RESEARCH ARTICLE

Tim-3 Expression by Peripheral Natural Killer Cells and Natural Killer T Cells Increases in Patients with Lung Cancer - Reduction after Surgical Resection

Li-Yun Xu[1], Dong-Dong Chen[1], Jian-Ying He[1], Chang-Chang Lu[2], Xiao-Guang Liu[1], Han-Bo Le[3], Chao-Ye Wang[3], Yong-Kui Zhang[2]*

Abstract

Background: The purpose of this study was to investigate Tim-3 expression on peripheral CD3-CD56+ natural killer (NK) cells and CD3+CD56+ natural killer T (NKT) cells in lung cancer patients. Materials and Methods: We analyzed Tim-3+CD3-CD56+ cells, Tim-3+CD3-CD56^{dim} cells, Tim-3+CD3-CD56^{bright} cells, and Tim-3+CD3+CD56+ cells in fresh peripheral blood from 79 lung cancer cases preoperatively and 53 healthy controls by flow cytometry. Postoperative blood samples were also analyzed from 21 members of the lung cancer patient cohort. Results: It was showed that expression of Tim-3 was significantly increased on CD3-CD56+ cells, CD3-CD56^{dim} cells and CD3+CD56+ cells in lung cancer patients as compared to healthy controls (p=0.03, p=0.03 and p=0.04, respectively). When analyzing Tim-3 expression with cancer progression, results revealed more elevated Tim-3 expression in CD3-CD56+ cells, CD3-CD56^{dim} cells and CD3+CD56+ cells in cases with advanced stages (III/IV) than those with stage I and II (p=0.02, p=0.04 and p=0.01, respectively). In addition, Tim-3 expression was significantly reduced on after surgical resection of the primary tumor (p<0.01). Conclusions: Tim-3 expression in natural killer cells from fresh peripheral blood may provide a useful indicator of disease progression of lung cancer. Furthermore, it was indicated that Tim-3 might be as a therapeutic target.

Keywords: Lung cancer - flow cytometry - natural killer cells - T cell immunoglobulin - mucin domain 3

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Introduction

Lung cancer is one of the most common and deadly malignant tumors worldwide, with high mortality rate, and still remaining a growing trend every year (Liu et al., 2013; Lu et al., 2013). Studies have shown that the development and progression of lung cancer are closely related with immunological dysfunction, especially cell-mediated immunity (Wang et al., 2013). Natural killer (NK) cells and natural killer T (NKT) cells are critical components of cellular immunity that are the first line of defense against chronic viral infection and tumor evasion, and involved in the amplification of the immune response to pathogens and cancer cells. In recent years, studies have demonstrated that T cell immunoglobulin and mucin domain 3 (Tim-3) is closely associated with dysregulation of innate immune cells, including macrophages/monocytes (Yang et al., 2013), dendritic cells (Chiba et al., 2012), and NK cells (Gleason et al., 2012). Abnormal expression of Tim-3 on these innate immune cells was found to be involved in the progression of many clinical diseases (Wu et al., 2012; Anderson et al., 2012; da Silva et al., 2014; Meggyes et al., 2014). However, it was not reported the relationship between Tim-3 expression on NK cells and NKT cells and lung cancer. This study is designed to examine the expression of Tim-3 on peripheral NK cells and NKT cells in lung cancer patients using flow cytometry, and to analyze the relationship between disease progression and Tim-3 expression. Furthermore, Tim-3 expression was also analyzed after surgery in a consecutive subset of patients to determine the effect of tumor debulking.

Materials and Methods

Human subjects and blood sample preparation

The study group included 79 lung cancer patients and 53 healthy volunteer donors from Zhoushan Hospital. The diagnosis of lung cancer was confirmed by histopathological examination. The healthy population was recruited from people who came for general health examination, and they were confirmed to be without any malignant disease and other diseases. Blood samples were obtained from patients with lung tumors before surgery or healthy volunteer donors and processed within 6 hours of collection. Postoperative sample collection was added late in the study, and these samples were collected consecutively. The postoperative sample stage distribution is 8 patients with I/II, and 13 patients with stage III/IV.

*[1]Cell and Molecular Biology Laboratory, [2]Lung Cancer Research Center of Zhoushan, [3]Department of Cardio-Thoracic Surgery, Zhoushan Hospital, Zhoushan, Zhejiang, China *For correspondence: zhoushanzhang1@126.com*

Staining and flow cytometric analysis

Every 50 μl peripheral blood sample was incubated for 30 min in 4°C in a dark room with 5 μl monoclonal antibodies or isotype matched control. Allophycocyanin (APC)-conjugated anti-human Tim-3 (Catalog Number. FAB2365A, Clone Number. 344823, R&D, Minneapolis, MN), APC-conjugated rat IgG2a Isotype control (Cat Number. IC006A, Clone Number. 54447, R&D, Minneapolis, MN), fluorescein isothiocyanate (FITC)-conjugated anti-human CD56 (Cat Number. 11-0569, Clone Number. MEM188, eBioscience, USA), FITC-conjugated anti-human Mouse IgG2a κ Isotype control (Cat Number. 11-4724, Clone Number. eBM2a, eBioscience, USA), phycoerythrin (PE)-conjugated anti-human CD3 (Cat Number. 12-0039, Clone Number. HIT3a, eBioscience, USA), and PE-conjugated anti-human Mouse IgG2a κ Isotype control (Cat Number. 12-4724, Clone Number. eBM2a, eBioscience, USA) were used for flow cytometric analysis according to the manufacture's instructions. 50 μl peripheral blood sample was submitted to 2 ml Red Blood Cell lysis using 1:10 FACS lysis solution (BD, Biosciences, San Jose, CA) and incubated for 10 min at room temperature. Centrifugation was set at 1200 rpm/min for 5 min and washed for three times with phosphate buffer saline (PBS). Then 500 μl PBS was added. At least 30, 000 cells were analyzed using a BD FACSCalibur™ Flow Cytometer (BD Biosciences) and the data were analyzed using the FlowJo software (TreeStar Inc, Ashland, OR, USA).

Statistical analysis

All data were analyzed using the GraphPad Prism 5 (GraphPad Software, Inc.). The student t-test and a paired Wilcoxon rank-sum test were used for comparison. Value of $p<0.05$ is considered as a significant difference.

Results

Characteristics of the study population

The distribution of selected characteristics of the cases and healthy controls are presented in Table 1. 79 lung cancer cases and 53 healthy controls did not reveal any significant difference in age ($p>0.05$), gender ($p>0.05$) and smoking history ($p>0.05$). Among 79 lung cancer patients, 40 (50.6%) cases were size of 0~3 cm and 39 (49.4%) were size of >3 cm. The histological type of 52 (65.8%) patients was adenocarcinoma, whereas squamous cell carcinoma accounted for 27 (34.2%). The Union for International Cancer Control/American Joint Committee on Cancer TNM Classification (7th ed) was also shown, in which 37 (46.8%) were in stage I ~ II and 42 (53.2%) were in stage III~IV.

Tim-3 expressions on peripheral CD3-CD56+ NK cells and CD3+CD56+ NKT cells in lung cancer

Although Tim-3 was first cloned in differentiated Th1 cells, studies have indicated that Tim-3 expression on various immune cells, such as CD8+ T cells, NK cells, NKT cells, which play roles in the development of diseases. We investigated Tim-3 expression on NK cells and NKT cells from peripheral blood of 79 lung cancer cases and 53 healthy controls. The gating strategy to distinguish the various lymphocyte populations is shown in Figure 1A. Increased proportion of Tim-3+ cells was detected on CD3-CD56+ cells in lung cancer patients than in healthy controls (73.8%±9.01% *vs* 70.4%±7.47%, $p=0.03$, Figure 1B). When NK cells were subtyped based on CD56 expression levels, the proportion of Tim-3+ cells in CD3-CD56^{dim} cells was elevated in lung cancer cases than in controls (75.3%±7.63% *vs* 72.4%±7.68%, $p=0.03$, Figure 1C). However, Tim-3 expression was not increased in CD3-CD56^{bright} cells in cases compared to controls (58.8% ± 13.1% *vs* 58.2% ± 6.43%, $p=0.91$, Figure 1D). Additionally, the proportion of Tim-3+ cells

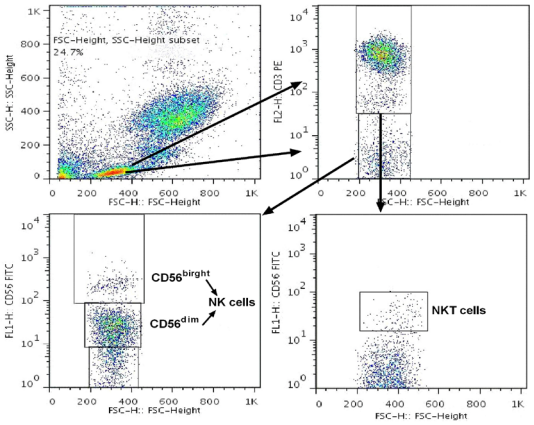

Figure 1. Tim-3 Expression on Peripheral CD3-CD56+ NK Cells and CD3+CD56+ NKT Cells in Lung Cancer. **A)** Representative dot plots of peripheral blood from one patient showing the gating strategy for the various lymphocyte populations; **B, C, D, E)** show the percentage of Tim-3+CD3-CD56+ cells, Tim-3+CD3-CD56^{dim} cells, Tim-3+CD3-CD56^{bright} cells and Tim-3+CD3+CD56+ cells (means ± SD) compared between lung cancer patients and healthy controls, respectively. Each dot represents one subject. The student t-test was used for comparison between groups. P-Values are shown. Value of $p<0.05$ is considered as a significant difference

Table 1. General Characteristics of the Subjects

	Lung Cancer (n=79)	Healthy Control (n=53)	*P* value
Age (years old)			NS
<60	34 (43.0%)	22 (41.5%)	
≥60	45 (57.0%)	31 (58.5%)	
Mean Age	63.5±10.5	62.1±12.8	NS
Gender			NS
Male	32 (40.5%)	20 (37.7%)	
Female	47 (59.5%)	33 (62.1%)	
Smoking History			NS
Yes	50 (63.2%)	30 (56.7%)	
No	29 (36.7%)	23 (43.4%)	
Tumor Size			
0~3 cm	40 (50.6%)		
>3 cm	39 (49.4%)		
Histology			
Ad	52 (65.8%)		
Sq	27 (34.2%)		
Stage			
I~II	37 (46.8%)		
III~IV	42 (53.2%)		

*Statistical comparisons were made by using the Student's t-test. Differences were considered significant when the value of P was equal to or less than 0.05. NS = not statistically significant.Ad, Adenocarcinoma; Sq, Squamous cell carcinoma

in CD3+CD56+ cells was also significantly increased in cases compared to controls (51.6%±14.1% *vs* 47.0%±8.32%, p=0.04, Figure 1E). These results indicate that Tim-3 may be involved in the pathogenesis of lung cancer by its regulation on various immune cells.

Tim-3 expressions on peripheral CD3-/CD56+ NK cells and CD3+CD56+ NKT cells in patients with different disease stages

To investigate whether Tim-3 may play roles in the spread of lung cancer, we examined Tim-3 expression with different stages of patients. Results showed that the proportion of Tim-3+CD3-CD56+ cells in patients with high stages (III/IV) was higher than those with low stages (I/II) (76.1%±9.46% *vs* 71.2%±7.94%, p=0.02, Figure 2A). Further analyses showed that Tim-3 expression of CD3-CD56^{dim} cells in cases with high stages (III/IV) was higher than those with low stages (I/II) (77.2%±7.36% *vs* 72.3%±7.14%, p=0.04, Figure 2B), while there was no difference in Tim-3 expression of CD3-CD56^{bright} cells between high stages and low stages (60.3%±14.8% *vs* 57.2%±10.9%, p=0.30, Figure 2C). Moreover, the expression of Tim-3+CD3+CD56+ cells in cases with high stages (III/IV) was higher than those with low stages (I/II) (55.2 ±13.6% *vs* 47.5%±13.8%, p=0.01, Figure 2D). These data suggest that Tim-3 may be used as a useful indicator of disease progression of lung cancer.

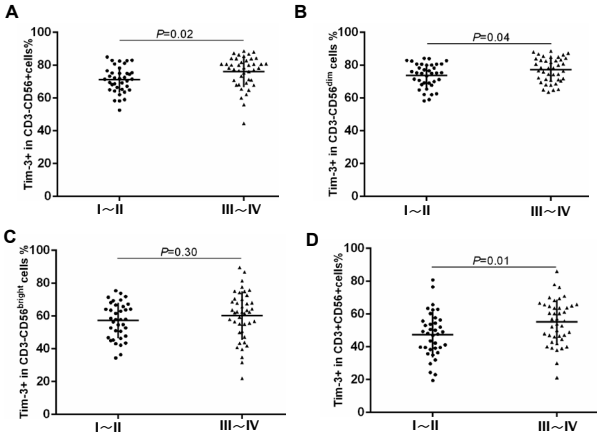

Figure 2. Comparison of Tim-3 Expressions on CD3-CD56+ Cells (A), CD3-CD56^{dim} Cells (B), CD3-CD56^{bright} Cells (C) and CD3+CD56+ cells (D) in Patients with Different Disease Stages. 79 patients were included, in which 37 patients were in stages I or II, and 42 patients were in stages III or IV. The student t-test was used for comparison. P-Values are shown. Value of p<0.05 is considered as a significant difference

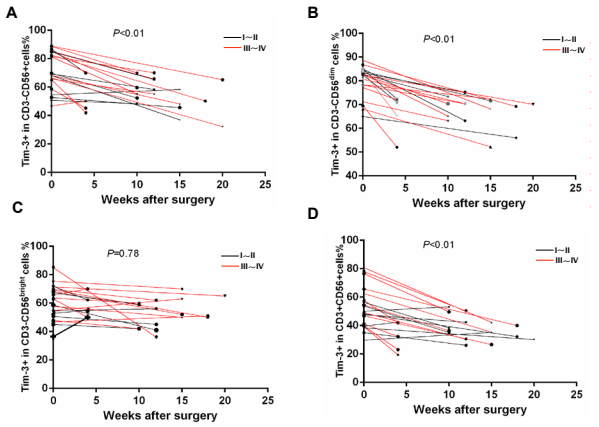

Figure 3. Tim-3 Expressions on CD3-CD56+ Cells (A), CD3-CD56^{dim} Cells (B), CD3-CD56^{bright} cells (C) and CD3+CD56+ Cells (D) after Resective Surgery. Tim-3 expression by cells is shown for 21 lung cancer patients before (0 weeks) and after resective surgery. Data from stage I or II patients are marked with black line and the date from stage III or IV patients are shown in red. A paired Wilcoxon rank-sum test was used to determine the statistical significance. P-Values are shown. Value of p<0.05 is considered as a significant difference

Effects of surgical resection on Tim-3 expression of CD3-CD56+ NK cells and CD3+ CD56+ NKT cells in patients with lung cancer

We examined Tim-3 expression in postsurgical blood samples from 21 of the patients with lung cancers in our cohort within 4 to 20 weeks after surgery, including 8 cases with stage I/II and 13 cases with stage III/IV, which stage were assessed when patients were in hospital and underwent surgery. The results showed that Tim-3 expression on CD3-CD56+ cells, CD3-CD56^{dim} cells, and CD3+CD56+ cells after surgery was significantly lower than those before surgery (CD3-CD56+ cells, p<0.01, Figure 3A; CD3-CD56^{dim} cells, p<0.01, Figure 3B; CD3+CD56+ cells, P < 0.01, Figure 3D). However, there was no difference in Tim-3 expression of CD3-CD56^{bright} cells before and after surgery of lung cancer (p=0.78, Figure 3C). Our data suggest that Tim-3-expressing immune cells are far more prevalent before resection of the primary tumor.

Discussion

Many reports have indicated that the dysfunction of NK cell and NKT cell is closely related to the occurrence and development of lung cancer (Hasegawa et al., 2014; Hodge et al., 2014), but the underlying molecular mechanisms involved in this process remain poorly understood. Tim-3, was originally identified for Th1-specific markers. Since then, it was also been found on CD8+ T cells, Treg, monocytes, dendritic cells, NK cells and NKT cells. Here, to our knowledge for the first time, we demonstrated that Tim-3 expression was significantly increased on CD3-CD56+ NK cells, CD3-CD56^{dim} NK cells and CD3+CD56+ NKT cells in lung cancer patients. In addition, Tim-3 expressions on these cells in cases with high stages (III/IV) were higher than those with low stages (I/II). More importantly, we found that Tim-3 expression on these cells after surgery was significantly lower than those before surgery. These suggest that Tim-3 could be a valuable marker for lung cancer. Further investigations with a larger scale of lung cancer from blood samples and the 5-year survival rate of patients are needed to establish to further confirm the role of Tim-3 as a potential diagnostic marker and a therapeutic target for the treatment of lung cancer.

It has been reported that Tim-3 has a negative regulatory role on the activity of NK cells, and chronic hepatitis B infection could upregulate Tim-3 expression on NK cells and subsequently suppressed NK cell function (Ju et al., 2010). It was also demonstrated that Tim-3

suppressed NK cell-mediated cytotoxicity when Tim-3 encountered target cells (Ndhlovu et al., 2012). NK cells in non-small cell lung cancer (NSCLC) patients is significantly lower than that of healthy controls (Wang et al., 2013), suggesting NK cell function was damaged in patients with lung cancer. Our data showed that Tim-3 expressions in patients with lung cancer were elevated on CD3-CD56+ NK cells, which might be an underlying mechanism for dysfunction of NK cells in lung cancer. When NK cells were subtyped based on CD56 expression levels, we found that Tim-3 expressions in patients with lung cancer were elevated on CD3-CD56^{dim} NK cells while Tim-3 expressions were not changed on CD3-CD56^{bright} NK cells. About 90% of human NK cells express low density CD56 (CD56^{dim}), which can effectively exert cytotoxicity, and about 10% NK cell phenotypes are CD56 bright, which function is to produce cytokines. CD56^{bright} NK cells may differentiate into CD3-CD56^{dim} NK cells over time, and recent data have shown that Tim-3 acts as an NK cell maturation marker (Ndhlovu et al., 2012) and is necessary for their cytotoxicity, which could explain the results in our study. Additionally, our data showed that Tim-3 expression was significantly increased on CD3+CD56+ NKT cells in lung cancer patients. Liu Y et al reported that there was a negative correlation between Tim-3 expression and TNF-α production by NKT cells (Liu et al., 2010), suggesting that Tim-3 may also play a negative role in regulating NKT cell function, and it was also consistent with our results. Cao X et al demonstrated that Tim-3 is highly upregulated on both CD4+ and CD8+ tumor-infiltrating lymphocytes (TILs) from human lung cancer tissues (Cao et al., 2012), which also supports our view on this, the increased Tim-3 expression may involved in the immunological dysfunctions of lung cancer.

Lung cancer is categorized by stages, depending on how far they have spread beyond the lung. The higher the stage number, the worse the situation. Our data showed that there was a positive correlation between disease stages and Tim-3 expression on CD3-CD56+ NK cells, CD3-CD56^{dim} NK cells and CD3+CD56+ NKT cells. It is the first report Tim-3 expression on immune cells was associated with stages of lung cancer patients. Lung cancers are divided into stage I, II, III, IV and high stage means the cancer is more serious and the disorder of immune cells is more serious. Our data demonstrated that cases with stages III/IV had significantly higher proportion of Tim-3+ CD3-CD56+ NK cells, Tim-3+CD3-CD56^{dim} NK cells and Tim-3+CD3+CD56+ NKT cells than those with stages I/II. These results suggested that Tim-3 on these immune cells may play roles in the progression of lung cancer. In addition, we show that surgical resection of primary tumor rapidly reverses Tim-3 expression on CD3-CD56+, CD3-CD56^{dim} and CD3+CD56+ populations, which has significant implications for the timing of Tim-3-based therapies, and also suggesting Tim-3 blocking therapies could be more effective if started before surgery.

In summary, the expression of Tim-3 is upregulated on NK and NKT cells from peripheral blood in lung cancer, and is positively correlated with stages. In addition, Tim-3 expression on NK and NKT cells is reduced after surgical resection. Our data suggest that Tim-3 might be a potential diagnostic marker for the progression of lung cancer and might be as a therapeutic target for the treatment of this disease.

Acknowledgements

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