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Indoleamine2,3-Dioxygenase as A Prognostic Factor in Patients with Non-Small Cell Lung Cancer

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Abstract

Background: Indoleamine 2,3-dioxygenase (IDO) is an immunomodulatory enzyme produced by tumor cells and some alternatively activated macrophages and other immunoregulatory cells. The purpose of the present study was to evaluate the prognostic value of the relative expression of the forkhead/winged helix transcription factor 3 (Foxp3) and IDO in non-small cell lung cancer (NSCLC) tissues.

Methods: The NSCLC tissues from 141 patients who underwent complete surgical resection were collected at the time of surgery. The relative expression levels of Foxp3 and IDO in the tissues were determined by quantitative RT–PCR.

Results: The histological types of cancer seen in these patients included 105 adenocarcinomas, 24 squamous cell carcinomas and 12 other types of carcinoma. The average expression levels of Foxp3 and IDO relative to that of β-actin in the NSCLC tissue were 0.052 ± 0.147% and 0.088 ± 0.157%, respectively. The relative expression of Foxp3 tended to increase with the relative expression of IDO (R=0.451, P=0.001). The five-year survival rates of the patients according to the relative expression of Foxp3 were 78.3% and 71.9% in the lower and higher groups, respectively. According to the relative expression of IDO, the five-year survival rate was 83.2% in the lower expression group, and 67.9% in the higher expression group. There was a significant difference between the lower and higher IDO expression groups (p=0.0389).

Conclusions: The expression of IDO tended to have a positive correlation with the expression of Foxp3. The higher expression of IDO was therefore a significantly unfavorable prognostic factor in patients with NSCLC.

Keywords: Indoleamine 2,3-dioxygenase; Foxp3; Regulatory T cells; NSCLC; Surgical resection

Abbrevations

IDO: Indoleamine 2,3-Dioxygenase; Foxp3: Forkhead/Winged Helix Transcription Factor 3; NSCLC: Non-Small Cell Lung Cancer; Tregs: Regulatory T Cells; CD25: Interleukin 2 Receptor Chain; MRI: Magnetic Resonance Imaging; UICC: International Union Against Cancer; qRT-PCR: Quantitative RT–Polymerase Chain Reaction; CT: Cycle Number; CTLs: Cytotoxic T Lymphocytes; CTLA-4: Cytotoxic T-Lymphocyte Antigen-4

Introduction

Non-small cell lung cancer (NSCLC) is one of most common malignant neoplasms, and one of the leading causes of cancer-related mortality world-wide [1]. Surgery remains the mainstay of treatment for patients with early stage or loco regional advanced disease that is resectable. However, local or distant failure often becomes clinically evident in the follow-up period after complete resection, suggesting that undetectable micro metastases are often present at the time of the diagnosis [2]. The survival rate after surgery is reported to be only 60% to 80% even in patients with stage I NSCLC [3]. Moreover, the outcome of treatment for recurrent disease remains dismal in spite of advances in radiotherapy and chemotherapy [4]. It is therefore necessary to establish reliable prognostic biomarkers and effective adjuvant treatment to improvement of the treatment and prognosis of NSCLC patients.

Many types of cancer suppress the immune system to escape from host immunosurveillance. Regulatory T cells (Tregs) make important contributions to the immune escape mechanisms in the tumor microenvironment [5,6]. An increasing number of studies have suggested that the Tregs surrounding or infiltrating a tumor impair the antitumor immune response of NK and T cells and promote tumor progression, invasion and metastasis [7]. Tregs constantly express high levels of the interleukin 2 receptor chain (CD25) and specifically express forkhead/winged helix transcription factor (Foxp3) [8]. Foxp3 is a master transcriptional factor that modulates the expression of many key immunoregulatory genes for Tregs [9].

Indoleamine 2,3-dioxygenase 1 (IDO) is the rate-limiting enzyme that mediates the catabolism of the essential amino acid, tryptophan. IDO is produced both in antigen-presenting cells and tumor cells [10]. It participates in tumor-induced immune tolerance, because the depletion of tryptophan and its toxic catabolites subsequently inhibit T cell proliferation and the T cell immune response [11]. In a mouse model, IDO facilitated the development of lung cancer metastasis

through interleukin 6-dependent inflammation and immune escape driven by myeloid-derived suppressor cells [12]. Furthermore, plasma IDO activity was reported to be an unfavorable prognostic factor for NSCLC patients receiving chemotherapy or chemoradiation therapy [13]. IDO has recently attracted attention as a potent mediator contributing to the immune escape of tumors. Yang et al. demonstrated that some tryptanthrin derivatives were potent inhibitors of IDO, and these reduced the number of regulatory T cells and showed therapeutic activity against Lewis lung cancer in a mouse model [14].

In the present study, we evaluated the correlation of the relative expression levels of Foxp3 and IDO in NSCLC, and evaluated the prognostic significance of these molecular biomarkers.

Materials and Methods

Patients

The study was approved by the Human and Animal Ethics Review Committee of the University of Occupational and Environmental Health, Japan, and a signed consent form was obtained from each patient before we collected the tissue samples used in this study. From 2005 to 2007, 181 patients with non-small lung cancer underwent surgery at the University of Occupational and Environmental Health. Among them, those who underwent induction chemotherapy or treatment with immunosuppressive agents were excluded from this analysis. The patients who did not undergo complete resection were also excluded. The remaining 141 patients were evaluated in this study, and their tumor tissues were collected at the time of surgery. The patients' records, including their clinical data, preoperative examination results, details of the surgery, histopathological findings and TNM staging were also reviewed. The preoperative assessments included chest roentgenography, computed tomography of the chest and upper abdomen, magnetic resonance imaging (MRI) of the brain, bronchoscopy and bone scintigraphy. The dissected hilar and mediastinal lymph nodes were examined pathologically to identify the extent of lymph node metastasis. Tumor tissues were soaked immediately in RNAlater (Ambion, Austin, TX) after surgical resection for over 24 h and were then frozen in a deep freezer at −120°C until use. The histopathological findings were classified according to the World Health Organization criteria, and the TNM staging system of the international union against cancer (UICC) was employed [15,16].

Follow-up information regarding each patient was obtained through office visits or by telephone interviews with the patient, a relative or the patient's primary physician. The patients were evaluated every three months using chest roentgenography, and chest CT scans and bone scintigraphy were each performed every six months for the first two years after surgery, and annually thereafter. The mean follow-up period after surgery was 46 months.

Quantitative RT–polymerase chain reaction (RT-PCR)

Total RNA from the frozen tissue specimens was obtained using the RNeasy kit (QIAGEN Science, Maryland, USA). RNA was converted to cDNA using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Tokyo, Japan). These cDNAs were used as templates for PCR amplification. Quantitative RT-PCR was carried out in an ABI Prism 7000 instrument (Applied Biosystems, Foster, CA). The relative amount of Foxp3 mRNA and IDO mRNA were measured by detecting intercalated SYBR green. The PCR was performing using 10 μl of SYBR GREEN PCR Master Mix (Applied Biosystems, Foster, CA), either 2 μl of cDNA or 7.4 μl of water and each primer set (described below) in a total volume of 20 μl. The PCR cycles were 95 for 20 seconds, followed by 45 cycles of 95 for three seconds and 60 for 30 seconds. The sense and antisense primer sequences for Foxp3 used for quantitative RT-PCR were: 5'- CTT CAA GTT CCA CAA CAT GCG-3'and 5'- CGT GGC GTA GGT GAA AGG G-3', respectively. The sense and antisense primer sequences for IDO used for the quantitative RT-PCR were: 5'- GGT CAT GGA GAT GTC CGT AA-3'and 5'- ACC AAT AGA GAG ACC AGG AAG AA-3', respectively. The quantitative PCR primers used for β-actin were β-actin Control Reagents (Applied Biosystems). The threshold cycle number (CT) was defined as the fractional cycle number at which the amount of amplified target product reached a fixed threshold. The ΔCT was obtained by comparing the CT of Foxp3 with the CT of β-actin in same amount of templates, and 2-ΔCT was defined as the fold-difference in the mRNA expression of the target gene compared to the β-actin expression in the same sample. The relative expression was calculated using the following formula:

$$\text{Relative expression} = 2^{-(\Delta CT\text{sample} - \Delta CT\text{ control})}$$

The median level of mRNA expression in all patients was 0.012 for Foxp3, and 0.030 for IDO. The patients were considered to be part of the higher expression group when their relative expression of Foxp3 and IDO exceeded 0.012 and 0.030, respectively.

Statistical analysis

The Mann-Whitney U-test was used to determine the significance of differences in the continuous variables between the two groups. The survival curves were calculated using the Kaplan-Meier method, and the data were compared using the Log-rank test for a univariate analysis. The prognostic factors were analyzed using a multivariate analysis with Cox's proportional hazard model to adjust for any potentially confounding factors. The categorical variables were compared using the chi-square test or Fisher's exact test. Differences in the findings were considered to be significant for values of $p<0.05$. The Statview V software package (Abacus Concept, Berkeley, CA) was used for all of the statistical analyses.

Results

Each of the 141 patients had undergone a complete resection for NSCLC. The patients included 93 males and 48 females. The mean age of the patients was 69.4 years (range: 18-86). The histological types of cancer seen in these patients included 105 adenocarcinomas (74.5%), 24 squamous cell carcinomas (17.0%) and 12 other types of carcinoma (8.5%). The pathological stage was diagnosed as stage IA in 67 patients (47.5%), stage IB in 29 patients (20.6%), stage II in 16 patients (11.3%), and stage III in 29 patients (20.6%). The average expression levels of Foxp3 and IDO relative to that of β-actin in the NSCLC tissues were 0.052 ± 0.147% and 0.088 ± 0.157%, respectively. The relative expression levels of Foxp3 and IDO according to the clinicopathological factors, such as the gender, age, histology and the pathological stage are shown in Table 1. A significant correlation with IDO expression was not observed for these clinical factors. However, the expression of Foxp3 in patients at stage IA was significantly lower than that of patients with more advanced disease (stage IB-III). The correlation between the relative expression levels of Foxp3 and IDO

are shown in Figure 1. The relative expression of Foxp3 tended to increase with the relative expression of IDO (R=0.451, P=0.001).

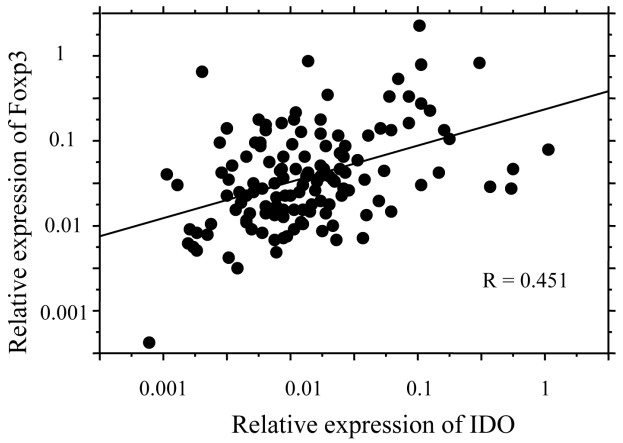

Figure 1: The correlation between the Foxp3 and IDO expression in NSCLC tissue specimens. The relative expression of Foxp3 tended to increase with the relative expression of IDO (Foxp3 = -0.638 + 0.427 x IDO; R2 = 0.203, R = 0.451).

	n	Foxp3 Mean + SD	p value	IDO Mean + SD	p value
All patients	141	0.052 + 0.147		0.087 + 0.157	
Gender					
Male	93	0.061 + 0.173	0.292	0.087 + 0.145	0.974
Female	48	0.037 + 0.071		0.086 + 0.179	
Age (years)					
< 75	88	0.062 + 0.175	0.291	0.101 + 0.181	0.161
> 75	53	0.035 + 0.081		0.063 + 0.102	
Histology					
Adenocarcinoma	105	0.036 + 0.079	0.061	0.077 + 0.147	0.224
Squamous cell carcinoma	24	0.089 + 0.236		0.120 + 0.199	
Other types of carcinoma	12	0.118 + 0.299		0.113 + 0.150	
Pathological stage					
IA	67	0.018 + 0.039	0.009	0.090 + 0.190	0.819
IB	29	0.050 + 0.078		0.075 + 0.055	
II	16	0.160 + 0.310		0.084 + 0.109	
III	29	0.071 + 0.196		0.093 + 0.163	

Table 1: The relative expression of Foxp3 and IDO in NSCLC tissue specimens

The five-year overall survival rates of the patients according to the relative expression of Foxp3 were 78.3% and 71.9% in the lower and higher groups, respectively (Figure 2). A significant difference was not observed in the survival rate between the higher and lower groups (p=0.2939). Regarding the relative expression of IDO, the five-year overall survival rate was 83.2% in the lower expression group and 67.9% in the higher expression group (Figure 3). There was a significant difference between the lower and higher IDO expression groups (p=0.0389).

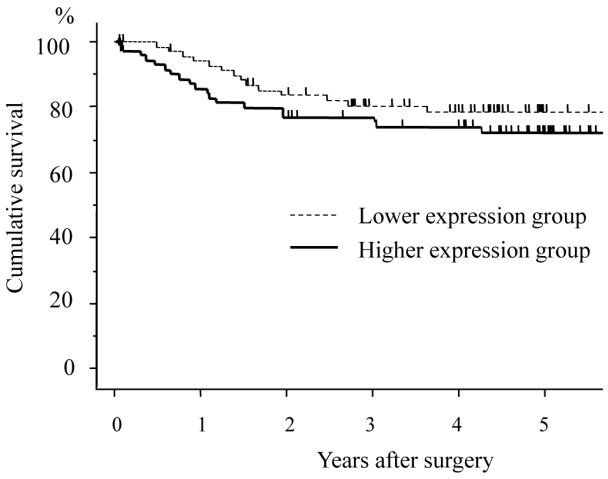

Figure 2: The overall survival of the patients according to the expression of Foxp3 in NSCLC tissue specimens. The five-year overall survival rate was better in the lower expression group (78.3%) than in the higher expression group (71.9%).

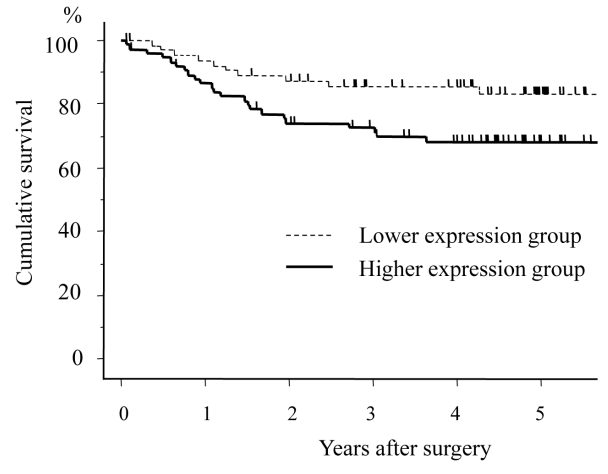

Figure 3: The overall survival of the patients according to the expression of IDO in NSCLC tissue specimens. A significant difference was observed in the survival rates between the patients with higher IDO expression than those with lower expression.

With respect to the patients with stage I disease, the five-year survival rate was 89.0% for those in the lower Tregs group and was

79.0% for those in the higher group. There was no significant difference in the five-year survival rates between the two groups (p=0.2608). Regarding the IDO expression in the NSCLC tissues, the five-year survival rates (89.9%) for patients at stage I in the lower groups tended to be better than in that of higher group (78.7%, p=0.0887). In both histological subgroups (adenocarcinoma and squamous cell carcinaoma), no significant difference in the prognosis was observed between the patients with lower and higher expression of Foxp3. The five-year survival rate in the lower IDO expression group in patients with adenocarcinoma was 83.4%, whereas it was 69.6% in the higher expression group (Figure 4, p=0.098). For patients with squamous cell carcinoma, the five-year survival rates in the lower and higher IDO expression groups were 87.5% and 65.5%, respectively (p=0.2435).

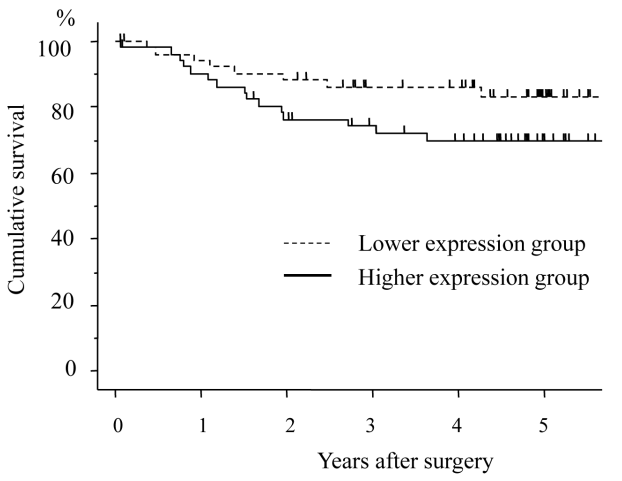

Figure 4: The overall survival of adenocarcinoma patients according to the expression of IDO. The five-year overall survival rate was better in the lower expression group (87.5%) than in the higher expression group (65.5%).

A univariate analysis for the overall survival showed that gender (female vs. male, p=0.0037), the T factor (T1 vs. T2-4, p=0.0030), the N factor (N0 vs. ≥ N1-2, p=0.0001) and the relative expression of IDO (Low vs. High, p=0.0432) were significant prognostic factors. A multivariate analysis using these significant variables (gender, T factor, N factor and IDO expression) showed that the hazard ratio of the relative expression of IDO was 0.541 (95% confidence interval 0.256–1.140, p=0.1060), thus indicating that it is not a significantly independent prognostic factor for patients with NSCLC who had undergone complete surgical resection.

Discussion

Tregs are critical for the maintenance of immune homeostasis, which is essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases [17]. While Tregs block beneficial responses against autoimmune or allergy disease, they also suppress anti-tumor immunity. Tregs are thought to be a major cause of tumor-driven immune evasion, which is a major obstacle to the development of effective immunotherapies. Our previous studies demonstrated that the Tregs in the regional lymph nodes of NSCLC patients inhibited the induction of cytotoxic T lymphocytes (CTLs) against autologous tumor cells, and the depletion of Tregs restored the induction of CTLs [18]. Foxp3 has been used as hallmark of Tregs, because it is a master regulator for the differentiation and function of Tregs [19]. The Foxp3 protein expression was previously noted to be increased according to the activation of CD4+, CD25+ regulatory T cells [20].

Tumor-infiltrating lymphocytes are often found in tumors, presumably reflecting a positive or negative immune response against the tumor. Tregs become elevated in the peripheral circulation of advanced-cancer patients, and advanced disease leads to their accumulation within the tumor microenvironment [21]. The enrichment of intra-tumoral Tregs might manipulate the balance of tumor-infiltrating T cells against anti-tumor effector cells [22]. Several investigators reported that a high frequency of tumor-infiltrating Tregs was an unfavorable prognostic factor in many kinds of cancer [23]. We also reported that a high frequency of Tregs in the regional lymph nodes was a significant unfavorable prognostic factor in NSCLC patients after surgery [24]. It has been reported that Foxp3 was overexpressed not only in tumor-infiltrating lymphocytes, but also in NSCLC cells, and the Foxp3 expression in cancer cells correlated with both an increase in tumor-infiltrating Tregs and the presence of lymph node metastasis [25]. However, in the present study, although the expression of Foxp3 was significantly increased in advanced stage tumors compared with stage IA tumors, it was not associated with a poor prognosis. The role of Foxp3 expression by tumor cells remains controversial, and several *in vitro* and *in vivo* mouse model studies clearly pointed to a critical role of Foxp3 as a tumor suppressor in breast, prostate and ovarian carcinoma [26]. These findings support the assertion that Foxp3 exhibits tumor suppressor activity in terms of the migration and proliferation of human glioblastoma [27].

IDO was originally described as contributing to maternal tolerance toward the fetus. It is expressed by fetal-derived syncytiotrophoblasts in the human placenta, and it prevents rejection during pregnancy, probably by inhibiting alloreactive T cells [28]. It has a key role in the rate-limiting step in tryptophan degradation, and the combination of the local reduction in tryptophan levels and production of bioactive tryptophan metabolites (kynurenine) suppresses T cells [11]. IDO is often induced or maintained by the exposure of cells to many inflammatory cytokines, such as endotoxin and IFN-γ; which may be present in cancer patients (29). Therefore, IDO is produced by the tumor-associated macrophages in the stroma of human tumors, and it is also expressed by the tumor cells themselves, including lung cancer cell lines [30,31]. It was found that lung cancer patients bearing malignant tumors had a 20-fold higher level of the enzyme activity of IDO compared to patients bearing benign lesions [32]. Because IDO plays an important role in the cancer immune escape mechanism by suppressing the T-lymphocyte function, IDO overexpression is related to a poor prognosis in cancer patients [33]. The present study showed that the higher expression of IDO in NSCLC tissues was a significant unfavorable prognostic factor in patients who underwent a complete resection.

Therefore, IDO may represent an important regulatory checkpoint influencing the Treg activity by both stabilizing and augmenting the suppressive phenotype [34]. IDO-expressing DCs trigger the differentiation of naive CD4[+] T cells toward a Foxp3+ [inducible Treg] phenotype, and also directly activate mature, pre-existing Tregs [34]. A recent study indicated that IDO expression in brain tumors increases the recruitment of Tregs and negatively impacts survival [35]. Sznurkowski reported that the expression of IDO also predicted

an unfavorable prognosis in patients with vulvar squamous cell carcinoma; however, it did not influence the recruitment of Foxp3-expressing Tregs in cancer nests [36]. The present study showed that a mild correlation was observed between the expression of Foxp3 and IDO.

Many concerns regarding novel cancer immunotherapy are currently focused on immune checkpoint blockade. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is one of the immune checkpoint receptors, and the CTLA-4 molecule contributes to the suppressor function of Tregs [37]. A monoclonal antibody against human CTLA-4, ipilimumab, was found to elicit objective tumor responses and prognostic benefits in clinical trials [38,39]. Selective depletion of Tregs enhances the immune responses to tumor antigen vaccination by targeting Tregs [40]. IDO is part of a critical resistance mechanism in antitumor T cell immunotherapy targeting CTLA-4, suggesting that IDO has an important role in the context of immunotherapies targeting immune checkpoint molecules [41]. An increasing number of studies have shown that the inhibition of IDO is a promising approach for therapeutic application in cancer patients [42,43].

In conclusion, this study indicated that there is a weak correlation between the expression of Foxp3 and IDO in NSCLC tissues, and that the higher expression of IDO was a significant unfavourable prognostic factor. Information about the IDO expression in the tumor environment might therefore be important for selecting patients who require adjuvant therapy. Further studies will be necessary to provide a better understanding of the immunosuppressive tumor environment, including the role of IDO, in order to facilitate the development of efficient anti-cancer immunotherapy.

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