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RRM1 gene expression in peripheral blood is predictive of shorter survival in Chinese patients with advanced non-small-cell lung cancer treated by gemcitabine and platinum*

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Abstract: Objective: To evaluate the predictive values of gene expressions of ribonucleotide reductase M1 (*RRM1*) and breast cancer susceptibility gene 1 (*BRCA1*) in peripheral blood from Chinese patients with non-small-cell lung cancer (NSCLC) treated with gemcitabine plus platinum. Methods: Forty Chinese patients with advanced NSCLC were recruited and received gemcitabine 1200 mg/m^2 on Days 1 and 8 plus carboplatin AUC 5 on Day 1. *RRM1* and *BRCA1* expression levels in peripheral blood were detected by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Kaplan-Meier survival curve and log-rank test were performed to evaluate the correlation between gene expression and overall survival for these subjects. Results: No correlation was observed between gene expression of *RRM1* and that of *BRCA1* (P>0.05), but there was a strong correlation between the expression of *RRM1* and the response to chemotherapy (P=0.003). Subjects with low *RRM1* expression levels in peripheral blood had longer survival time than those with high *RRM1* expression levels (16.95 vs. 12.76 months, log-rank 3.989, P=0.046). However, no significant association between *BRCA1* expression levels and survival time was found (16.80 vs. 13.77 months, log-rank 0.830, P=0.362). Conclusions: Patients with low *RRM1* expression levels in peripheral blood have a greater response to chemotherapy and longer survival time. Advanced NSCLC patients with low *RRM1* expression levels may benefit from gemcitabine plus platinum therapy. *RRM1* mRNA expression in peripheral blood could be used to predict the prognosis of NSCLC treated by gemcitabine and platinum.

Key words: Gemcitabine, Ribonucleotide reductase M1 (*RRM1*), Breast cancer susceptibility gene 1 (*BRCA1*), Non-small-cell lung cancer (NSCLC), Gene expression

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1 Introduction

Lung cancer remains the leading cause of cancer death (Jemal *et al.*, 2007), and its overall five-year survival rate is still unsatisfactory. More than 75% of lung cancers are non-small-cell lung cancers (NSCLCs) at diagnosis. Recently, the combinations of third-generation cytotoxic agents (such as gemcitabine, vinorelbine, and taxane) and platinum have emerged as a new chemotherapy standard for NSCLC. In our previous phase II clinical trials in advanced NSCLC (Wang *et al.*, 2007b), the median survival time and one-year survival for the combination chemotherapy

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of platinum and gemcitabine were 8.2–14.8 months and 38.1%–41.2%, respectively. Retrospective studies of stage IV NSCLC have reported that patients with low ribonucleotide reductase M1 (*RRM1*) or excision repair cross completion gene 1 (*ERCC1*) messenger RNA (mRNA) levels had a median survival up to 15 months when treated with gemcitabine plus cisplatin, with more significant differences in survival according to *RRM1* levels (Rosell *et al.*, 2003; 2004a; Ceppi *et al.*, 2006).

Resistance to gemcitabine has been associated with *RRM1* overexpression (Goan *et al.*, 1999; Davidson *et al.*, 2004). RRM1 is a key enzyme involved in DNA synthesis, which could catalyze the biosynthesis of deoxyribonucleotide, and previous data showed that higher levels of RRM1 are associated with chemoresistance to gemcitabine-based therapies. A close correlation has also been observed between expression levels of *RRM1* and breast cancer susceptibility gene 1 (*BRCA1*) (Taron *et al.*, 2004; Rosell *et al.*, 2004b; 2007). *BRCA1* expression confers differential chemosensitivity in cancer cell lines (Quinn *et al.*, 2003; 2007). Low levels of *BRCA1* also correlated with increased survival in NSCLC patients treated with gemcitabine plus cisplatin (Rosell *et al.*, 2004b).

Hopefully, the search for genetic markers for predicting a better response may lead to customized chemotherapy. In order to further investigate the relationship between the *RRM1*/*BRCA1* expression in peripheral blood and the sensitivity to platinum plus gemcitabine, we collected the peripheral blood samples from 40 Chinese patients with advanced NSCLC treated by gemcitabine plus platinum, and analyzed the expressions of *RRM1* and *BRCA1*, as well as their clinical predictive significance.

2 Materials and methods

2.1 Patients and samples

Forty Chinese patients with histologically-confirmed stages III and IV NSCLC were recruited and the peripheral blood samples were collected. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the expression levels of *RRM1* and *BRCA1*. Subjects received gemcitabine (Gemzar; Eli Lilly, USA) 1200 mg/m^2 on Days 1 and 8 and carboplatin AUC 5 on Day 1. Patient evaluations were carried out at baseline and after every two cycles of chemotherapy (Wang *et al.*, 2007a; 2007b). The study protocol was approved by the Ethical Committee of the First Affiliated Hospital of Zhejiang University, China. All subjects signed an informed consent before entry into the study.

2.2 Gene expression analysis

Total RNA was purified from peripheral blood by column chromatography method (E.Z.N.A® Blood RNA Mini Kit; Omega, Berkeley, USA). Complementary DNA (cDNA) synthesis was performed using reverse transcription system (Promega, Madison, WI, USA) and the products were stored at −80 °C. Relative quantifications of gene expressions for *RRM1*, *BRCA1*, and *β-actin* (internal reference gene) were performed using quantitative RT-PCR [RealMasterMix (SYBR Green) Kit; TIANGEN, Beijing, China]. The PCR was carried out as follows: 50 °C for 2 min, an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 s, and annealing at 60 °C for 1 min. The sequences of the primers used were as follows: *RRM1* forward 5′-ACTAAGCACCCTGACTATGCTATCC-3′, reverse 5′-CTTCCATCACATCACTGAACACTTT-3′; *BRCA1* forward 5′-CCCATTTTCCTCCCGCA-3′, reverse 5′-GGACCTTGGTGGTTTCT-3′; *β-actin* forward 5′-TGAGCGCGGCTACAGCTT-3′, reverse 5′-TCCTTAATGTCACGCACGATTT-3′.

2.3 Statistical analysis

Statistical analyses were performed with SPSS software (Version 15.0; SPSS Inc., Chicago, IL, USA). For statistical analyses, the responses to the therapy were simply split into “responders” (partial responders) and “non-responders” (stable and progressive diseases). Mann-Whitney *t*-test was used to evaluate associations between the gene expression and clinical and pathological factors (including gender, tumor stage, smoker, and performance status; Kruskal-Wallis test for histology). Correlation between *RRM1* and *BRCA1* mRNA levels was evaluated by Spearman correlation coefficients. Log-rank test and Kaplan-Meier survival curves were used to analyze the potential association between each clinical factor and the survival rate. Factors with significant influence on survival in univariate analysis were further analyzed by multivariate Cox regression analysis.

3 Results

3.1 Patient characteristics

Three subjects dropped out of treatment due to drug side effects, and a total of 37 patients with a median age of 64 (range 24–77) years were included. The baseline characteristics of the 37 subjects are shown in Table 1. All of these subjects received gemcitabine plus platinum as first-line chemotherapy, including 4 subjects treated with gefitinib (an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor) as further therapy and 2 subjects with a concomitant radiotherapy treatment. Thirteen subjects were diagnosed with stage III NSCLC (including 5 of IIIA and 8 of IIIB) and 24 with stage IV. Overall mean survival time (MST) was 15.23 months (stage III 15.79 months, stage IV 14.55 months).

Table 1 Baseline characteristics of 37 Chinese patients with NSCLC

Characteristics	Number	Percentage (%)
Smoker		
Yes	15	40.5
No	22	59.5
Gender		
Male	27	73.0
Female	10	27.0
Performance status		
0	9	24.3
1	26	70.3
2	2	5.4
Stage		
III	13	35.1
IV	24	64.9
Histology		
Squamous cell carcinoma	12	32.4
Adenocarcinoma	25	67.6
Response to therapy		
Partial	12	32.4
Stable	16	43.3
Progressive	9	24.3
Metastasis sites		
Lung	7	18.9
Liver	3	8.1
Bone	13	35.1
Nodes	11	29.7
Others	8	21.6
Number of metastasis sites		
1	13	35.1
2	8	21.6
≥3	4	10.8

3.2 *RRM1* and *BRCA1* mRNA expression levels

RRM1 and *BRCA1* mRNA expression levels were analyzed by quantitative RT-PCR. Median mRNA expression levels were 1.14×10^{-2} (range 0.03×10^{-2}–20.15×10^{-2}, mean 3.46×10^{-2}, standard deviation 4.59×10^{-2}) for *RRM1*, and 7.87×10^{-2} (range 0.09×10^{-2}–98.47×10^{-2}, mean 17.68×10^{-2}, standard deviation 24.53×10^{-2}) for *BRCA1*. By adopting cut-off values according to median expression levels, no correlation was found between *RRM1* expression and the clinical factors including gender (P=0.122), stage (P=0.405), smoker (P=0.092), performance status (P=0.596), and histology (P=0.412). Similar results were observed between *BRCA1* expression and the clinical factors. However, a strong correlation between gene expression levels of *RRM1* and the response to chemotherapy was observed (P=0.003). Ten of nineteen subjects with low *RRM1* mRNA levels got partial response to chemotherapy (52.6%), and only two of eighteen patients with high *RRM1* mRNA levels got partial response (11.1%). Also, the percentages of responses in subjects with low and high *BRCA1* mRNA levels were 33.3% (6/18) and 31.6% (6/19), respectively, and no statistical significance was found (P>0.05).

3.3 Correlation between *RRM1* and *BRCA1* mRNA levels

No correlation was found between *RRM1* and *BRCA1* expression levels (P>0.05) by means of the Spearman's rank correlation method.

3.4 Overall survival time associated with clinical and pathological factors and gene expression

In the univariate analysis model of survival, the median value of *RRM1*/*BRCA1* gene expression was used as the cut-off value. It was found that MST in subjects with low *RRM1* mRNA levels was significantly longer than that in subjects with high *RRM1* mRNA levels (16.95 vs. 12.76 months, log-rank 3.989, P=0.046; Fig. 1a). However, no significant association between *BRCA1* expression levels and survival was found (16.80 vs. 13.77 months, log-rank 0.830, P=0.362; Fig. 1b), and no significant difference was observed on the overall survival time between patients with low *RRM1* and *BRCA1* expression levels and the others (18.01 vs. 13.92 months,

log-rank 1.801, P=0.180; Fig. 1c). Other selected factors including gender, stage, smoker, performance status, and histology were also not statistically correlated with the overall survival time (Table 2). In multivariate Cox regression analysis, mRNA expression levels of *RRM1* in peripheral blood appeared to be a possible predictive factor for response to chemotherapy with gemcitabine and cisplatin, and possibly for survival (Hazard ratio 2.586, 95% confidence interval (CI) 0.967–6.916, P=0.058; Table 2).

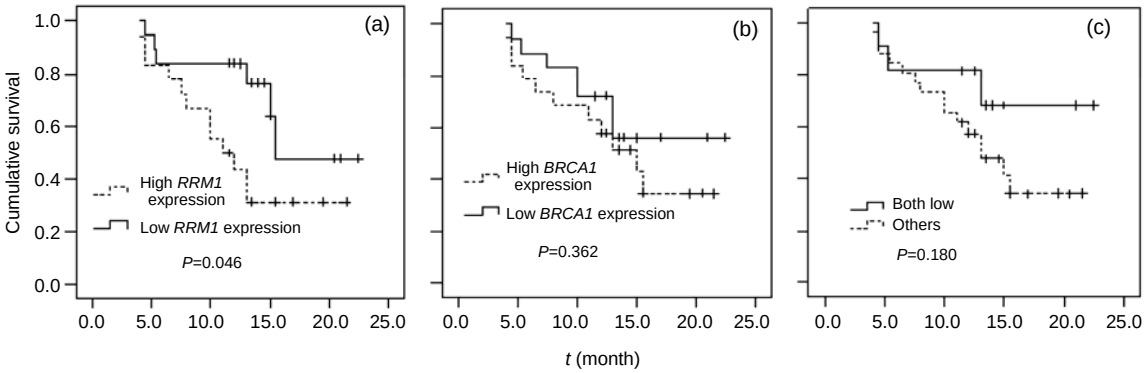

Fig. 1 Kaplan-Meier survival analyses for *RRM1* (a), *BRCA1* (b), and the concomitant low expressions of *RRM1* and *BRCA1* (c) in Chinese subjects with NSCLC according to median values of gene expression in overall population

Table 2 Factors associated with overall survival time of Chinese subjects with NSCLC

Factor and gene expression	Number of patients	Mean survival time (month)	Univariate analysis		Multivariate analysis		
			Log-rank	*P*	Hazard ratio	95% CI	*P*
Gender							
Male	27	15.11	0.044	0.833			
Female	10	15.10					
Stage							
III	13	15.79	0.005	0.943			
IV	24	14.55					
Smoker							
Yes	15	15.29	0.008	0.927			
No	22	14.36					
Performance status							
0	9	15.20	0.062	0.803			
1 or 2	28	14.88					
Histology							
Squamous cell carcinoma	12	13.95	0.484	0.487			
Adenocarcinoma	25	15.78					
Response							
Partial	12	19.50	8.392	0.004	10.55	1.40–79.41	0.022
Stable/progressive	25	12.88					
RRM1							
Low	19	16.95	3.989	0.046	2.586	0.967–6.916	0.058
High	18	12.76					
BRCA1							
Low	18	16.80	0.830	0.362			
High	19	13.77					
RRM1/*BRCA1*							
Both low	11	18.01	1.801	0.180			
Others	26	13.92					

4 Discussion

Many researchers have proven that DNA repair capacity is associated with the pathogenesis and drug-resistance of lung cancer. As one of the targets of gemcitabine, ribonucleotide reductase (RR) is a rate-limiting enzyme in the DNA synthesis pathway and is responsible for transforming bisphosphate nucleotide to bisphosphate deoxynucleotide. The latter is the necessary material for DNA synthesis and repair. Thus, RR is key for cell survival.

It was presumed that *RRM1* expression may be related to the resistance to gemcitabine that could interfere with the function of ribonucleotide reductase. Clinical studies have also shown that high expression level of *RRM1* was correlated with a poor prognosis in NSCLC patients. Gautam *et al*. (2003) detected the *RRM1* mRNA expression in tumor tissues of 22 NSCLC patients receiving the combination chemotherapy of cisplatin/gemcitabine by means of quantitative RT-PCR, and found that low expression level of *RRM1* mRNA of patients was associated with better responses to chemotherapy, higher impact on the time to progression (P=0.05), and higher survival rate (P=0.0028). In another retrospective study (Rosell *et al.*, 2004a), the survival time of NSCLC patients receiving cisplatin/gemcitabine chemotherapy with low expression levels of *RRM1* mRNA was found to be significantly longer than that for those with higher *RRM1* mRNA levels (13.7 vs. 3.6 months, P=0.009).

BRCA1 may also play an important role in NSCLC not only as a prognostic factor, but also as a valuable tool for choosing the optimal treatment regimen. Patients with negative or low expression of *BRCA1* were more sensitive to cisplatin combined with gemcitabine or etoposide, while those with positive expression of *BRCA1* were resistant to antimicrotubular drugs.

Several clinical studies have already shown that quantitative analyses of *RRM1* and *BRCA1* mRNA expression levels were mainly applied to tumor tissue from an operative resection and with sufficient amount. Given that the definite diagnosis of advanced NSCLC was mainly by cellular pathology, the main obstacle for detection was the difficulty of tumor tissue extraction from bronchoscopic biopsy and insufficient tissue samples. Therefore, clinical practice may need a simpler and more convenient detection method to realize individualized treatment for patients with NSCLC. A simple, convenient, and non-invasive method, such as assessment of *RRM1* and *BRCA1* mRNA expression levels in peripheral blood lymphocytes from NSCLC patients, could provide guidance for the treatment of NSCLC especially in advanced stage. Isla *et al*. (2004) detected *ERCC1* expression in peripheral blood by means of fluorescence quantitative PCR, but did not find a correlation between *ERCC1* expression and prognosis of advanced NSCLC. Dong *et al*. (2010) detected *RRM1* mRNA expression of peripheral blood mononuclear cells by fluorescence quantitative PCR method, and found that it showed a higher sensitivity and specificity and was suitable for clinical practice. Whether the mRNA expression levels of *RRM1* and/or *BRCA1* reflect the tolerance status of tumor cells to gemcitabine plus platinum is unclear.

In the present study, we recruited patients with advanced NSCLC and not suitable for operative treatment. All subjects received gemcitabine plus platinum as follows: intravenous gemcitabine 1200 mg/m^2 on Days 1 and 8; intravenous carboplatin AUC 5 on Day 1. The therapeutic effects were evaluated after every two cycles of chemotherapy, including gene expression levels of *RRM1* and *BRCA1* in peripheral blood and their prognostic significance. *RRM1* and *BRCA1* mRNA expression levels relative to housekeeping gene expression in peripheral blood of NSCLC subjects were assessed by fluorescence quantitative RT-PCR. Results showed that there was a significant difference in patients' survival due to the variable sensitivities to chemotherapy, in which low *RRM1* expression conferred longer survival. Furthermore, the results of multivariate Cox regression analysis also indicated that mRNA expression levels of *RRM1* in peripheral blood appear to be a possible predictive factor for response to chemotherapy with gemcitabine and cisplatin, and possibly for survival. Though it failed to reach statistical significance due to the small number of subjects, the trend could still be observed.

In summary, this study preliminarily validated the mRNA level of *RRM1* in peripheral blood as a determinant factor for the prognosis of NSCLC treated by gemcitabine and platinum. In addition, we would continue to investigate other possible factors in

peripheral blood such as *ERCC1* to further explore more potential molecular markers in predicting the prognosis of advanced NSCLC, and to supply a simple and convenient detection method to realize individualized treatment in clinical practice.

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