Original Article

Preoperative Peripheral Naïve / Memory Ratio and Prognosis of Nonsmall-Cell Lung Cancer Patients

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Purpose: This study focused on the relationship between preoperative peripheral blood CD4$^+$ naïve/memory ratio and prognosis following surgery for patients with nonsmall-cell lung cancer.

Methods: After measuring CD3, CD4, CD8, CD45RA, CD45RO, CD25, and intracellular CTLA4 levels, CD4$^+$ naïve/memory ratios were determined for 13 controls and 38 patients.

Results: Although we observed no significant difference in the ratios, the patients exhibited a wider range of values than the controls. Of the 38 patients, 24 subsequently underwent surgery and were divided into three groups based on their preoperative CD4$^+$ naïve/memory ratio: Group I: <0.3; Group II: 0.3–0.8; and Group III: >0.8. Survival times were then evaluated. Group II survival was significantly better than Groups I and III.

Conclusion: Our data suggest that the preoperative CD4$^+$ naïve/memory ratio may serve as a prognostic factor for nonsmall-cell lung cancer patients. (Ann Thorac Cardiovasc Surg 2007; 13: 384–390)

Key words: CD4, CD45RA, CD45RO, nonsmall-cell lung cancer, prognosis

Introduction

Tumor cells have tumor-specific antigens that are capable of eliciting host immune response.[1)] When presented in the context of a major histocompatibility (MHC) antigen class I complex either on the tumor cell itself or on antigen-presenting cells, these antigens are capable of inducing tumor-specific cytotoxic T lymphocytes.[2,3)] Recently, tumor antigens presented by MHC class II molecules and recognized by CD4$^+$ T cells have also been identified.[4,5)] Thus the antitumor response appears to be mediated not only by CD8$^+$ T lymphocytes, which are directly cytotoxic, but also by CD4$^+$ T cells, which help CD8$^+$ T cells augment the immune response, e.g., with the secretion of cytokines and/or with CD40-CD40L interactions.[6–8)] In many immunocompetent cases, however, tumor growth occurs with an inadequate immune response.

The T cell population is a heterogeneic group of cells. The intracellular domain of CD45 is a haemopoietic cell–specific tyrosine phosphatase that is essential for efficient T and B cell antigen receptor signal transduction.[9,10)] Multiple CD45 isoforms can be generated by complex alternative splicing of the exons 4(A), 5(B), and 6(C) in the extracellular domain of the molecule.[11,12)] The expression of different CD45 isoforms is cell-type specific and depends on the stage of differentiation and on the state of activation of the cells. In humans, CD45RA and CD45RO are thought to be naïve and memory T cells, respectively.[13)]

The function of the CD45 isoform extracellular domain, however, remains obscure. Importantly, human CD45 polymorphic variants that alter CD45 isoform expression are associated with autoimmune and infectious diseases. One study indicates that CD45 may be a valuable immunomodulator with a significant influence on disease load.[12)] In our study, we focused on the relationship between the preoperative peripheral blood CD4$^+$

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naïve/memory ratio and prognosis following curative surgery in patients with nonsmall-cell lung cancer (NSCLC).

Methods

NSCLC patients and controls

Peripheral blood samples were obtained from 38 NSCLC patients (25 men, 13 women) from June 2001 to April 2002 at the University of Miyazaki Hospital prior to surgery, radiotherapy, or chemotherapy. Of these, 24 NSCLC patients (15 men, 9 women) subsequently underwent surgery, and survival times were then evaluated. Control samples (n=13) were obtained from normal healthy volunteers and from patients undergoing surgery for benign diseases (pneumothorax, thymic cyst). Patients with inflammatory diseases were excluded from this study. Written consent was obtained from patients and controls prior to blood being drawn.

Fluorescence activated cell sorting (FACS) reagents

The following mAbs were used for flow cytometry: UCHT1 (anti-CD3) PE, RPA-T4 (anti-CD4) APC, SK1 (anti-CD8) PerCP, M-A251 (anti-CD25) FITC, HI100 (anti-CD45RA) FITC, UCHL1 (anti-CD45RO) PE, and BNI3 (anti-CD152) PE. Isotype controls were purchased from BD Bioscience (San Diego, CA, USA).

Isolation of peripheral blood mononuclear cells (PBMC), immunofluorescence labeling, and FACS analysis

Heparinized blood was centrifuged using the Ficoll-Hystopaque gradient (Sigma, St. Louis, MO, USA). Leukocyte containing interface was collected and washed twice in cold phosphate buffered saline (PBS) supplemented with 1% fetal calf serum (FCS) (PBS/FCS) and 0.02% sodium azide (Sigma). The cells were then incubated with PBS/FCS containing anti-CD4-APC, anti-CD8-PerCP, and several panels of FITC/PE directly conjugated mAbs for 30 min at 4°C. The cells were then washed twice, resuspended in PBS containing 2% v/v formaldehyde, and stored in darkness at 4°C until acquisition. To prepare the cells for intracellular staining, we washed them again in PBS and then incubated them with the permeabilization buffer PBS/1%FCS/0.5% saponin (Sigma) for 30 min at 4°C. They were then incubated with anti-CD152-PE or isotype control-PE mAbs for 30 min at 4°C. After two washes with permeabilization buffer, the cells were then washed with PBS/FCS. Data were acquired with the FACSCalibur flow cytometer and analyzed using the CellQuest software package (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

All data are expressed as mean ± SD. The unpaired Student t-test or ANOVA test was used for statistical analysis. Categorical data were compared using the χ^2 test. Survival was evaluated by the Kaplan-Meier method, and differences among survival curves were tested with the log-rank test. For all tests, a probability value (p) of <0.05 was considered significant.

Results

Distribution of $CD4^+$ and $CD8^+$ T-cell subsets

Table 1 summarizes the clinicopathological characteristics of 38 NSCLC patients (LC Group) and 13 controls (C Group). There was no significant difference between patient and control groups with respect to gender (p=0.8203). The mean age of the controls was younger than that of the lung cancer patients ($p<0.01$).

The heterogeneity of the peripheral T cell pool is illustrated in Fig. 1, which shows the distribution of naïve and memory T cells based on the surface expression of CD45RA (naïve) and CD45RO (memory) isoforms in patients and controls. Percentages for naïve T cells, memory T cells, and naïve/memory ratios are displayed in Fig. 2. The mean $CD4^+$ for naïve T cells was 29.9% (11.9–38.5%) for controls and 25.5% (2.4–57.8%) for patients. $CD4^+$ memory T cells represented 56.1% (44.1–81.7%) of circulating $CD4^+$ cells in controls and 57.7% (26.9–92.8%) in patients. The $CD4^+$ naïve/memory ratio was 0.56 (0.15–0.79) for controls and 0.56 (0.03–2.15) for patients. Although we observed no significant differences in the percentage of $CD4^+$ naïve T cells (p=0.2791) or $CD4^+$ memory T cells (p=0.7494) and none in the $CD4^+$ naïve/memory ratio (p=0.9991) between the two groups, the ranges for the lung cancer patients were clearly wider than the controls in all categories.

Furthermore, we found no significant difference in the percentage of $CD8^+$ naïve T cells (p=0.6347) or $CD8^+$ memory T cells (p=0.9224) and also none in the $CD8^+$ naïve/memory ratio (p=0.9485) between patients and controls (Fig. 2). Unlike the $CD4^+$ T cells, however, the ranges within the three categories were relatively similar between patients and controls. The mean $CD8^+$ for naïve T cells was 37.9% (18.5–54.1%) for controls and 35.9% (14.8–58.9%) for patients. $CD8^+$ memory T cells represented 43.6% (18.5–70.4%) of circulating $CD8^+$ cells in the con-

Table 1. Clinicopathological parameters of NSCLC patients and controls

Group		C group (n=13)	LC group (n=38)
Sex (M/F)		9/4	25/13
Age (years)		46.5±19.7	67.2±10.8
Stage	I		24
	II		3
	III		7
	IV		4
Histology	Ad		22
	Sq		13
	LC		3

M, male; F, female.

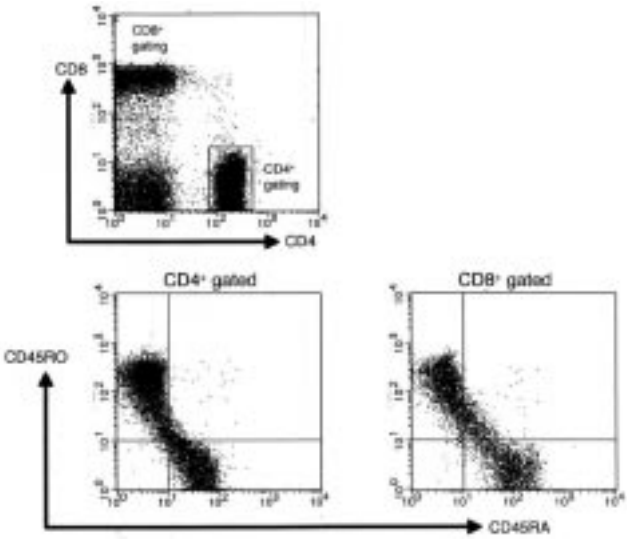

Fig. 1. Flow cytometry profiles of CD45RA and CD45RO. Isolated PBMC labeled with anti-CD4, anti-CD8, anti-CD45RA, and anti-CD45RO conjugated with APC, Per-CP, FITC, and PE, respectively.

trols and 47.5% (8.9-68.3%) in the patients. The $CD8^+$ naïve/memory ratio was 0.56 (0.15–0.79) for controls and 0.56 (0.03–2.15) for patients.

We next evaluated the influence of cancer stage, tumor size, and patient age on the heterogeneity of the peripheral T cell pool. As shown in Fig. 3, we found no significant difference in the percentage of $CD4^+$ naïve T cells (p=0.5281) or in $CD4^+$ naïve/memory ratio (p=0.5850) in the individual cancer stages. We also found no significant difference with regard to the percentage of $CD4^+$ memory T cells, $CD8^+$ naïve T cells, $CD8^+$ memory T cells, and $CD8^+$ naïve/memory ratio in the individual cancer stages (data not shown). As further seen in Fig. 3, we found no correlation between tumor size and percentage of $CD4^+$ naïve T cells (r=–0.012, p=0.9450). The frequency of $CD4^+$ naïve T cells decreased with age, but was not significant in the patient group (r=–0.249, p=0.1318).

$CD4^+$ naïve/memory ratio and postoperative survival

Although there was no significant difference in the $CD4^+$ naïve/memory ratios between the two groups, the range of values in the patients was wider than in the controls (Fig. 2). Although the ratios of all but one of the controls were from 0.32 to 0.79, patient values ranged from 0.03 to 2.15. Using control ratio data, we established three groups: Group I: <0.3; Group II: 0.3–0.8; and Group III: >0.8. To evaluate the relationship between $CD4^+$ naïve/memory ratio and postoperative survival, we assigned to these groups 24 NSCLC patients who had undergone surgery based on their $CD4^+$ naïve/memory ratio, and survival times were evaluated.

There was no significant difference with regard to gender, age, tumor size, cancer stage, or histology among the three groups (Table 2). As seen in Table 3, we found no significant difference in the PBMC percentage of $CD3^+$, $CD4^+$, $CD4^+CD25^{high}$, and $CD8^+$ cells among the three NSCLC groups. The total cell number in Group II, however, was significantly higher than that in Group I (p=0.0005) and Group III (p=0.0034). It is interesting that in Table 3 we see that the intracellular $CD152^+$ expression in $CD4^+$ cells in Group III was significantly higher than in Group I (p=0.0008) or Group II (p=0.0027).

Finally, with regard to disease-specific survival rates among the three groups and as shown in Fig. 4, we see that the overall survival of Group II was significantly better than Group I (p<0.05) or Group III (p<0.05). There was no difference in survival between Groups I and III.

Discussion

With regard to the analysis of survival predictors, some studies have focused on the correlation between CD45R and the malignancy state based on the relationship between isoform expression and the functional status of T cells. Tosi et al. reported that high tumor infiltrating $CD45RO^+$ lymphocytes were associated with improved prognosis for patients with lung cancer.[14] Furthermore, Takenoyama et al. showed that regional lymph node lymphocytes in primary lung cancer patients contained a significantly higher percentage of $CD45RO^+$ T cells and a

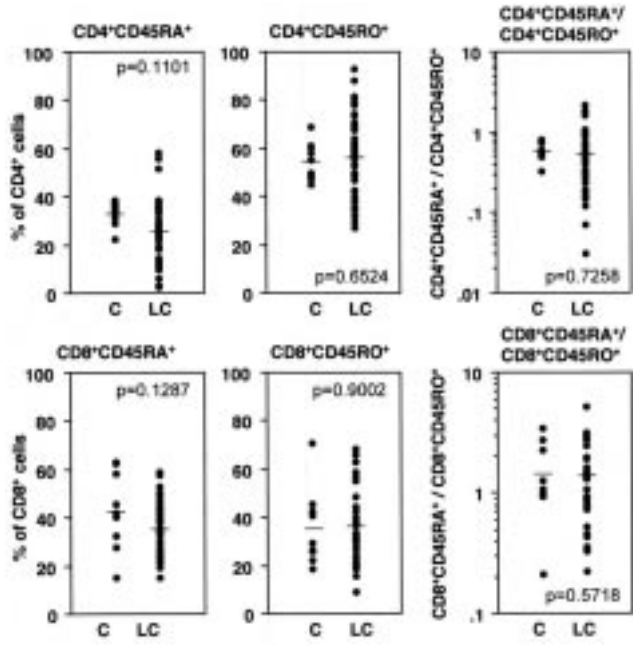

Fig. 2. Dotted plot showing percentage of CD45RA$^+$ (naïve) cells, CD45RO$^+$ (memory) cells, and the naïve/memory ratio in CD4$^+$ T cells (upper panels) and CD8$^+$ T cells (lower panels) in controls (C) and NSCLC patients (LC).
Horizontal bars are means.

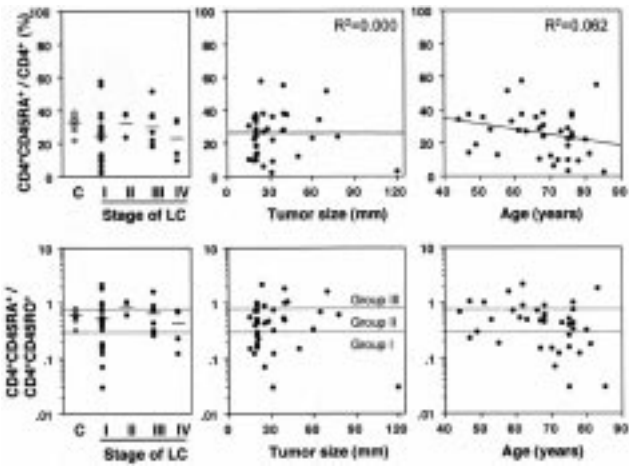

Fig. 3. Correlation of cancer stage, tumor size, and patient age with percentage of CD45RA$^+$ cells in CD4$^+$ T cells and with the CD4$^+$ naïve/memory ratio in PBMC of NSCLC patients and controls.
Horizontal bars are means.

Table 2. Clinicopathological parameters of NSCLC patient groups included in the survival study

Group		I (n=8)	II (n=8)	III (n=8)	p
Sex (M/F)		5/3	7/1	3/5	NS
Age (years)		72.0±8.8	64.3±9.2	63.4±12.1	NS
Size (mm)		39.1±34.4	29.6±14.2	35.1±16.9	NS
Stage	I	7	7	4	NS
	II	0	0	2	
	III	1	1	2	
Histology	Ad	6	5	4	NS
	Sq	2	3	2	
	LC	0	0	2	

M, male; F, female; NS, not significant.

lower percentage of CD45RA$^+$ T cells in comparison to the corresponding peripheral blood lymphocytes.[15] Studies on the relationship between peripheral CD45R and cancer prognosis, however, have not been reported. To our knowledge, this study is the first report to analyze the prognostic influence of preoperative peripheral blood CD4$^+$ naïve/memory ratio in NSCLC patients.

Studies have shown that the percentage of CD45RA$^+$ T cells in immunologically naïve healthy newborns is >90%; it decreases to 60% in childhood, and decreases further to 40% in adults.[16,17] Furthermore, De Paoli et al. report that the absolute number of CD4$^+$CD45RA$^+$ cells in adults decreases gradually with age.[16] In our study, however, the range of CD4$^+$ naïve and CD4$^+$ memory T cells in lung cancer patients was much wider when compared with control data (Fig. 2). There are several possible explanations for these results. Since the general consensus is that the percentage of CD4$^+$ naïve and CD4$^+$ memory T cells is maintained by a homeostasis mechanism, our results may be due to a failure of the homeostatic mechanism in lung cancer patients.

A second possibility is based on two studies that reported on the importance of the thymus in reconstituting naïve T-helper cells. Heitger et al. reported the requirement of the thymus for reconstituting CD4$^+$CD45RA$^+$ T cells,[18] and Kuss et al. found that the number of peripheral recent thymic emigrant T cells was significantly lower in head and neck cancer patients than in the control group.[19] Thus the dysfunction of the thymus may account for our results.

Another possible explanation is the existence of CD45 polymorphisms.[12] Several studies have reported that human CD45 alleles have several polymorphisms, located primarily in the variably spliced exons 4, 5, and 6.[20–22] The exon 6 A138G allele is found at a frequency of approximately 20% in Japanese samples.[21] A138G carriers have more T cells capable of producing IFN-γ following PMA and ionomycin stimulation in vivo.[23] The high frequency of the allele suggests that it might confer a selective advantage, and indeed, it shows a protective effect in

Table 3. Phenotypic profile of NSCLC patient groups included in the survival study

Group	I	II	III	*p*
Cell count ($\times 10^5$/mL)	13.0±3.7	21.8±6.3	14.7±3.0	<0.01
$CD3^+$ (%)	41.8±13.3	49.3±8.3	53.0±9.6	NS
$CD4^+$ (%)	25.7±9.7	31.7±7.3	36.4±7.8	NS
$CD4^+CD25^{high}$ (% of $CD4^+$)	10.4±4.4	11.2±5.1	10.2±2.1	NS
$CD4^+CD152^+$ (a% of $CD4^+$)	16.1±6.1	9.8±1.9	8.3±3.1	<0.05
$CD8^+$	14.4±6.3	14.8±4.0	14.7±4.1	NS

NS, not significant.

hepatitis B virus infection and autoimmune Graves' thyroiditis.[23] There is no evidence, however, that a high frequency of the A138G allele exhibits a protective effect in cancer production. On average, A138G carriers have 20% more $CD8^+CD45RO^+$ and 10% more $CD4^+CD45RO^+$ cells, but because percentages of CD45RO cells vary in normal individuals, they can be reliably detected only by the use of molecular methods. Further study is necessary to determine the exact relationship between the naïve/memory ratio and polymorphisms.

One additional factor that is vital for the survival of naïve $CD4^+$ T cells is cytokines. The IL-7 receptor is expressed on resting $CD4^+$ and $CD8^+$ T cells and is responsible for providing signals that promote survival of the resting cells, including upregulation of the Bcl-2 expression.[24] It is important to note that the level of IL-7 in the serum of older people has been shown to significantly reduce as compared to younger individuals.[25] Moreover, a direct correlation between CD45 expression and IL-6 dependency has been found.[26]

Several explanations for the failure by the body to eliminate tumor cells have been put forth. In one study, Foss discusses suppressive cytokines, loss of surface MHC class I expression, and lack of costimulatory molecules.[27] Stephens and Mason.[28] and Baecher-Allan et al.[29] attribute the failure to the existence of $CD4^+CD25^{high}$ regulatory T cells (Treg), and some researchers have reported an increase in the prevalence of Treg in the peripheral blood of cancer patients.[30,31] Since most Treg expresses CD45RO,[32] we speculated that its presence might result in a poor prognosis for cancer patients. In our study, however, we found no significant difference in the number of $CD4^+CD25^{high}$ cells among the three NSCLC groups (Table 3).

Other studies have shown that CTLA-4 is essential for the in vivo suppressive activity of Treg[33,34] and that anti-CTLA-4 therapy induced tumor regression in mice[35] and humans.[36] In our study we observed that the intracellular

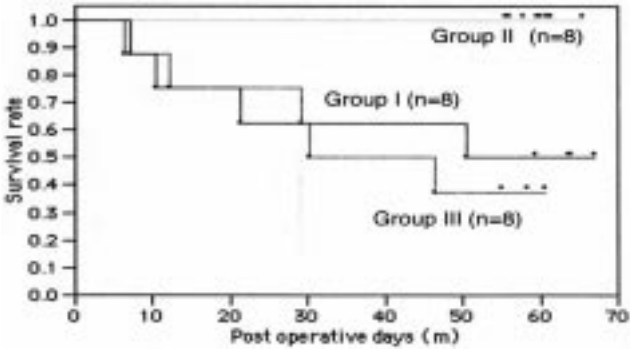

Fig. 4. Overall survival based on preoperative $CD4^+$ naïve/memory ratio in PBMC.

CD152 expression in $CD4^+$ cells in Group III was significantly higher than in Group I or II (Table 3). This result suggests that high CTLA-4 expression in the peripheral blood $CD4^+CD45RO^+$ enriched group may suppress tumor rejection and contribute to a poor prognosis.

Since most malignant cells express MHC class I but not class II molecules on the surface, $CD8^+$ T cells are believed to play an important role in attacking malignant cells. In our study, however, we observed no significant difference between controls and patients with regard to $CD8^+$ naïve/memory T cells (Fig. 2), confirming an earlier study by Prado-Garcia et al.[37] Furthermore, the preoperative peripheral blood $CD8^+$ naïve/memory ratio in NSCLC patients did not influence the prognosis in this study (data not shown).

Our data suggest that the preoperative $CD4^+$ naïve/memory ratio may be a prognostic factor for NSCLC patients and that the characterization of the role of the immune system in tumor control may have implications on cancer treatment in general.

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