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The potential use and clinical significance of plasma RIPK3 in distinguishing malignant from benign lung lesions

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Abstract: **To evaluate the potential value of the plasma level of receptor- interacting protein kinase 3 (RIPK3), a key protein involved in the activation of necroptosis, as a biomarker for the differential diagnosis of lung cancer in patients with lung lesions detected by chest computed tomography. The subjects of this study were divided into 3 groups: lung cancer (n = 30), benign disease (n = 13), and healthy controls (n = 33). The measurement of plasma RIPK3 was performed by enzyme-linked immunosorbent assay. The plasma RIPK3 level in patients with lung cancer (1467.4 ± 347.4 pg/ml) was significantly higher than that in patients with benign disease (1219.8 ± 156.4 pg/ml) and healthy controls (746.2 ± 255.3 pg/ml). Besides, plasma RIPK3 levels that were greater than 970.06 pg/ml provided 96.7% sensitivity and 84.8% specificity for lung cancer. Our results show that plasma RIPK3 levels are higher in lung cancer patients. We suggest that plasma RIPK3 level could be used as an auxiliary tool for distinguishing lung cancer from benign lesions and healthy lungs.**

Keywords: Lung cancer; Necroptosis; Receptor-interacting protein kinase 3

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1. Introduction

Lung cancer is the major cause of cancer-associated mortality. Millions of new cases occur every year, causing lung cancer to be a leading health challenge globally[1]. Chest computed tomography (CT) is increasingly used in patient evaluation, leading to the rising detection rates of lung lesions, which may be caused by various pathological processes[2]. Often, clinicians are faced with the challenges of accurate diagnosis of lung cancer and avoidance of invasive evaluation methods. An increasing number of studies have discovered a programmable form of necrosis characterized as "necroptosis", initially identified as a caspase-independent cell death induced by tumor necrosis factor (TNF)[3]. The core necroptotic pathway involves a complex called "necrotomic," mainly composed of receptor-interacting protein kinase 1 (RIPK1/RIP1), RIPK3, and mixed lineage kinase domain-like protein (MLKL)[4]. RIP1 activates RIPK3 through their RIP homotypic interaction motif (RHIM), while RIPK3 is the molecular switch for necroptosis[5]. MLKL, the key downstream component of RIPK3, directly eliminates cells through downstream events such as reactive oxygen species (ROS) burst, plasma membrane permeabilization, and cytosolic ATP reduction[6-8].

RIPK3-dependent necroptosis is involved in many pathophysiological processes and diseases such as ischemia-reperfusion injury, stroke, necrotizing pancreatitis, autoimmune disorders, some types of neoplastic diseases, and inflammatory conditions[9]. Accumulating evidence indicates that necroptosis also plays a role in the regulation of cancer therapy[4,10].

However, little is known about the role of plasma RIPK3 in lung cancer patients. In this study, we measured and compared the plasma RIPK3 levels in patients with pulmonary lesions detected by chest CT and healthy volunteers, in order to examine the differential diagnosis and prognosis assessment value of plasma RIPK3 in lung cancer. Additionally, we analyzed the association between plasma RIPK3 and clinicopathological characteristics of lung cancer patients.

2. Materials and methods

2.1. Subjects

The protocol used in this study was approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University, China. All patients and healthy volunteers recruited from the above hospital between September 2017 and March 2018 provided written informed consent. Patients enrolled were without any of the following: antitumor or anti-inflammation treatment in 6 months; other malignancies or severe organic disorders; receipt of any blood products within 30 days. Age- and sex-matched volunteers without a significant medical history were enrolled as controls. All lung lesions were diagnosed by pathology obtained through CT-guided transthoracic biopsy or trans-bronchial needle aspiration. The diagnosis of cancer was in accord with World Health Organization criteria [11]. Age, sex, and smoking status of each patient were extracted from medical records.

2.2. Plasma collection

Blood samples were collected into EDTA-2K

containing tubes (Becton Dickinson, Beijing, China), before lung biopsy and other treatments, following an overnight fasting period of 12 hours.

2.3. Clinical and laboratory tests

An automatic biochemistry analyzer (SYSMEX, KOBE, Japan) was used in the clinical laboratory of our hospital for biochemistry tests including blood routine, liver function, and carcinoembryonic antigen (CEA) tests. The biochemical determinations were performed on the same day the blood was collected. For plasma RIPK3 measurements, another aliquot of each blood sample was centrifuged at 3000 rpm for 10 min, and the cell-free samples were stored at -80°C. After total enrolment, enzyme-linked immunosorbent assay (ELISA, kits from CUSABIO, Wuhan, China) was used to detect plasma RIPK3 according to the manufacturer's instructions.

3.3. Statistical analysis

Data were presented as means ± SD. The student's t-test and one-way ANOVA were used to compare the data. Correlations were examined by Pearson's correlation tests. For assessing the diagnostic value of RIPK3, receiver operating characteristics (ROC) curves were constructed to calculate the cut-off values for best sensitivity and specificity. All P values shown were two-sided with statistical significance established at a P-value less than 0.05. Prism 7 (GraphPad software) and SPSS 23.0 (SPSS Inc, Chicago, IL, USA) software were used for statistical analysis.

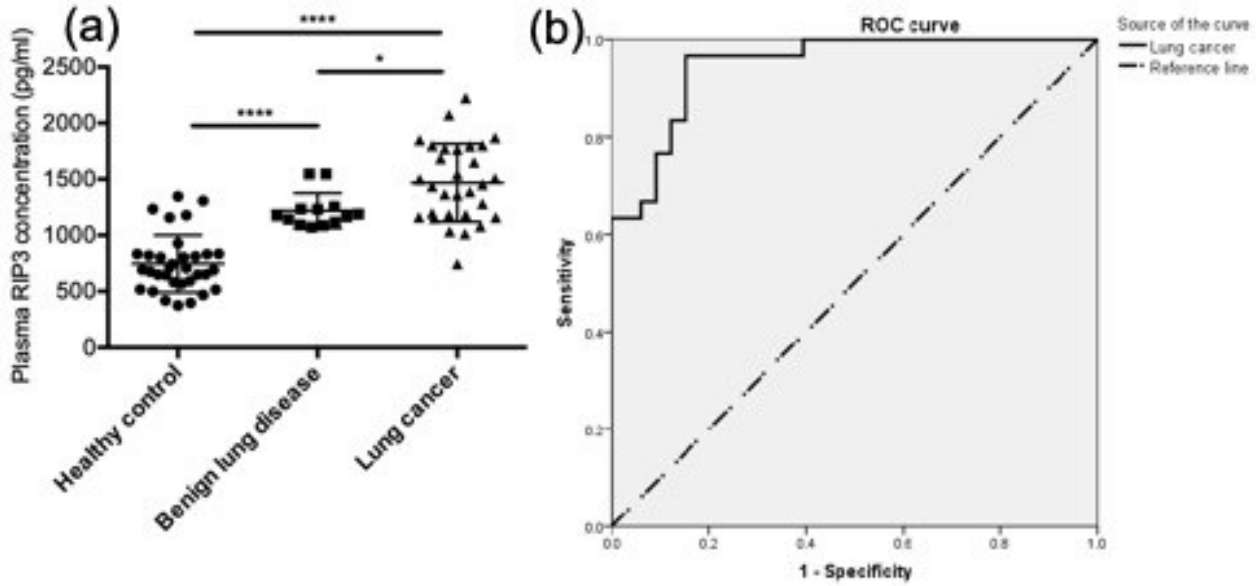

Figure 1. Plasma RIPK3 levels in patients and healthy controls (a) Levels of plasma RIPK3 were significantly higher in patients with lung cancer than that in patients with benign disease (*P < 0.05) and in healthy controls (**P < 0.0001). (b) The receiver operating characteristic curve indicated plasma RIPK3 could be used for identification of lung cancer (AUC = 0.95).**

3. Results

3.1. Patient demographics

In total, 43 patients with initially unknown pulmonary lesions, and 33 healthy controls participated in this study. Table 1 shows the clinicopathological characteristics of patients. There were no statistical differences in sex, age, and smoking status among these three groups (P = 0.13, P = 0.10, and P = 0.82, respectively). Among 43 patients, 30 were diagnosed with lung cancers and 13 were diagnosed with benign diseases by pathology. The pathologic types of lung cancers were as follows: 11 adenocarcinomas (ADC), 9 squamous cell carcinomas (SCC), 8 small cell lung cancer (SCLC), and 2 large cell lung cancer (LCLC). There were 4 patients with pneumonia, 4 with inflammatory nodules, 2 with tuberculosis, and 3 with lung abscess in the benign disease group.

3.2. The differential diagnosis value of plasma RIPK3

As shown in Figure 1a, plasma RIPK3 levels were significantly higher in patients with lung cancer (1467.4 ± 347.4 pg/ml) than those in patients with benign disease (1219.8 ± 156.4 pg/ml, P = 0.0186), and healthy controls (746.2 ± 255.3 pg/ml, P < 0.0001). Additionally, according to ROC curves (Figure1b), plasma RIPK3 levels could help to make a preliminary diagnosis of lung cancer with an area under the curve (AUC) value of 0.95 (95% CI, 0.89–0.99). Using a

cut-off value of 970.06 pg/ml, the sensitivity and specificity predictive values were 96.7% and 84.8%, respectively.

Table 1. Characteristics of all patients and healthy controls

	Lung cancer (n=30)	Benign disease (n=13)	Healthy control (n=33)	P value
Age (year)	61.63(1.88)	56.46(3.56)	56.73(1.34)	0.10
Male gender(%)	19(63.3)	8(61.5)	20(60.6)	0.13
Smoker(%)	11(36.7)	7(53.8)	12(57.1)	0.82
WBC(x 10^9 L)	6.85(0.49)	6.81(0.56)		0.96
Neutrophil(x 10^9 L)	4.53(0.42)	4.03(0.49)		0.49
CEA(ng/ml)	8.96(1.73)	8.45(5.89)		0.91
HGB(g/l)	131.80(3.2)	133.84(4.74)		0.73
ALB(g/l)	38.24(1.48)	38.08(1.26)		0.95

WBC: white blood cell, CEA: carcinoembryonic antigen, HGB: hemoglobin; ALB: albumin

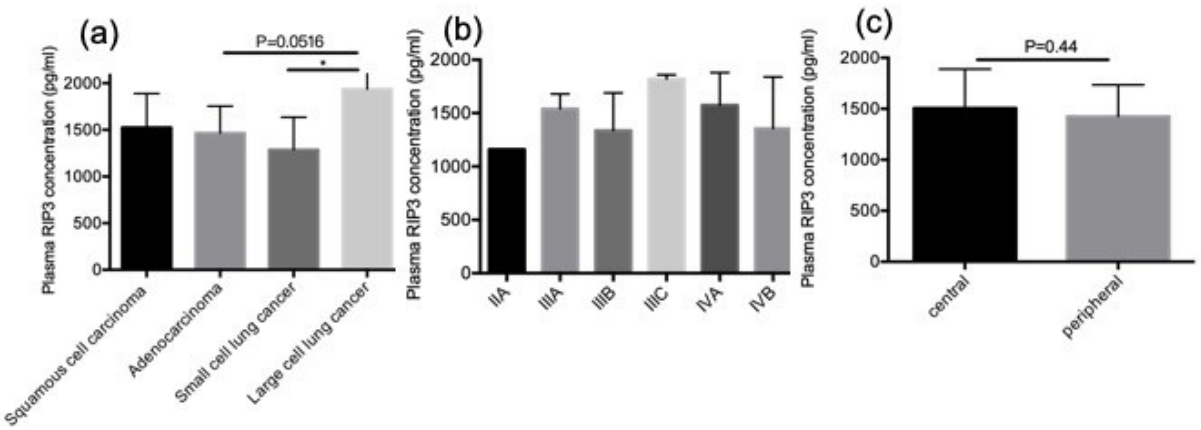

Figure 2. Different plasma RIPK3 levels in various lung cancer types (a) In analysis by tumor histology, plasma RIPK3 was significantly higher in patients with LCLC than SCLC (*P＜0.05). (b) Statistical difference of plasma RIPK3 was not observed according to TNM stage (P＞0.05). (c) There was no statistical difference of plasma RIPK3 levels in terms of the location of lung cancer (P＞0.05).

3.3. Different plasma RIPK3 levels in various lung cancer types

Plasma RIPK3 levels were further analyzed according to the lung cancer classification and staging. In cases categorized by histology, a statistical significance was found between patients with LCLC and SCLC (1287.5 ± 347.2 pg/ml versus 1287.5 ± 347.2 pg/ml, P = 0.0383; Figure 2a). As shown in Figure 2b and 2c, no statistical differences were observed in plasma RIPK3 levels in terms of tumor, node, and metastasis (TNM) staging and the general tumor location.

3.4. The prognostic value of plasma RIPK3 in lung cancer

Patients with lung cancer were followed up until August 2018. The endpoint was extracted from their medical records, and attempts were made to contact patients by telephone to obtain information of their present survival status since the last inquiry.

According to Figure 3, plasma RIPK3 levels were negatively associated with the prognosis of lung cancer patients. Patients with an improved condition had a significantly lower level of plasma RIPK3 (1101.1 ± 84.1 pg/ml), compared with patients with an unchanged condition (1400.1± 250.8 pg/ml; P = 0.0322), and patients with a worsened condition (1633.7 ± 262.2 pg/ml; P =0.0003). However, no statistical significances were observed between patients who died and patients with an unchanged or improved condition.

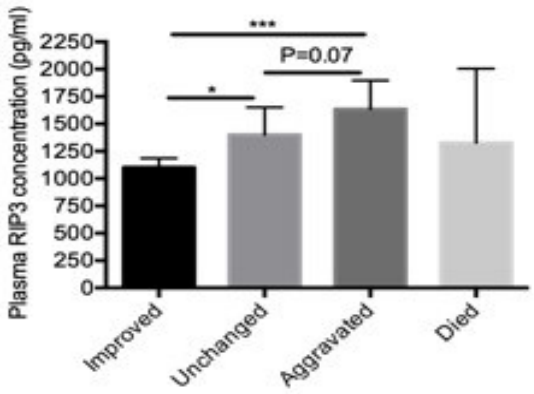

Figure 3. Roughly, plasma RIPK3 was negatively related with the prognosis of patients.

3.5. The association of plasma RIPK3 with clinicopathological characteristics of lung cancer patients

As shown in Figure 4, there were no significant differences among plasma RIPK3 levels in terms of sex, age, smoking status, and plasma CEA levels. However, a significantly positive relationship was observed between plasma RIPK3 levels and the white blood cell (WBC) count, and the neutrophil count (P=0.0337, P=0.0303, respectively). Moreover, we found plasma RIPK3 levels to be negatively correlated with the hemoglobin (HGB) count, and albumin (ALB) level (P=0.0970, P=0.0019, respectively).

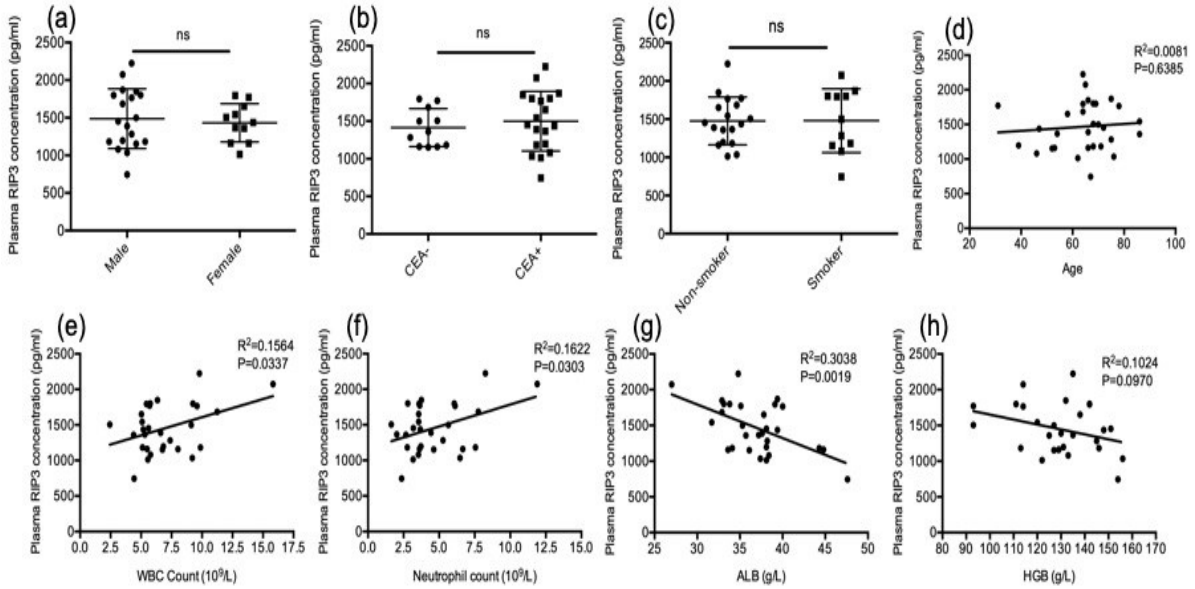

Figure 4. The association of plasma RIPK3 with clinicopathological characteristics of lung cancer patients (a-d) Plasma RIPK3 levels were not significantly different in terms of sex, age, status of smoking, and plasma CEA levels in patients with lung cancer (P＞0.05）. (e-f) Plasma RIPK3 levels were positively related to the count of WBC and Neutrophils (R^2= 0.1564, P < 0.05; R^2=0.1622; P < 0.05; respectively). (g-h) Plasma RIPK3 levels were negatively correlated with ALB and HGB levels.

4. Discussion

Evasion of cell death, an important feature of tumors, is a dominant cause of therapeutic resistance and failure. RIPK3-dependent necroptosis is involved in various pathological states, and the potential therapeutic role of necroptosis in cancers has aroused wide attention[12-13]. According to our data, plasma RIPK3 levels were elevated in patients with lung cancer. While a significant difference was observed in plasma RIPK3 levels between lung cancer patients and healthy controls (P < 0.0001), plasma RIPK3 levels could also help distinguish lung cancers from benign lesions (P < 0.05), which may be limited by the type of benign disease. The ROC curve we constructed also indicated that plasma RIPK3 had a high value in predicting the lung cancer risk. Next, we compared plasma RIPK3 levels in different stages and types of lung cancer. Even though the differences in plasma RIPK3 levels among lung cancers in different TNM stages were not statistically significant, we saw a tendency of higher staged cancers to have a higher level of plasma RIPK3. While there was a statistical significance between the different RIPK3 levels in SCLC and LCLC, no such difference was found between other histological types. Furthermore, plasma RIPK3 levels were not affected by the lung tumor location.

We also found that in lung cancer patients, the higher levels of plasma RIPK3 were negatively

correlated with the prognosis, while the mortality rate may be limited by the sample size. Conversely, a recent study demonstrated that RIPK3-dependent necroptosis was positively associated with clinical prognosis of non-small cell lung cancer patients after radiation therapy. Particularly, patients with high expression levels of RIPK3 in cancer cells had a significant trend toward improvement in survival[14]. However, another study reported that RIPK3 was highly expressed in pancreatic ductal adenocarcinoma compared with the normal pancreas, and demonstrated that in vivo RIPK3 deletion was protective against oncogenic progression[15]. Similarly, in renal malignant cells, high-grade tumors displayed a higher expression of RIPK3 compared with the normal tubular epithelium and low-grade tumors, and RIPK3 levels were up-regulated by TNF[16]. Nevertheless, a majority of studies reported that RIPK3 was significantly down-regulated in tumors, and lung tumor growth could be suppressed by inducing necroptosis[17-19]. The contradictory results on RIPK3 expression and RIPK3-dependent necroptosis in cancers may be attributed to different cancer cell lines or sample types used in the above studies. For instance, a study that evaluated the expression levels of RIPK3 in 6 different lung cancer cell lines using western blot assays reported that RIPK3 expression was higher in LTEP- A2 and SPC-A1 cells, whereas no RIPK3 expression was detected in A549, H520, H460 and H292 cells[14].

We postulated that the given discrepancies in RIPK3 expression levels may be due to the complex mechanisms of necroptosis involved in tumorigenesis. Since evading death is a basic mechanism tumors use for survival, undoubtedly, cells expressing lower levels of RIPK3 may have a greater potential to survive. Additionally, it has been proposed that necroptotic tumor cells could release factors that induce tumor-stimulating immune-suppression, thus leading to the development or progression of tumors. As an intracellular protein, RIPK3 in plasma could indicate the presence of cell programmed necrosis. Although the intracellular materials of necrotic cells will be released into the internal environment, all of the RIPK3 present may not be completely due to necroptosis[20]. Besides, a study conducted on mice with an endothelial cell-specific deletion of RIPK3, lead to reduced tumor cell-induced endothelial necroptosis and tumor cell metastasis[21]. The study explained that dying endothelial cells provide a gap through which tumor cells can pass and extravasate. It is also probable that damage-associated molecular pattern (DAMP) molecules, which could be released from necroptotic cells, act on nearby cells and then promote tumor development. It is conceivable that a tissue or organ invaded or infiltrated by a tumor could be damaged, and necroptosis of these cells is likely to contribute to increased plasma RIPK3 levels. However, if tumor cells could tolerate necroptosis, they may gain promoting factors from recruited inflammatory cells, thus developing to worse conditions. Moreover, it is widely accepted that necroptosis is a pro-inflammatory process. Changes in systemic inflammation can be assessed by detecting levels of hematological parameters. Our results consistently showed that plasma RIPK3 levels were positively related to the WBC and neutrophil counts. It is known that the neutrophil count in blood is higher than any other kind of blood cells, but the survival period of neutrophils is short with 2-3 days. We postulated that part of the increased plasma RIPK3 may be derived from neutrophils. Emerging studies have indicated that inflammation is of increasing importance in malignancies and could act as an independent prognostic factor[22]. Interactions between inflammation and cancer are much too complex, and the role of inflammation in tumorigenesis is still under investigation. It is thought that the excess number of circulating neutrophils has a direct or indirect role in the progression of tumors and tumor angiogenesis. Moreover, the immune inflammatory cells recruited by the inflammatory factors released by necrotic cells may promote cancer cell proliferation[23].

We also conducted correlation analysis between plasma RIPK3 levels, and clinical characteristics of patients with lung cancer, such as sex, age, status of smoking, and plasma CEA levels, which demonstrated that RIPK3 levels in peripheral blood were not affected by these parameters. However, RIPK3 levels were negatively correlated with levels of HGB and ALB, low levels of which could reflect the poor nutritional status of cancer patients. Consistently, increasing studies show that anemia and nutritional deficiency are common in cancer patients, while their presence predicts inferior overall prognosis[24-25]. Interestingly, it has shown that ALB could modulate inflammatory reactions and protect against cancer because of its antioxidant effects[26]. While RIPK3 is involved in reactive oxygen species (ROS) production, the relationship between RIPK3 and ALB merits further investigation.

Our study is limited by the relatively small number of patients and controls, and especially those with proven benign lesions. Besides, lack of analysis of overall survival could also abate the prognostic value of plasma RIPK3. In addition, we cannot exclude that, in some cases, biopsies of unclear lesions were collected according to different means, which might have introduced further bias.

5. Conclusion

Although these factors limit us in drawing a definite conclusion, we provide useful information as the first study to evaluate the diagnostic and discriminative power of plasma RIPK3. In conclusion, our results indicate that plasma RIPK3 could serve as a

non-invasive diagnostic biomarker for lung cancer, which may not only distinguish cancer patients from the healthy population, but may also discriminate lung cancers from non-malignancies. Additionally, a negative relationship between plasma RIPK3 levels and prognosis of cancer patients was also suggested. However, further investigations to validate the clinical application of RIPK3 in tumorigenesis are presently under way.

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Availability of data and materials

All data generated and/or analyzed during the present study are included in this published article.

Ethical approval

All procedures performed in studies were in accordance with the ethical standards of the ethics committee of the Qingdao University.

Patient consent for publication

Informed consent was obtained from all individual participants included in the study.

Competing interests

The authors declare that they there is no conflict of interest.

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