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Increased serum kynurenine/tryptophan ratio correlates with disease progression in lung cancer

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ABSTRACT

Background: Indoleamine 2,3-dioxygenase (IDO) catalyzes the rate-limiting step of tryptophan (Trp) degradation along the kynurenine (Kyn) pathway. By depleting tryptophan, IDO is considered to be a fundamental immune escape mechanism for tumor cells. However, IDO expression in lung cancer has not been explored thoroughly. Thus, the present study investigated IDO activity determined by serum Trp and Kyn concentrations in lung cancer and the correlation between the IDO activity and clinical parameters.
Method: The concentrations of Trp and Kyn were measured simultaneously by liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI/MS/MS) in the sera of 123 patients with lung cancer and 45 healthy controls. The IDO activity was estimated by calculating the serum Kyn-to-Trp ratio (Kyn/Trp ratio).
Results: Trp concentrations were significantly lower in patients with lung cancer than in healthy controls (62.6 ± 15.8 μM vs. 71.1 ± 11.8 μM, respectively; $p = 0.0007$), while Kyn concentrations were significantly higher in patients compared with the controls (2.82 ± 1.17 μM vs. 2.30 ± 0.56 μM, respectively; $p = 0.0036$). The IDO activity determined by the Kyn/Trp ratio was significantly higher in the patients than in the controls (47.1 ± 21.3 vs. 32.9 ± 9.10, respectively; $p < 0.0001$). In addition, patients in the advanced stages of lung cancer had significantly lower Trp concentrations and higher IDO activity than those in the early stages ($p = 0.0058$ and $p = 0.0209$, respectively).
Conclusions: IDO activity was increased in lung cancer patients, and higher IDO activity was associated with more advanced stages. These results suggest that increased IDO activity is involved in disease progression of lung cancer, possibly through its immunosuppressive effect.

1. Introduction

The immune system is highly specialized to recognize non-self-pathogens or tissues and efficiently eliminate them, but tumors can often escape host immunity in various ways. Accumulating evidence in tumor immunology has revealed that tumors generate an immunosuppressive microenvironment that protects the tumor from host immunity, promotes tumor growth, and attenuates immunotherapeutic efficacy [1,2]. The molecular mechanisms underlying tumor-induced immunosuppression include the generation of regulatory T cells (Treg), impairment of dendritic cell (DC) function, and production of immunosuppressive cytokines, such as IL-10 and TGF-β [1]. More recently, indoleamine 2,3-dioxygenase (IDO), a tryptophan (Trp)-catabolizing enzyme, has attracted special attention in tumor-induced immunosuppression [3–9]. IDO catalyzes the rate-limiting step of Trp degradation along the kynurenine (Kyn) pathway [5]. Both the reduction in local Trp concentration and production of immunomodulatory Trp metabolites, such as Kyn, by IDO activity potently inhibit T cell proliferation and induce T cell apoptosis, leading to immune tolerance. Surprisingly, recent studies have demonstrated IDO expression in cancer cells as well as tumor-infiltrating antigen-presenting cells (APCs) in tumor tissues, indicating that this molecule is essential for the immune escape of tumor cells [4,6,10]. Indeed, IDO expression by tumor cells has been shown to correlate with a poor prognosis in several types of cancers [7–9].

Recent advances in mass spectrometry enable the simultaneous measurement of Trp and Kyn in human serum or plasma using liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI/MS/MS). In human serum or plasma, the IDO

Abbreviations: Treg, regulatory T cells; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; Trp, tryptophan; Kyn, kynurenine; APCs, antigen-presenting cells; LC–ESI/MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; Kyn/Trp ratio, Kyn-to-Trp ratio; LDH, lactate dehydrogenase.

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activity can be estimated by the Kyn-to-Trp ratio (Kyn/Trp ratio), because Kyn is the first product formed through catabolizing Trp, which is tightly regulated by IDO. To date, several studies have shown that the Kyn/Trp ratio increases in the serum or plasma of patients with cancers, such as malignant melanoma [11] and gynecological cancers [12], suggesting that enhanced IDO activity play a role in immunosuppression observed in cancer patients. However, no data are available about the IDO activity determined from the Kyn/Trp ratio in serum of lung cancer patients using LC–ESI/MS/MS. The present study measured the serum concentrations of Trp and Kyn by LC–ESI/MS/MS and estimated the IDO activity in lung cancer patients. In addition, the correlation between the IDO activity and the clinical parameters of the patients was examined.

2. Methods

2.1. Subjects

One hundred and thirty-six patients with histologically confirmed primary lung cancer were referred to our institutions from 2002 to 2008. Among them, 13 patients were excluded because of chronic renal failure, which increased the serum Kyn/Trp ratio [13], and concomitant cancer other than lung cancers. Thus, a total 123 patients with lung cancer (99 men and 24 women with a mean age of 66.4 ± 10.1 years) were enrolled in the present study. No patients had autoimmune diseases, viral hepatitis, or human immunodeficiency virus (HIV) infection. All patients were staged according to the 1997 International Union Against Cancer (UICC) criteria [14], and were divided into two subgroups: early and advanced lung cancers. Early and advanced lung cancers were defined as stages I and II and stages III and IV, respectively. Histological typings were classified according to the World Health Organization (WHO) criteria [15]. The study subjects also included 45 healthy blood-donors as a control group (34 men and 11 women with the mean age of 63.4 ± 9.4 years). This study was approved by the Ethics Committee of our Institution, and informed consent was obtained in accordance with the institution guidelines.

2.2. Laboratory examinations

At the time of blood sampling, all of the patients had not received any treatment, including operation, chemotherapy, or radiation therapy. Serum samples of patients were collected in the morning and frozen at −20 °C until analysis. Routine laboratory examinations, such as blood cell counts and biochemical analysis, were performed.

2.3. Measurements of serum tryptophan and kynurenine

The concentrations of Trp and Kyn were determined by LC–ESI/MS/MS (TSQ 7000 LC-quadrupole mass spectrometer, ThermoFisher, San Jose, CA, USA) as described previously [16–18]. In brief, frozen serum samples were thawed at room temperature. The serum samples were spiked with standards and deproteinized with 0.5N perchloric acid for 10 min on ice. Then, the samples were centrifuged (15,000 × *g*, 10 min) and the supernatants were vortex-mixed with an equal volume of 1 M ammonium formate and injected onto a capillary column for LC–ESI/MS/MS. Analyses were separated by an isocratic elution of the injected samples. Detection was performed using sheathless electrospray tandem mass spectrometry in the multiple reaction monitoring mode. The IDO activity was determined by dividing the serum concentration of Kyn by that of Trp.

2.4. Measurements of serum IFN-γ

IFN-γ is the most potent inducer of IDO expression in various types of cells, such as monocytes/macrophages and DC [3,4], and increased production of IFN-γ was reported to be responsible for high serum Kyn/Trp ratios through enhanced IDO expression under certain chronic inflammatory conditions [19]. To determine any association between the IDO activity and IFN-γ in the present patient groups, the serum levels of IFN-γ were examined. Serum concentrations of IFN-γ were measured using ELISA kits from R&D Systems (Minneapolis, MN, USA).

2.5. Statistical analysis

Statistical analysis was performed using the Wilcoxon/Kruskal–Wallis test and Spearman's rank correlation technique. Cox proportional hazards regression analysis was used to identify significant valuables associated with prognosis. *p* values less than 0.05 were considered significant.

3. Results

3.1. Clinical characteristics

The clinical characteristics of the 123 patients with lung cancer and 45 healthy controls are summarized in Table 1. Twenty six patients were classified as early lung cancer, and 97 patients were classified as advanced lung cancer. One hundred and nine patients had non-small cell carcinoma (72 adenocarcinoma, 23 squamous cell carcinoma, 4 large cell carcinoma, 3 adenosquamous cell carcinoma, 7 unclassified carcinoma), while 14 patients had small cell carcinoma.

3.2. Serum concentrations of Trp and Kyn

Concentrations of Trp, Kyn, and the Kyn/Trp ratio are presented in Table 2. Patients with lung cancer had significantly lower levels of Trp ($p = 0.0007$) together with significantly higher levels of Kyn ($p = 0.0036$) than healthy controls, resulting in significantly higher Kyn/Trp ratios ($p < 0.0001$). No significant difference was found between the concentrations of Trp, Kyn, and the Kyn/Trp ratio and histologic types or differentiations of lung cancer.

Table 1
Clinical characteristics of patients with lung cancer and healthy controls.

	Lung cancer	Controls	*p*-Value
Sex (M/F)	99/24	34/11	n.s.
Age (year)	66.4 ± 10.1[a]	63.4 ± 9.4	n.s.
Body mass index	21.1 ± 3.4		
Staging			
Early lung cancer	26		
Stage I	19		
Stage II	7		
Advanced lung cancer	97		
Stage III	43		
Stage IV	54		
Histological type			
Non-small cell cancer	109		
Adenocarcinoma	72		
Squamous cell carcinoma	23		
Large cell carcinoma	4		
Adenosquamous cell carcinoma	3		
Unclassified carcinoma	7		
Small cell carcinoma	14		

[a] Mean ± SD; n.s., not significant.

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Table 2
Serum concentrations of kynurenine and tryptophan.

	Lung cancer ($n=123$)	Controls ($n=45$)	p-Value
Tryptophan (Trp) (μmol/l)	62.6 ± 15.8[a]	71.1 ± 11.8	0.0007
Kynurenine (Kyn) (μmol/l)	2.82 ± 1.17	2.30 ± 0.56	0.0036
Kyn/Trp (μmol/(l mmol))	47.1 ± 21.3	32.9 ± 9.10	<0.0001

[a] Mean ± SD.

3.3. Correlation of serum Trp, Kyn, and Kyn/Trp ratio with disease stages

Comparing patients with early lung cancer, those with advanced lung cancer, and the healthy controls, significant increases in serum concentrations of Kyn and the Kyn/Trp ratio were found in patients with early and advanced lung cancers than healthy controls (Kyn: 2.63 ± 0.67 μM, 2.86 ± 1.27 μM, and 2.30 ± 0.56 μM, respectively; Kyn/Trp ratio: 39.0 ± 10.6, 49.2 ± 22.9, and 32.9 ± 9.10, respectively) (Fig. 1). The serum concentrations of Trp in early lung cancer patients did not differ from healthy controls (68.6 ± 11.8 μM and 71.1 ± 11.8 μM, respectively), whereas the concentrations of Trp were significantly lower in advanced lung cancer patients (61.0 ± 16.3 μM) than in healthy controls ($p < 0.0001$). Interestingly, patients with advanced lung cancer had a significantly lower serum concentrations of Trp ($p = 0.0058$) with a significantly higher Kyn/Trp ratio ($p = 0.0209$), compared with those of early lung cancer (Fig. 1).

Among each factor of TNM classification, the serum concentrations of Trp and Kyn were significantly lower in T4 than T1, without a significant difference in the Kyn/Trp ratio (Table 3). Regarding N factors, the serum concentrations of Kyn were significantly lower in N0 than N3, and in N2 than N3. Kyn/Trp ratio was significantly higher in N3 than N0 or N2. There was no significant difference in the serum concentrations of Trp, Kyn, or the Kyn/Trp ratio between M0 and M1.

3.4. Correlation of serum Trp, Kyn, and Kyn/Trp ratio with prognosis

Next, we determined whether serum concentrations of Trp, Kyn, or Kyn/Try ratio were significant variables predicting survival by Cox proportional hazards regression analysis. Among the variables, disease stage was a significant variable, but the serum concentrations of Trp, Kyn, or Kyn/Trp ratio were not significantly associated with the prognosis (Table 4).

Table 3
Comparison between serum concentrations of kynurenine, tryptophan, and kynurenine/tryptophan ratio and TNM classification.

	Trp (μmol/l)	Kyn (μmol/l)	Kyn/Trp (μmol/(l mmol))
T			
T1 ($n=21$)	70.8 ± 11.7	3.28 ± 1.98	47.1 ± 31.4
T2 ($n=45$)	63.1 ± 13.7	2.82 ± 0.71	46.5 ± 14.3
T3 ($n=12$)	64.7 ± 21.7	2.72 ± 1.01	43.9 ± 15.2
T4 ($n=45$)	57.8 ± 16.2[*]	2.63 ± 1.06[*]	48.4 ± 23.4
N			
N0 ($n=34$)	62.4 ± 13.3	2.59 ± 0.75	43.0 ± 13.4
N1 ($n=13$)	62.1 ± 17.3	2.62 ± 0.61	45.1 ± 15.0
N2 ($n=46$)	63.4 ± 18.2	2.74 ± 1.31	45.2 ± 21.7
N3 ($n=30$)	62.0 ± 14.2	3.28 ± 1.41[†,‡]	55.5 ± 28.0[†,‡]
M			
M0 ($n=69$)	63.5 ± 13.8	2.80 ± 1.18	45.7 ± 20.4
M2 ($n=54$)	61.6 ± 18.1	2.84 ± 1.17	48.8 ± 22.5

[*] $p < 0.05$, T1 vs. T4.
[†] $p < 0.05$, N0 vs. N4.
[‡] $p < 0.05$, N2 vs. N3.

3.5. Correlation of serum Trp, Kyn, and Kyn/Trp ratio with clinical parameters

The correlation between the serum concentrations of Trp, Kyn, and the Kyn/Trp ratio and clinical parameters was examined. The serum concentrations of Trp were positively associated with total protein, albumin, and hemoglobin, and negatively with platelet, while those of Kyn did not correlate with any clinical parameters (Table 5). The Kyn/Trp ratio was negatively associated with albumin and hemoglobin, and positively correlated with lactate dehydrogenase (LDH).

3.6. Serum concentrations of IFN-γ

To determine whether endogenous IFN-γ production in patients with lung cancer is associated with high serum Kyn/Trp, the serum concentrations of IFN-γ were measured. Only 8 of the patients with lung cancer had measurable levels of serum IFN-γ (14.7 ± 3.47 pg/ml), and no IFN-γ was detected in the remaining patients and normal controls (detection limit <8.0 pg/ml). No correlation was found between the serum Kyn/Trp ratio and serum concentrations of IFN-γ.

4. Discussion

The present study simultaneously measured the serum concentrations of Trp and Kyn using LC–ESI/MS/MS and calculated the Kyn/Trp ratio to determine the IDO activity in patients with lung cancer. It was found that patients with lung cancer had significantly lower Trp concentrations and higher Kyn concentrations than healthy controls, resulting in significantly higher IDO activity. Moreover, degradation of Trp and increased levels of the IDO activity correlated with disease progression. These data suggest that Trp catabolism by the IDO activity is associated with the progression of lung cancer.

To date, there have been two reports demonstrating IDO expression in lung cancer. Initially, Uyttenhove et al. found IDO expression in 9 of 11 non-small cell lung cancers and in 2 of 10 small cell lung cancers by immunohistochemistry [6]. Most recently, Karanikas et al. indicated increased levels of IDO mRNA expression, relative to the reference level in normal lung tissues, in lung cancer tissues as well as in adjacent non-malignant tissues [20]. They concluded that IDO is expressed constitutively by lung cancer cells, but the higher IDO expression level observed in patients with lung cancer can also contribute to other cells, such as macrophages and DC, expressing this enzyme that recruited in the peri-tumoral lung tissues. Taken together, these two studies suggest that increased expression of IDO in lung cancer is associated with tumor-induced immunosuppression or tolerance. However, there has been no study to examine the IDO activity assessed by serum concentrations of Trp and Kyn in lung cancer. Although the present study did not assess IDO expression in lung cancer tissues directly, it was demonstrated that the IDO activity determined by serum concentrations of Trp and Kyn was significantly higher in patients with lung cancer than in healthy controls.

Little is known about a correlation of IDO activity and clinicopathlogical parameters in lung cancer. We found that patients with advanced lung cancer had significantly higher IDO activity than those with early lung cancer, indicating a correlation between this enzyme activity and disease progression. In contrast, Karanikas et al. found no significant correlation between IDO mRNA expression by lung cancer tissues and disease staging in 28 patients with lung cancer [20]. This discrepancy may be partially attributable to the differences in the sample sizes and the methods to assess IDO expression or activity between these studies. Regarding TNM

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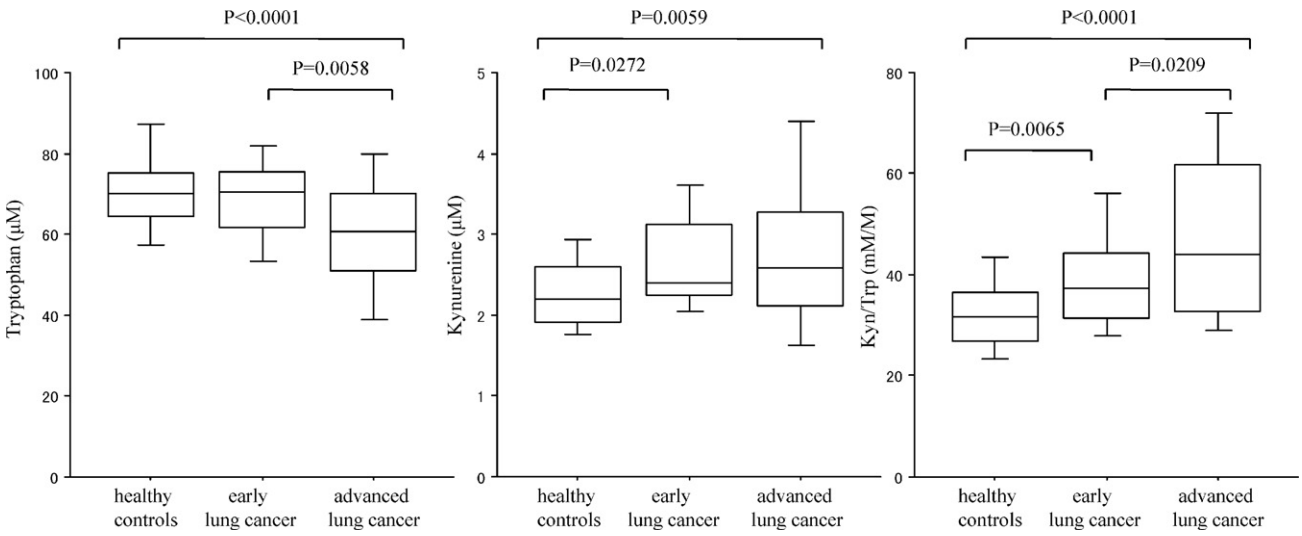

Fig. 1. Comparison of serum concentrations of kynurenine, tryptophan, and kynurenine/tryptophan ratio between early lung cancer, advanced lung cancer, and healthy controls.

Table 4
Univariate Cox proportional hazard model predicting survival.

Variables	Categories	HR	95% CI	*p*-Value
Age (year)		0.997892	0.970551–1.026472	0.8824
Gender	Female	0.787963	0.531795–1.103518	0.1736
Histological type	Adeno	0.767359	0.460254–1.330448	
	Small	1.165676	0.529075–2.366503	
	Large	0.468857	0.101348–1.318796	0.5132
	Adenosquarmous	2.023365	0.604168–5.011776	
	Non-small	1.027451	0.22169–2.907179	
Stage	Advance	2.267006	1.36725–4.609834	0.0005
Trp (μmol/l)		1.001541	0.985381–1.017248	0.8501
Kyn (μmol/l)		1.16692	0.897541–1.476225	0.2381
Kyn/Trp (μmol/(l mmol))		1.005792	0.99014–1.020058	0.4553

HR: hazard ratio, CI: confidence interval.

classifications, we found a significant increase in IDO activity in patients with N3, suggesting that higher IDO activity is associated with the extent of lymph node metastasis. However, T factors, including tumor sizes, were not related to IDO activity. We also examined a correlation between IDO activity and survival. Cox proportional hazards regression analysis showed that IDO activity was not a significant factors predicting survival. In colorectal cancers, however, Brandacher et al. recently demonstrated that higher IDO protein expression in cancer tissues significantly correlated a worse prognosis, indicating that IDO protein expression in tumors was a significantly prognostic variable [8]. The reason for this difference is not clear between Brandacher's and our studies, but measurements of IDO expression in tumor tissues, rather than those in serum, may be associated with prognosis more closely. Further study to assess IDO protein expression in the tissues of lung cancer will clarify this. Collectively, the present data suggest that a higher degree of IDO activity observed in lung cancer plays a role in its disease progression.

Table 5
Correlation between serum concentrations of kynurenine, tryptophan, and kynurenine/tryptophan ratio and clinical parameters.

	Kynurenine (Kyn)		Tryptophan (Trp)		Kyn/Trp	
	R	*p*	*R*	*p*	*R*	*p*
TP (g/dl)	0.0296	0.746	0.2164	0.0167	−0.1311	0.15
Alb (g/dl)	−0.0967	0.2892	0.2613	0.0036	−0.2885	0.0013
BUN (mg/dl)	0.0912	0.3177	−0.1046	0.2516	0.1285	0.1584
Cre (mg/dl)	0.1407	0.122	−0.0002	0.9984	0.1319	0.1476
T.Bil (mg/dl)	0.0202	0.8256	0.1414	0.1203	−0.0598	0.5126
AST (IU/l)	0.1695	0.062	0.1312	0.1498	0.0687	0.4523
ALT (IU/l)	0.047	0.6072	0.0818	0.3701	−0.0174	0.8494
LDH (IU/l)	0.1209	0.1845	−0.0379	0.6787	0.1799	0.0474
CRP (mg/dl)	0.0529	0.583	−0.169	0.0627	0.1619	0.0747
WBC (μl^{-1})	−0.0197	0.8296	−0.1125	0.2173	0.0677	0.4586
HB (g/dl)	−0.0005	0.996	0.3358	0.0002	−0.2612	0.0037
Plt ($\times 10^4$ μl^{-1})	−0.1027	0.2601	−0.1953	0.0311	0.0044	0.9616

TP, total protein; Alb, albumin; BUN, blood urea nitrogen; Cre, creatinine; T.Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CRP, C reactive protein; WBC, white blood cell count; HB, hemoglobin; Plt, platelet.

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The mechanism of the enhancement of IDO activity determined by serum Kyn/Trp ratio in cancer patients is not fully determined, but two possible explanations are considered. One explanation is that increased IDO activity in cancer cells as well as other cells infiltrating tumors directly catabolizes Trp into Kyn. Second, an increase in endogenous production of IFN-γ, which is the most potent inducer of IDO, may augment IDO activity. In cancer patients, higher concentrations of serum neopterin, which is produced in large amounts by human monocytes/macrophages upon stimulation by IFN-γ, were reported, and measurement of serum neopterin allows assessment of the endogenous production of IFN-γ [11,12,19,21]. Fuchs et al. reported a significant positive correlation between neopterin concentration and Kyn/Trp ratio in serum of patients with several cancers, suggesting that increased IFN-γ production in cancer patients is responsible for higher IDO activity, in particular, in macrophages and DC [11,12,19,21]. However, in the present study no association was found between the serum concentrations of IFN-γ and the IDO activity in patients with lung cancer. To clarify the true cause of increased IDO activity assessed by serum Kyn/Trp ratio in cancer patients, further studies will be required.

A significantly negative correlation was noted between the IDO activity and hemoglobin and albumin. Consistent with the present results, a previous study revealed that the serum Kyn/Trp ratio was inversely correlated with hemoglobin in patients with chronic inflammatory diseases, such as rheumatoid arthritis [22]. They suggest that Trp depletion induced by increased IDO activity limits Trp availability in erythroid progenitors, leading to erythropoiesis suppression. Additionally, in patients with a hematologic neoplasm, Denz et al. showed that low Trp concentrations with high IDO activity were shown to be associated with low serum albumin, which is similar to the present results [23]. They also showed that cachexia, including body weight loss, was closely linked to increased endogenous activity of IFN-γ, which raised IDO activity [23]. This enhanced IDO activity turned to decrease serum tryptophan. In our patients with lung cancer, a mean body mass index was not so low (21.1 ± 3.4), and no elevation of serum IFN-(was found. Thus, cachexia might not largely contribute to increase IDO activity observed in our patients with lung cancer. A positive correlation was also found between the IDO activity and serum LDH, but the precise reason remains unclear. Taken together, these data suggest that accelerated catabolism of Trp through IDO activity plays a role in pathogenesis of several pathologic conditions, such as anemia and low albuminemia.

In summary, the present study showed that patients with lung cancer had higher IDO activity, as assessed by the serum concentrations of Kyn and Trp, than healthy controls, and that enhanced IDO activity was related to more advanced stages of lung cancer. These results suggest increased IDO activity is involved in disease progression of lung cancer, possibly through its immunosuppressive effect.

Conflict of interest

All authors have no conflicts of interest to disclose.

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