti-tumor T-cell responses.[6] The recent success of clinical trials concerning antibodies targeting inhibitory receptors (so called "immune checkpoint receptors"), such as programmed death 1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4, has highlighted the importance of tumor-specific T cell responses in controlling the development of cancer.[7]

Tumor-specific reactivity has been detected exclusively in CD39+ cells, in particular in CD39+CD103+ tumor-infiltrating T cells.[8] CD39 is the rate-limiting enzyme in the conversion of adenosine triphosphate to immunomodulatory adenosine, and CD103 is an integrin that triggers bidirectional signaling events that cooperate with T cell receptor signals to enable T cell migration and optimal cytokine production. CD39 expression has recently been proposed as a marker for tumor-specific T cells,[9-12] and was shown to distinguish tumor-specific T cells from bystander CD8+ tumor-infiltrating lymphocytes with diverse phenotypes that overlap with tumor-specific cells, such as cytomegalovirus and flu-specific T cells.[9,10] In this study, we used CD39 as a specific marker to detect tumor-specific T cells in patients with NPC, and investigated the predictive value of CD39+CD8+ T cells for metastasis in patients with late-stage NPC post-chemoradiotherapy.

Methods

Ethics approval

This study was designed in accordance with the guidelines outlined in the *Declaration of Helsinki* and approved by the Institutional Ethics Committee of the Affiliated Tumor Hospital of Xinjiang Medical University (No. K-2019001). Informed consent was obtained from all patients.

Study population

A total of 55 patients with NPC admitted to the Affiliated Tumor Hospital of Xinjiang Medical University between March 2015 and July 2019 were recruited in this study. Eligibility criteria included newly diagnosed patients with stage III–IVa NPC according to the 7th edition Union for International Cancer Control and American Joint Committee on cancer tumor, node, metastasis staging system, without other types of cancer, autoimmune diseases, or infectious diseases. No patients had received previous surgery, radiotherapy, chemotherapy, or other oncologic treatments before enrollment in this study. All enrolled patients received standard combined chemoradiotherapy and had blood samples taken before treatment. Twenty-four of those also had blood samples taken at 1 month and 6 months post-treatment, including 21 without distant metastasis. The patients included 40 males and 15 females, aged 17 to 78 years (median age 47 years), and 29 patients classified as stage III and 26 as stage IVa. By the end of follow-up, a total of ten patients had developed distant metastases post-treatment, two of whom also had recurrent diseases at the primary site.

Lymphocyte isolation from blood samples

Blood from each patient was collected from the median basilic vein into vacutainers containing ethylenediaminetetraacetic acid. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by Ficoll-Hypaque density gradient centrifugation.

Multichromatic flow cytometry analysis

PBMCs were initially stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFischer Scientific, Waltham, MA, USA) for 20 min before surface staining with conjugated antibodies in washing buffer for 20 min and fixed with CellFix solution (BD Biosciences, San Jose, CA, USA). Commercial conjugated antibodies used included: BV785-CD3, BV510-CD14, BV510-CD16, BV510-CD19, PE/Dazzle594-CD39, BV711-CD103, BV421-CCR7, PE/Cy7-CD27 (BioLegend Inc, San Diego, CA, USA); PE-CD4, PerCP/Cy5.5-CD8, BV650-PD1, BB515-Tim3, APC/H7-CD45RA (BD Bioscience, San Jose, CA, USA). All samples were acquired by a four-laser BD LSRFortessa flow cytometer (BD Company, Franklin Lakes, NJ, USA) and analyzed by FlowJo software v.10.6 (FlowJo Co, San Diego, CA, USA) [Figure 1].

Statistical analysis

Statistical analyses and graph plotting were carried out using GraphPad PRISM v.8.0 (GraphPad Software Inc., San Diego, CA, USA) and SPSS v. 21.0 (IBM, Armonk, NY, USA). Continuous variables were shown as mean ± standard deviation and compared using a two-tailed Student's *t* test, while medians (Q_1, Q_3) and Mann-Whitney *U* tests were used for non-normally distributed variables. Categorical variables were shown as counts (percentages) and between-group comparisons were made using the Chi-square test or Fisher exact test. Differences between paired groups were compared by paired *t* test or Wilcoxon paired signed-rank test. The strengths of the linear relationships were evaluated by Pearson correlation coefficient. The median percentage of CD39+/CD39+CD103+ in T cells was used for the cut-off to define high *vs.* low group. PFS was defined from the date of beginning cancer treatment to the date of local or distant relapse confirmed by radiological examination, or the last day of follow-up. Survival rates were analyzed by the Kaplan–Meier method and the differences were compared by log-rank test. Univariate and multivariate proportional hazards models were applied to analyze the prognostic factors. All variables with $P < 0.05$ in univariate analysis were included in the multivariate Cox regression model to analyze the prognostic factors. Hazard ratios (HRs) with 95% confidence intervals (CIs) were calculated for risk estimation. A *P* value of <0.05 was considered statistically significant.

Results

Lack of CD39 expression on CD8 T cells correlated with distant metastasis in NPC patients post-chemoradiotherapy

The baseline characteristics of NPC patients (stage III–IVa) with or without metastasis after treatment are shown in Table 1. Flow cytometry analysis of CD39 and CD103

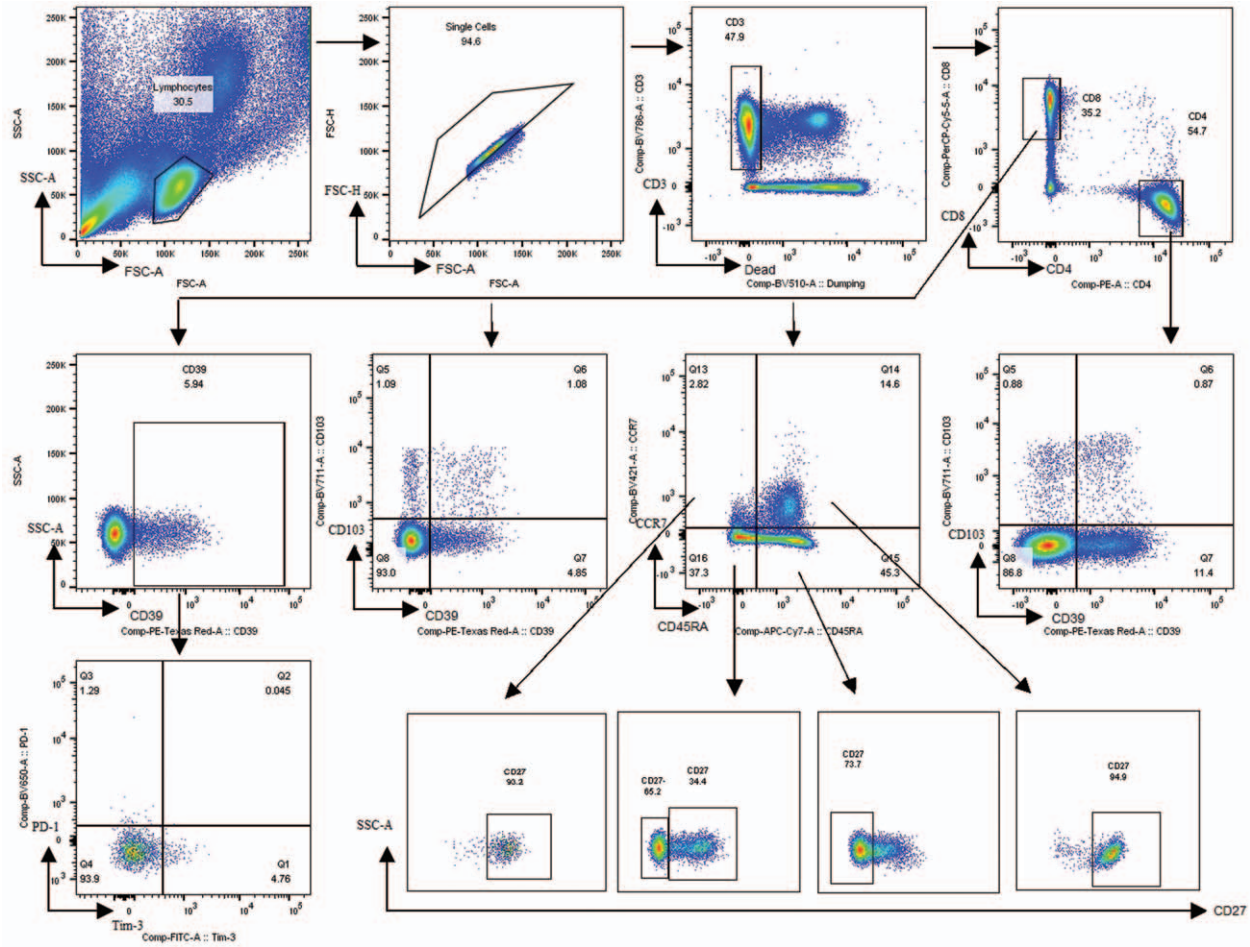

Figure 1: Gating strategy for CD8+ T cells by flow cytometry. CCR7: CC-chemokine receptor 7; FITC: Fluorescein isothiocyanate; FSC-A: Forward scatter-area; FSC-H: Forward scatter-height; PD-1: Programmed death-1; PE: Phycoerythrin; SSC-A: Side scatter-area; Tim-3: T cell immunoglobulin and mucin domain-containing protein 3.

expression on CD4+ and CD8+ T cells revealed that 6.08% (0.91%, 10.79%) of CD8+ T cells were CD39+ and 0.64% (0.23%, 1.50%) were CD39+CD103+, while 7.03% (1.84%, 11.88%) of CD4+ T cells were CD39+ and 1.12% (0.88%, 1.97%) were CD39+CD103+ before treatment. We compared the percentage of CD39+ cells in CD8+ and CD4+ T cells in patients with and without distant metastasis post-chemoradiotherapy. The percentages of CD39+CD8+ and CD39+CD103+CD8+ T cells in patients with metastasis were 2.41% (0.58%, 5.31%) and 0.26% (0.12%, 0.64%), and those in patients without metastasis were 6.52% (1.24%, 12.58%) and 0.72% (0.26%, 2.05%), respectively. We found that the expression of CD39+CD8+ ($Z = -2.073$, $P = 0.038$) and CD39+CD103+CD8+ T cells ($Z = -2.313$, $P = 0.021$), but not CD4+ T cells, was significantly higher in patients without metastasis [Table 1].

Kaplan–Meier survival curves demonstrated that the PFS of patients with high expression of CD39+CD103+CD8+ T cells was significantly higher than those with low expression (log rank value = 4.854, $P = 0.028$) [Figure 2]. Multivariate analyses confirmed that high expression of CD39+CD103+CD8+ T cells was significantly associated with better PFS (HR, 0.147; 95% CI: 0.028–0.781; $P = 0.024$) [Table 2].

We further evaluated the expression levels of the well-known exhaustion markers/inhibitory receptors PD-1 and Tim-3 on CD39+CD8+ T cells. The results showed that most CD39+CD8+ T cells did not express PD-1 and/or Tim-3, with 11.46% (7.11%, 16.52%) of PD-1+CD39+ CD8+ T cells and 4.77% (3.73%, 11.18%) of Tim-3+ CD39+CD8+ T cells.

CD39+CD8+ T cells exhibited higher percentages of central and effector memory phenotypes compared with CD39-CD8+ T cells

Distinct differentiation stages of CD8+ T cells based on the expression of CD27, CD45RA, and CCR7 on CD39+ CD8+ T cells have been defined according to a linear differentiation model,[13,14] including naïve (CD27+CCR7+CD45RA+), early differentiated (CD27+CCR7+CD45RA–), intermediate differentiated (CD27+CCR7–CD45RA–), advanced differentiated (CD27–CCR7–CD45RA–), and late differentiated T cells (CD27–CCR7–CD45RA+). We analyzed the differentiation statuses of CD39+CD8+ and CD39–CD8+ T cells before treatment. The percentages of naïve, early, intermediate, advanced and late differentiated phenotypes on CD39+ CD8+ T cells were 5.07% (2.12%, 6.01%), 6.32% (3.03%, 9.28%), 41.05% (32.70%, 51.05%), 27.95% (20.25%, 34.47%), and 5.64% (3.37%, 10.64%), respectively; and

Table 1: Baseline characteristics of NPC patients (stage III–IVa) with or without metastasis after treatment.

Characteristics	Without metastasis ($n = 45$)	With metastasis ($n = 10$)	Statistical values	P
Age (years)	48.1 ± 13.7	42.7 ± 13.6	1.134*	0.262
Gender			<0.001†	1.000
Male	33 (73.3)	7 (70.0)		
Female	12 (26.7)	3 (30.0)		
Pathology			0.099†	0.753
Differentiated	14 (31.1)	2 (20.0)		
Undifferentiated	31 (68.9)	8 (80.0)		
T stage			4.116†	0.128
T1-2	9 (20.0)	0 (0.0)		
T3	23 (51.1)	7 (70.0)		
T4	13 (28.9)	3 (30.0)		
N stage			4.079†	0.130
N1	6 (13.3)	0 (0.0)		
N2	31 (68.9)	6 (60.0)		
N3	8 (17.8)	4 (40.0)		
TNM stage			1.541†	0.214
III	26 (57.8)	3 (30.0)		
IVa	19 (42.2)	7 (70.0)		
Laboratory findings				
CD39+CD8+ (%)	6.52 (1.24, 12.58)	2.41 (0.58, 5.31)	−2.073‡	0.038
CD103+CD8+ (%)	3.02 (2.00, 4.44)	2.27 (1.39, 3.78)	−1.277‡	0.202
CD39+CD103+CD8+ (%)	0.72 (0.26, 2.05)	0.26 (0.12, 0.64)	−2.313‡	0.021
CD39+CD4+ (%)	7.09 (2.02, 12.07)	3.81 (1.41, 8.11)	−1.648‡	0.099
CD103+CD4+ (%)	1.30 (0.90, 2.05)	1.01 (0.82, 1.37)	−1.342‡	0.180
CD39+CD103+CD4+ (%)	0.29 (0.08, 0.53)	0.12 (0.03, 0.61)	−0.895‡	0.371

Data are shown as mean ± SD, median (Q_1, Q_3) or n (%). *t value. †Chi-square value. ‡Z value. N: Lymph node; NPC: Nasopharyngeal carcinoma; T: Tumor; TNM: Tumor, node, metastasis.

those on CD39–CD8+ T cells were 22.05% (11.47%, 34.35%), 2.33% (1.69%, 3.89%), 21.40% (12.25%, 24.97%), 14.95% (8.08%, 18.42%), and 21.35% (12.90%, 35.80%), respectively. We found significantly higher percentage of early, intermediate and advanced CD39+CD8+ T cells and significantly lower levels of naïve and terminally differentiated CD39+CD8+ T cells (naïve: $Z = -4.454$, $P < 0.001$; early: $Z = -3.485$, $P < 0.001$; intermediate: $Z = -5.227$, $P < 0.001$; advanced: $Z = -4.444$, $P < 0.001$; late: $Z = -4.701$, $P < 0.001$) [Figure 3].

Expression of CD39+CD8+ T cells increased post-chemoradiotherapy in NPC patients without metastasis

We examined the cell differentiation stage of overall CD8+ T cells in the blood of patients with NPC and no distant metastasis pre-treatment and 1 month and 6 months post-treatment ($n = 21$). The percentage of advanced and late CD8+ T cells increased at 1 month (advanced: 33.10% [21.60%, 43.05%] *vs.* 21.00% [11.65%, 43.00%], $Z = -2.155$, $P = 0.031$; late: 23.80% [12.10%, 31.35%] *vs.* 12.00% [4.50%, 27.15%], $Z = -3.076$, $P = 0.002$) and late CD8+ T cells increased at 6 months (28.20% [10.50%, 36.80%] *vs.* 12.00% [4.50%, 27.15%], $Z = -2.215$, $P = 0.027$) post-treatment compared with pre-treatment. However, the expression of naïve CD8+ T cells was reduced at 1 month (2.23% [0.84%, 14.54%] *vs.* 22.00% [3.59%, 34.95%], $Z = -4.029$, $P < 0.001$) and 6 months (4.74% [1.10%, 9.16%]) *vs.* 22.00% [3.59%, 34.95%], $Z = -3.237$, $P = 0.001$) post-treatment compared with pre-treatment [Figure 4A].

The percentage of CD39+ cells in CD8+ T cells was 5.91% (0.61%, 10.23%) pre-treatment, which increased to 10.02% (0.98%, 17.42%) at 1-month post-treatment ($Z = -2.943$, $P = 0.003$) and then reduced to 4.27% (0.58%, 17.09%) at 6 months post-treatment ($Z = -2.272$, $P = 0.023$) [Figure 4B]. We then analyzed the association between the changes of CD39+CD8+ T cells at 1-month post-treatment and the changes of various differentiation phenotypes. There was a significant correlation between elevated CD39+CD8+ T cells and increased effector memory T cells (intermediate stage: $r = 0.469$, $P = 0.031$; advanced stage: $r = 0.508$, $P = 0.019$) [Figure 4C].

Discussion

In this study, we found that the expression of circulating CD39+ and CD39+CD103+ CD8 T cells is significantly higher in NPC patients without distance metastasis post-treatment. The majority of the CD39+ T cells do not express exhaustion markers PD-1 and Tim-3; interestingly, patients with a high percentage of CD39+CD103+CD8+ T cells had a better PFS than those with a low percentage of CD39+CD103+CD8+ T cells. The percentage of CD39+ CD8+ T cells and advanced differentiated CD8+T cells significantly increased after 1 month of standard treatment in patients with no metastasis and there was a significant

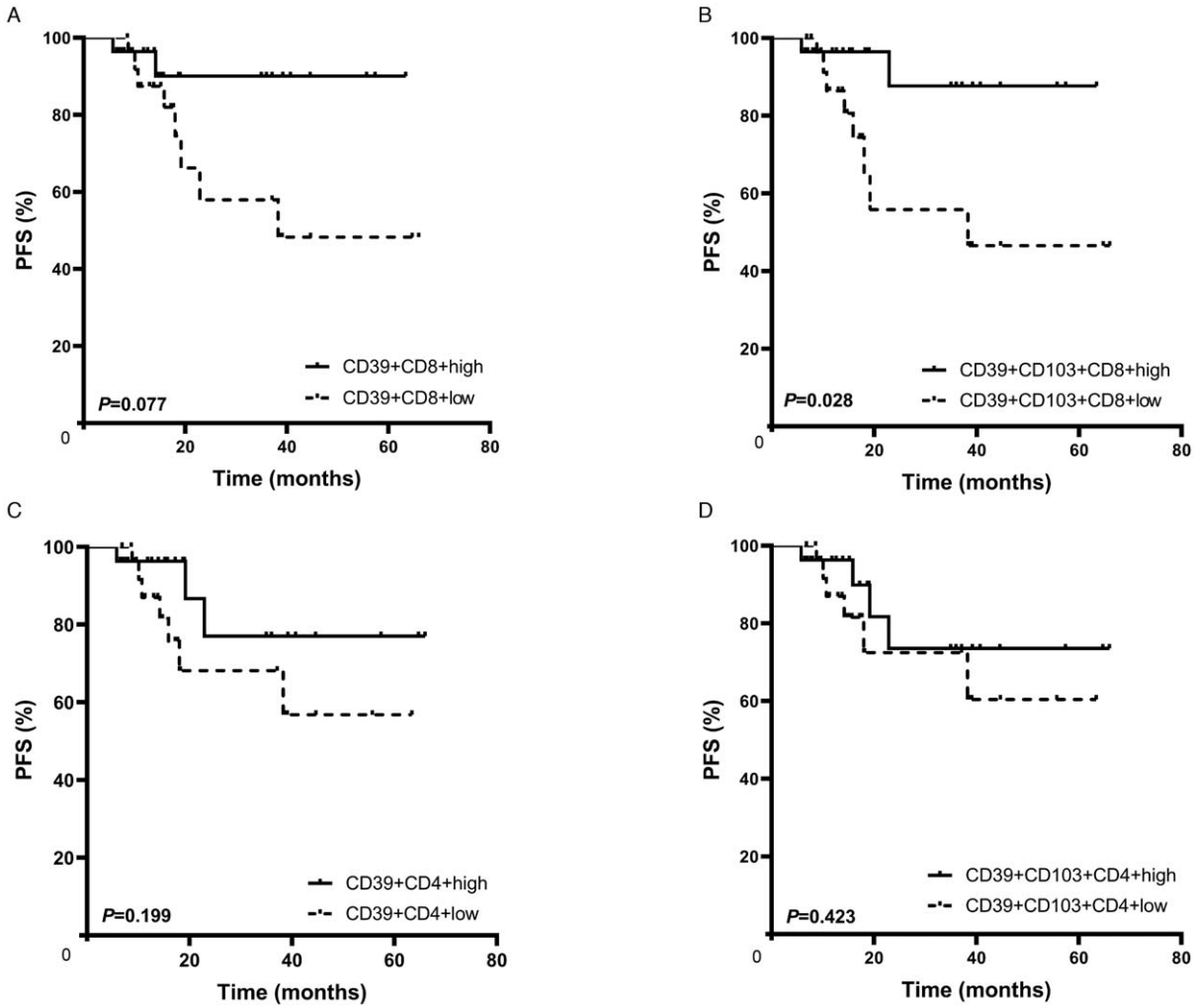

Figure 2: Kaplan-Meier analysis of PFS based on different expression levels of CD39+CD8+ T cells (A), CD39+CD103+CD8+ T cells (B), CD39+CD4+ T cells (C), and CD39+CD103+CD4+ T cells (D). PFS: Progression-free survival.

Table 2: Univariate and multivariate Cox regression analysis of possible predictors for progression in NPC patients.

	Univariate			Multivariate		
Variables	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age	0.987	0.941–1.034	0.574			
Gender	1.240	0.319–4.822	0.756			
Pathology	2.249	0.476–10.631	0.306			
T stage	1.541	0.623–3.810	0.350			
N stage	8.922	2.311–34.528	0.002	12.187	2.561–57.995	0.002
TNM stage	3.883	0.993–15.183	0.051			
CD39+CD8+	0.271	0.057–1.280	0.099			
CD39+CD103+CD8+	0.205	0.043–0.972	0.046	0.147	0.028–0.781	0.024

HR: Hazard ratio; CI: Confidence interval; N: Lymph node; NPC: Nasopharyngeal carcinoma; T: Tumor; TNM: Tumor, node, metastasis.

correlation between elevated CD39+CD8+ T cells and increased effector memory T cells.

Chemotherapy and/or radiotherapy are thought to have the capacity to generate immunogenic cancer antigens in

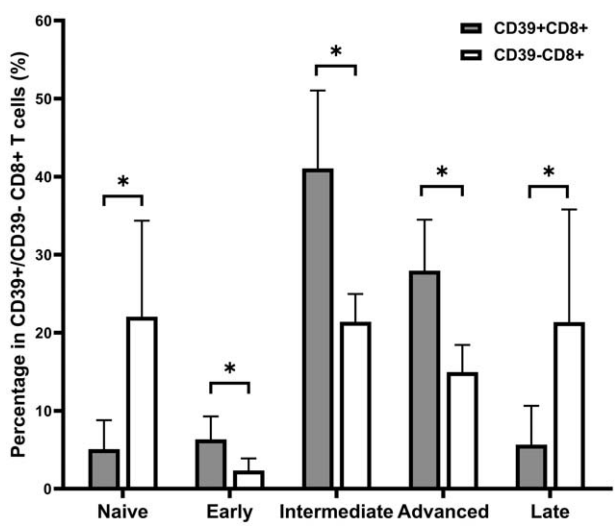

Figure 3: Percentages of naïve, early, intermediate, advanced, and late differentiation phenotypes in CD39+/CD39- CD8+ T cells before treatment. $^{*}P < 0.001$.

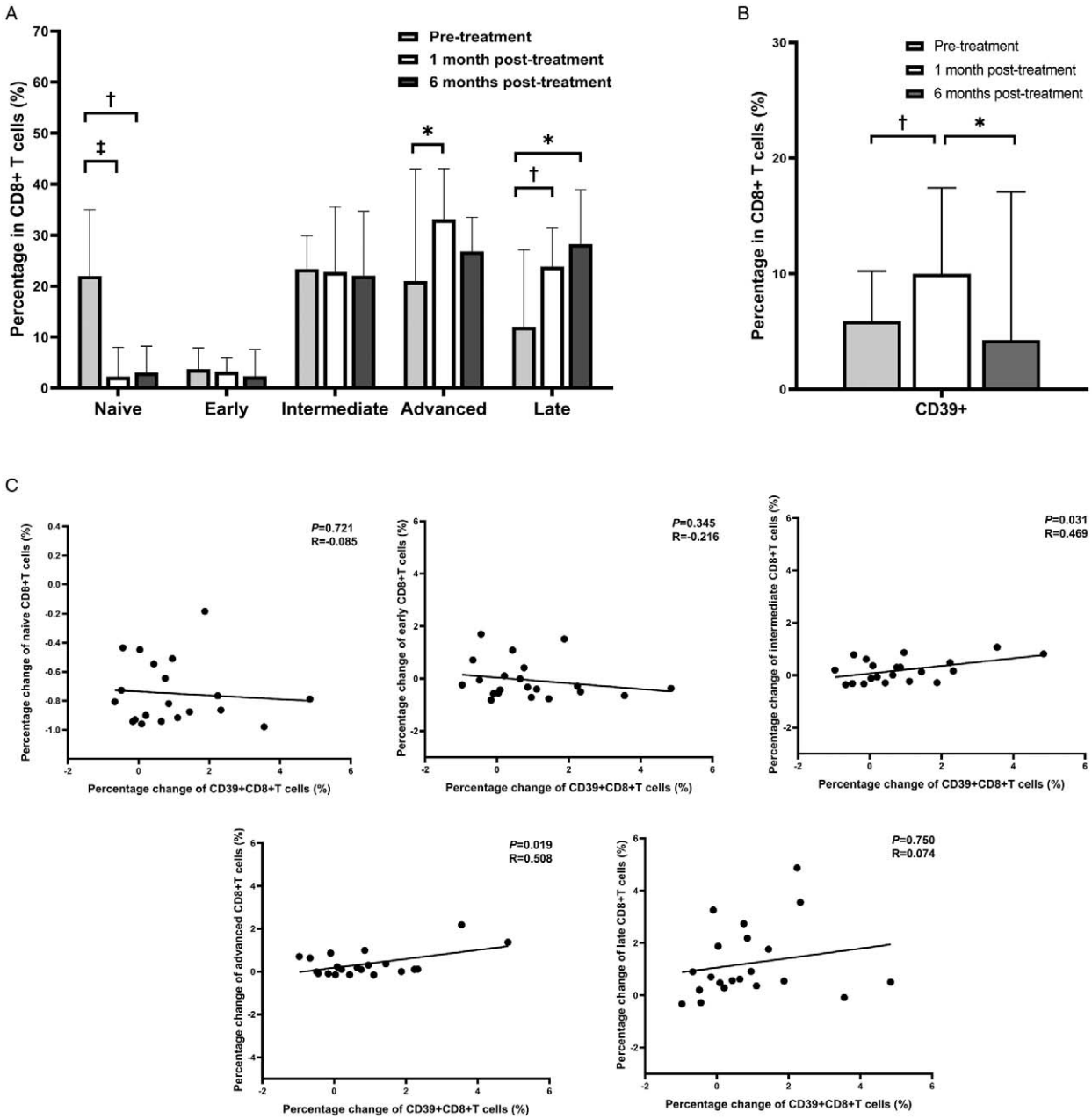

Figure 4: Changes of different differentiation statuses of CD8+ T cells and CD39+CD8+ T cells before treatment and 1 month and 6 months after treatment. (A) Percentages of naïve, early, intermediate, advanced, and late differentiation phenotypes in CD8+ T cells before treatment and 1 month and 6 months after treatment. (B) Percentages of CD39+ cells in CD8+ T cells before treatment and 1 month and 6 months after treatment. (C) Correlation between the changes of different differentiation statuses of CD8+ T cells and CD39+CD8+ T cells before treatment and 1 month after treatment ($n = 21$). $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.

dying tumor cells through the induction of immunogenic cell death (ICD).[15] The observed activation of CD39+ CD8+ T cells post-chemoradiotherapy could be explained by increased antigenic stimulation as a result of chemo-radiotherapy, and the induction of ICD together with tumor antigen cross-presentation.[16]

Effector memory $CD8^{+}$ T cells exhibit remarkable phenotypic and functional heterogeneity. The heterogeneity of such responses (quality) has important implications for whether they are protective or not. The quality of the T cell response is known to be important in efficient control of cancer development with the most efficacious and polyfunctional T cells producing high levels of cytokines on a per-cell basis.[17] Such cells are CCR7– with an effector memory phenotype, at an intermediate/advanced but not terminally differentiated stage. Importantly, these cells are optimized to carry out effector functions such as specific cytokine productions, proliferative capacity, and ability to eliminate cells carrying "foreign" antigens (such as virus infected or cancer cells).[14] Correlation between effector T cells and the better treatment efficacy has been reported in several studies.[18-20] A study in advanced pancreatic cancer revealed that poorer clinical response was correlated with higher level of CD8+ naïve/memory ratio after the chemotherapy, and patients with a lower level of CD8+ naïve/memory ratio had longer PFS.[18] Another study reported that the better tumor response of

lung cancer a month after radiotherapy correlated with high levels of peripheral memory CD8+ T cells.[19]

It is reported that CD39+CD103+ tumor-infiltrating CD8+ T cells were enriched for tumor-reactive cells both in primary and metastatic tumors, those cells could efficiently kill autologous tumor cells in a major histocompatibility complex (MHC)-class I-dependent manner, and higher percentage of CD39+CD103+CD8+ tumor-infiltrating T cells in patients with head and neck cancers was associated with better overall survival.[10] However, no study so far evaluated the role of these cell populations in head and neck cancer metastasis and treatment outcome. Our results indicated that a lack of distant metastasis was correlated with higher expression of effector memory T cells without terminal differentiation in patients with late-stage NPC post-chemoradiotherapy. CD39+CD8+ T cells were also significantly elevated at 1-month post-treatment. There was a significant correlation between elevated CD39+CD8+ T cells and increased effector memory T cells. This suggests that CD39+CD8+ T cells are stimulated and expanded during treatment, with preserved polyfunctional properties. Importantly, these cells do not co-express the exhaustion/inhibitory molecules PD-1 and Tim-3, indicating that they are high-quality functional effector cells able to kill cancer cells effectively, and are therefore likely to be a major contributor to the good control of metastasis in NPC patients post-chemoradiotherapy.

There were several limitations to this study. The sample size for patients with metastasis after treatment was relatively small, and the follow-up period was short. Therefore, it is necessary to continue to expand the sample size as well as extending follow-up period in subsequent studies.

In conclusion, the results of this study support the potential of circulating CD39+CD8+ T cells as prognosis markers for chemoradiotherapy and their potential role in protecting NPC patients from distant metastasis post-chemoradiotherapy, which merits further investigation. The data also suggest that these cells may have potential for future adoptive immunotherapy strategies in patients with NPC at high risk of metastasis.

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Conflicts of interest

None.

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