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Original Article

Ribonucleotide reductase small subunit M2 expression and its clinical significance in lung adenocarcinoma tissues

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Abstract: Lung cancer is one of the most lethal cancers worldwide. At present, cancer cells metastasis becomes the hot spot. Related studies showed that ribonucleotide reductase small subunit M2 (RRM2) is associated with tumor cell invasion, metastasis, and angiogenesis. It can activate a series of proto-oncogenes to promote tumor growth. This study investigates RRM2 expression and clinical significance in lung adenocarcinoma tissue. RT-PCR and immunohistochemistry were applied to test RRM2 gene and protein expression in 27 cases of lung adenocarcinoma tissue and para-carcinoma tissue. Correlation analysis was performed between its expression and clinicopathlogical characteristics. The relative RRM2 mRNA expression was 0.87 ± 0.62 in adenocarcinoma and 0.21 ± 0.77 in para-carcinoma ($P<0.05$). RRM2 positive expression rate in adenocarcinoma was obviously higher than that in normal control ($P<0.05$). RRM2 protein overexpression was correlated with TNM stage and metastasis ($P<0.05$). RRM2 highly expressed in lung adenocarcinoma. Its elevation is related to TNM stage and metastasis. It may be treated as new biomarker for lung cancer early diagnosis.

Keywords: RRM2, lung adenocarcinoma, RT-PCR, immunohistochemistry, biomarker

Introduction

Tumor tissue pathology classification plays a key role in treatment effect [1]. The complexity and heterogeneity of tumor histologic classification affect conventional treatment method [2]. Lung adenocarcinoma (LUAD) is the most common pathological type of lung cancer. It belongs to non-small cell lung cancer and is the leading cause of cancer related death [3]. In Europe, its five-year survival rate is only 11.5% [4]. In spite of treatment technology progress, the number of patients died of lung cancer did not decreased significantly that mainly due to tumor metastasis and recurrence [5]. Related research demonstrated that earlier treatment can elevate lung cancer five-year survival rate. The five-year survival rate of NSCLC in stage IA and IB stage is significantly higher than that in stage IIIA and IIIB, whereas the advanced patients often received chemotherapy [6]. Thus, early diagnosis and treatment of lung cancer is really important. Currently, the key and difficult point for lung cancer research is early diagnosis, recurrence, and metastasis. There are many scholars committed to lung cancer biomarkers investigation, but only a few can be applied in clinic. Ribonucleotide reductase small subunit M2 (RRM2) is one of human nucleotide reductase subunits that plays an important role in tumor development. This study investigates RRM2 expression and clinical significance in lung adenocarcinoma tissue using RT-PCR and immunohistochemical method to detect RRM2 expression in lung adenocarcinoma tissue, and analyzing its correlation with clinicopathological features.

Materials and methods

General information

A total of 27 cases of LUAD patients received surgery between Feb. 2015 and Dec. 2015 in First Affiliated Hospital of Henan University of Science and Technology were enrolled, including 12 males and 15 females. The mean age was 61.83 ± 8.59 (52-75) years old. There were 10 cases in stage I-II and 17 cases in stage III-

Table 1. RRM2 protein expression in LUAD tissue and normal lung tissue

Group	Cases	RRM2 protein expression				U/χ^2 value	*P* value
		-	1+	2+	3+		
LUAD tissue	27	0	4	10	13	-3.809	<0.01
Normal lung tissue	27	6	10	8	3		

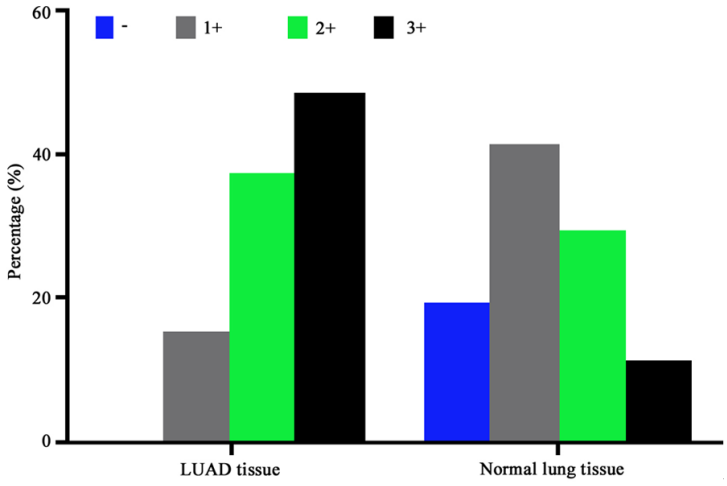

Figure 1. Immunohistochemistry detection of RRM2 expression in LUAD and normal lung tissue.

IV. All the patients had been diagnosed as LUAD without therapy before surgery.

This study was approved by ethics committee in First Affiliated Hospital of Henan University of Science and Technology and all the enrolled objects had signed informed consent.

RT-PCR

Tissue RNA was extracted using Trizol according to the manual. Its integrity was identified by agarose gel electrophoresis. RNA was reverse transcripted to cDNA by 42°C for 60 min and 95°C for 5 min [7]. The cDNA was used for PCR amplification. The RRM2 primers sequences were as follows: F-TGAACTGAAGATGTGCCCTTAC, R-TTACGGACAATTCATGGTGTG [8]. Real time PCR reaction condition was composed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s [9] and performed on ABI 7500 Real time PCR system. GAPDH was selected as internal reference. The relative expression level was calculated by $2^{-\Delta\Delta t}$ method.

Immunohistochemistry

The tissue was sliced at 4-5 pm after paraffin embedding and fixed. After roasted at 70°C for 10-20 min, the section was dewaxed by dimethyl benzene and ethanol. After repaired by sodium citrate buffer (pH 6.0), the section was treated with 3% H_2O_2 (80% methanol) to quench endogenous peroxidase. Nonspecific noise was blocked by goat serum. Next, the section was incubated in RRM2 primary antibody at 4°C overnight and secondary antibody. After redyed by hematoxylin for 40 s and hydrochloric acid alcohol differentiation, the section was examined under microscope [10]. The results were evaluated by semi-quantitative method. Two investigators judged the results under 200 times microscope, respectively. Tan granule appeared in the cell was considered as positive. Judgment criteria included positive cell number and staining intensity: (1) positive cells <10%, 0 point; 10-50%, 1 point; >50%, 2 points. (2) staining intensity: light yellow, 1 point; tan, 2 points; brown, 3 points. Protein expression was calculated as the product of two scores, 0 point, "-"; 1 point, "1+"; 2-3 points, "2+"; 4-6 points, "3+" [11].

Statistical analysis

All data was analyzed using SPSS 19.0 software. Measurement data was compared by t test or ANOVA. Ranked data was compared by rank sum test. P<0.05 was considered as statistical significance.

Results

RRM2 protein expression in LUAD tissue and normal lung tissue

Immunohistochemistry was applied to detect RRM2 protein level in LUAD tissue and normal lung tissue. The results were evaluated according to the dyeing depth. Expression intensity was divided as "negative", "1+", "2+", and "3+". As shown in **Table 1**, the positive expression rate of RRM2 in LUAD tissue was 100%, while it was 77.8% in the normal lung tissue, RRM2 protein expression in normal lung tissue was obviously lower than that in LUAD tissue (P<0.01) (**Figure 1**).

Table 2. Correlation analysis of RRM2 protein expression and clinicopathological features

Clinicopathological features		Cases	RRM2 protein expression			U/χ^2 value	*P* value
			1+	2+	3+		
Gender							
Male		12	2	4	6	-0.053	>0.05
Female		15	2	6	7		
Age							
<60		10	1	4	5	-0.302	>0.05
≥60		17	3	6	8		
Tumor size							
T1	d≤3 cm	11	2	5	4	0.893	>0.05
T2	3 cm<d ≤7 cm	9	1	3	5		
T3	d>7 cm	7	1	2	4		
Clinical stage							
I		5	3	1	1	7.895	0.019
II		9	4	4	1		
III		13	1	4	8		
Mediastinal lymph node metastasis							
+		18	0	7	11	-2.56	0.017
-		9	4	3	2		

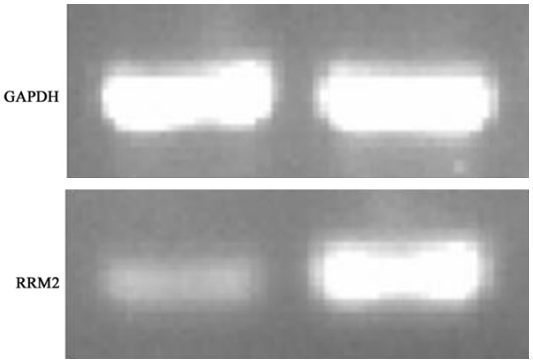

Figure 2. RRM2 mRNA expression in LUAD tissue and normal lung tissue.

Table 3. RRM2 mRNA expression in LUAD tissue and normal lung tissue

Sample	$2^{-\Delta\Delta Ct}$	*P* value
LUAD tissue	0.87 ± 0.62	<0.01
Normal lung tissue	0.21 ± 0.77	

Clinicopathological features analysis

RRM2 protein expression correlation analysis was performed with clinicopathological features, including gender, age, tumor size, clinical stage, and metastasis. The results revealed that RRM2 elevation was related to clinical stage and metastasis (P<0.05) (**Table 2**).

RRM2 mRNA expression in LUAD tissue and normal lung tissue

RT-PCR was used to determine RRM2 mRNA expression in LUAD tissue and normal lung tissue (**Figure 2**). $2^{-\Delta\Delta Ct}$ method was performed to calculate its level (**Table 3**). The relative RRM2 mRNA expression was 0.87 ± 0.62 in adenocarcinoma and 0.21 ± 0.77 in para-carcinoma (P<0.01).

Clinicopathological characteristics analysis

RRM2 mRNA expression correlation analysis was applied with clinicopathological characteristics, including gender, age, tumor size, clinical stage, and metastasis. The results demonstrated that RRM2 elevation was related to clinical stage and metastasis (P<0.05) (**Table 4**).

Discussion

NSCLC is a major histological type of lung cancer, while LUAD accounts for >40% [12]. Su-rgical treatment is usually the early stage treatment, whereas most of the patients are in advanced stage at diagnosis. Only about 30-40% of patients do not appear metastasis at diagnosis [8]. In addition, most patients will appear relapse in the following next two years, and the five-year survival rate is extremely low. Therefore, searching for new biomarkers is really important for early diagnosis and prognosis improvement. Up to now, numerous potential markers are discovered. For instance, Zhao J found that cytidine and uridine guanosine binding protein (CUGBP1) overexpression could increase adenocarcinoma recurrence rate and worse prognosis make the patients with poor prognosis [13]. It was found that CUGBP1 could be used to predict patients' prognosis in stage IB. Moreover, EGFR, ERCC1, RRM1, and TUBB-3 were also found abnormally distributed in

Table 4. Correlation analysis of RRM2 mRNA expression and clinicopathological characteristics

Clinicopathological features		Cases	RRM2 mRNA expression $2^{-\Delta\Delta Ct}$	F value	*P* value
Gender					
Male		12	0.84 ± 0.36	0.031	>0.05
Female		15	0.89 ± 0.27		
Age					
<60		10	0.83 ± 0.49	0.936	>0.05
≥60		17	0.88 ± 0.62		
Tumor size					
T1	d≤3 cm	11	0.81 ± 0.39	0.816	>0.05
T2	3 cm<d≤7 cm	9	0.85 ± 0.71		
T3	d>7 cm	7	0.88 ± 0.38		
Clinical stage					
I		5	0.61 ± 0.73	10.013	<0.01
II		9	0.79 ± 0.39		
III		13	0.97 ± 0.54		
Mediastinal lymph node metastasis					
+		18	0.96 ± 0.81	8.035	0.011
-		9	0.73 ± 0.94		

NSCLC [14]. To increase lung cancer diagnosis, more biomarkers need to be excavated. This study applied RT-PCR and immunohistochemistry to test RRM2 mRNA and protein expression in LUAD tissue, and analyze its level with clinicopathological features.

The main role of ribonucleotide reductase is to catalyze four types of ribonucleotide reduction and generate the corresponding enzymes. Study revealed that nucleotide reductase activity was closely related to cancer cells division and differentiation. As the component of ribonucleotide reductase, RRM2 is the key enzyme of cell cycle DNA replication. It plays a vital role in activating tumorigenesis and development process [15]. Related studies reported that RRM2 was highly expressed in multiple tumors, including ovarian cancer, stomach cancer. Furthermore, its overexpression was related to tumor grade and survival time [16, 17]. Tumor angiogenesis plays a key role in deterioration process. Zhang K showed that RRM2 can promote tumor angiogenesis by regulating thrombospondin 1 (TSP-1) and vascular endothelial growth factor (VEGF), thus accelerate cancer deterioration [18]. In addition, other studies demonstrated that RRM2 can be used as biomarker for tumor diagnosis and prognosis evaluation. Su YF found that RRM2 participated in cervical cancer development and could be used to predict survival through cell biology experiments and tissue microarray [19]. Morikawa T and Liu X demonstrated that RRM2 was associated with tumor invasion and metastasis, while RRM2 siRNA can obviously inhibit tumor cell growth and weaken invasive ability [20, 21].

Following the development of molecular biological tec hnology, how to improve the survival rate of lung cancer patients becomes a hotspot. Finding new early biomarkers is imminent to achieve early diagnosis and early treatment. RRM2, as a biomarker, is widely investigated in other cancers. Grossi F used microarray found that RRM2 was significantly associated with patient overall survival, and RRM2 overexpression can be used to predict poor prognosis [8]. Whether it could be used as molecular marker in lung cancer early diagnosis still needs further investigation. This study applied RT-PCR and immunohistochemistry to test RRM2 gene and protein expression in LUAD tissue, and analyzed its correlation with clinicopathological features. The results revealed that RRM2 positive expression rate in LUAD tissue reached 100%, and its mRNA and protein levels in LUAD tissue were obviously higher than that in normal lung tissue. Furthermore, its elevation was related with clinical stage and metastasis. It can be predicted that lower RRM2 level may lead to decreased DNA repair function, which is in favor of disease control. In addition, our results confirmed that RRM2 expression was related to metastasis, which supported that RRM2 may promote tumor angiogenesis and accelerate deterioration [18]. RRM2 could be used as a treatment target to elevate lung cancer curative effect.

To sum up, DNA repair plays an important role in cancer occurrence and development. This study found that RRM2 protein and mRNA

upregulated in lung cancer tissue, suggesting its level was closely relate to lung cancer. RRM2 overexpression may cause patient deterioration. It could be treated as a biomarker for lung cancer early diagnosis. This study provide basis for molecular marker investigation in lung cancer early diagnosis. The specific mechanism of RRM2 in lung cancer still needs further discuss.

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Disclosure of conflict of interest

None.

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