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Prognostic significance of the mRNA expression of *ERCC1*, *RRM1*, *TUBB3* and *TYMS* genes in patients with non-small cell lung cancer

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Abstract. The present study aimed to investigate the prognostic value of excision repair cross-complementing 1 (ERCC1), ribonucleotide reductase subunit M1 (RRM1), class III β-tubulin (TUBB3) and thymidylate synthase (TYMS) in patients with non-small cell lung cancer (NSCLC) receiving platinum-based adjuvant chemotherapy. The mRNA expression of these genes was assessed in 72 tumor tissue samples obtained following surgery, using multiplex branched-DNA technology. Subsequent to surgery, all 72 patients with NSCLC were treated with platinum-based chemotherapy. The expression of these five genes was analyzed and the correlation with clinical characteristics and patient survival was investigated. Among the 72 samples, the incidence rate of mRNA expression of *ERCC1* was 38.9% (28/72), *RRM1* was 55.6% (40/72), *TUBB3* was 47.2% (34/72) and *TYMS* was 62.5% (45/72). The incidence rate of *ERCC1* expression in adenocarcinoma (34.2%) was significantly lower than that in non-adenocarcinoma (44.1%; P<0.05). Furthermore, the incidence rates of *TYMS* and *TUBB3* expression in the high-median differentiation tissue samples were significantly lower than those in the low differentiation tissue samples (P<0.05). When the correlation of gene expression and patient survival was analyzed, high expression of *ERCC1, RRM1, TUBB3* or *TYMS* was found to be associated with poor prognosis (P<0.001, P=0.001, P=0.001 and P=0.001, respectively). ERCC1, RRM1, TUBB3 and TYMS are key factors involved in survival following surgical treatment in patients with NSCLC. The mRNA expression of these genes may have prognostic value for patients with NSCLC treated with platinum-based chemotherapy.

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Introduction

Lung cancer is one of the most prevalent types of malignant tumor in China and the overall five-year survival rate remains unsatisfactory. Approximately 80-85% of all lung cancer diagnoses are non-small cell lung cancer (NSCLC) (1). Adjuvant chemotherapy, for example platinum agents combined with cytotoxic agents, including vinorelbine, gemcitabine and taxane, has become a treatment standard for NSCLC (2). However, the resistance of tumor cells to these drugs leads to a poor prognosis and survival rate. Therefore, the investigation of tumor biomarkers associated with resistance to chemotherapeutic drugs is required.

Excision repair cross-complementing 1 (ERCC1) is a structure-specific DNA repair endonuclease responsible for the 5'-incision during DNA excision repair. Clinical studies have indicated that high *ERCC1* expression is associated with resistance to platinum-based chemotherapy (3). The ribonucleotide reductase subunit M1 (*RRM1*) gene, located on chromosome 11p15.5, encodes one of two non-identical subunits of the ribonucleoside-diphosphate reductase. The ribonucleoside-diphosphate reductase enzyme is essential for the production of deoxyribonucleotides prior to DNA synthesis. It has been reported that NSCLC patients with low *RRM1* expression have improved survival when treated with gemicitabine-based therapy (4). Thymidylate synthase (TYMS) is the enzyme used to generate thymidine monophosphate (dTMP), which is subsequently phosphorylated to thymidine triphosphate for use in DNA synthesis and repair. TYMS inhibitors, including fluorinated pyrimidine derivatives, are capable of inhibiting the activity of TYMS. Thus, TYMS expression is associated with *in vivo* chemosensitivity to TYMS inhibitors (5). Class III β-tubulin (TUBB3) encodes a class III member of the β-tubulin protein family. The overexpression of *TUBB3* is associated with resistance to docetaxel and paclitaxel (6,7).

Changes in the mRNA expression of the above tumor biomarkers during tumor development may have an impact on the prognosis of patients with NSCLC. In the present study, multiplex branched-DNA liquidchip technology was used to analyze the mRNA expression of the *ERCC1, RRM1, TUBB3* and *TYMS* genes in tumor tissue samples from patients with resected NSCLC prior to chemotherapy. The multiplex branched-DNA technology is a sandwich nucleic

acid hybridization method in which targets are captured through cooperative hybridization of multiple probes, then coupled with a fluorescence signal amplification system (8,9). The signals were detected using the Luminex® 200 system (Luminex, Austin, TX, USA). It is a multiplex assay that enables the measurement of the mRNA expression of >30 biomarkers in one test well (8). The present study focused on: i) the analysis of the correlation between gene expression and different clinical characteristics; and ii) the analysis of the correlation between gene expression and patient survival.

Materials and methods

Patient samples. A total of 72 patients with NSCLC at the General Hospital of People's Liberation Army (Beijing, China) who underwent curative surgery between March and October 2011 were enrolled in the present study. Informed consent was obtained from all patients. Patient information and clinical characteristics are shown in Table I. A total of 72 tissue samples were obtained following surgery and the tissues were fixed in 10% neutral formalin for 24 h, desiccated and embedded in paraffin. We confirmed the hematoxylin and eosin staining results of the 72 formalin-fixed paraffin-embedded (FFPE) tissue samples from the pathology department, which revealed that the tumor sections contained large numbers of tumor cells (usually >60%). For each FFPE tissue sample, the tumor tissue was cut using microdissection techniques and sent to Guangzhou Surexam Medical Test Center (SurExam Bio-Tech Co., Ltd., Guangzhou, China) for *ERCC1*, *RRM1*, *TUBB3* and *TYMS* mRNA expression analysis. This study was approved by the ethics comittee of the General Hospital of People's Liberation Army.

mRNA expression analysis of ERCC1, RRM1, TUBB3 and TYMS using multiplex branched-DNA liquidchip technology. Multiplex branched-DNA liquidchip technology measures mRNA expression without RNA extraction, reverse transcription or polymerase chain reaction amplification. FFPE tissue samples were homogenized at 65˚C for 1 h, then the supernatant was used for hybridization. Sandwich nucleic acid hybridization was performed for 16 h using magnetic capture beads containing probes for *ERCC1*, *RRM1*, *TUBB3* and *TYMS*, the homogenate and another set of capture probes for *ERCC1*, *RRM1*, *TUBB3* and *TYMS*. The hybridization reaction was performed in a 96-well plate. The unbound RNA was removed by washing three times with 250 μl wash buffer (0.1X saline sodium citrate and 0.03% lithium lauryl sulfate) under a vacuum system. Signal amplification steps were performed through incubation with 100 μl pre-amplifier solution for 1 h at 50˚C, followed by incubation with 100 μl amplifier solution for 1 h at 50˚C, then incubation with probes labeled with biotin for 1 h at 50˚C. Following washing, the samples were incubated with 100 μl streptavidin conjugated phycoerythrin solution at 50˚C for 30 min, prior to analysis using the Luminex 200 system (Luminex).

The fluorescence intensity value of *ERCC1*, *RRM1*, *TUBB3* and *TYMS* generated using the Luminex 200 system was normalized to that of the house keeping genes, including β 2-microglobulin, box binding protein and the transferrin receptor. The normalized values of *ERCC1*, *RRM1*, *TUBB3* and *TYMS* were compared to the cut-off value of each gene, which was provided by Guangzhou Surexam Medical Test Center. *ERCC1*, *RRM1*, *TUBB3* and *TYMS* mRNA expression was considered to be positive (high expression) if the normalized values were equal or higher than the cut-off value; otherwise, the result was considered to be negative (low expression).

Table I. Patient information and clinical characteristics.

Variable	No. of patients	Percentage
Age		
<60 years	42	58.3
≥60 years	30	41.7
Gender		
Male	47	65.3
Female	25	34.7
Smoking		
Yes	42	58.3
No	30	41.7
Histology		
Adenocarcinoma	38	52.8
Non-adenocarcinoma	34	47.2
Differentiation		
High-Mid	40	58.3
Low	32	41.7
Staging		
I/II	47	65.3
III	20	27.8
IV	5	6.9
Node metastasis		
Yes	25	34.7
No	47	65.3

Statistical analysis. Data were analyzed using the SPSS version 19.0 software package (SPSS, Inc., Chicago, IL, USA). The association between gene expression and different clinical characteristics was analyzed using the χ^2 test. The correlation between mRNA expression levels was analyzed using Spearman correlation coefficients. The Kaplan-Meier method was used to analyze the correlation between gene expression and patient survival. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Correlation between ERCC1, RRM1, TUBB3 or TYMS mRNA expression and clinical characteristics. Tumor specimens from 72 patients with NSCLC were subjected to mRNA expression analysis. Among the 72 samples, the incidence rate of a high mRNA expression level of the *ERCC1* gene was 38.9% (28/72), *RRM1* was 55.6% (40/72), *TUBB3* was 47.2% (34/72) and *TYMS* was 62.5% (45/72; Table II). The correlation between the expression of these four genes and different clinical characteristics was assessed. The incidence rate of

Table II. Correlation between mRNA expression of *ERCC1*, *RRM1*, *TUBB3* and *TYMS* and clinical characteristics.

Parameter	No. of patients	*ERCC1* high expression subgroup, n, (%)	*ERCC1* low expression subgroup, n, (%)	*RRM1* high expression subgroup, n, (%)	*RRM1* low expression subgroup, n, (%)	*TUBB3* high expression subgroup, n, (%)	*TUBB3* low expression subgroup, n, (%)	*TYMS* high expression subgroup, n, (%)	*TYMS* low expression subgroup n, (%)
Age									
<60 years	42	14 (33.3)	28 (66.7)	25 (59.5)	17 (40.5)	23 (54.8)	19 (45.2)	30 (71.4)	12 (28.6)
≥60 years	30	14 (46.7)	16 (53.3)	15 (50)	15 (50)	11 (36.7)	19 (63.3)	15 (50)	15 (50)
Gender									
Male	47	19 (40.4)	28 (59.6)	22 (46.8)	25 (53.2)	21 (44.7)	26 (55.3)	30 (63.8)	17 (36.2)
Female	25	9 (36.0)	16 (64.0)	15 (60.0)	10 (40.0)	13 (52.0)	12 (48.0)	15 (60.0)	10 (40.0)
Smoking									
Yes	42	15 (35.7)	27 (64.3)	17 (40.5)	25 (59.5)	16 (38.1)	26 (61.9)	24 (57.1)	18 (42.9)
No	30	13 (43.3)	17 (56.7)	18 (60.0)	12 (40.0)	18 (60.0)	12 (40.0)	21 (70)	9 (30.0)
Histology									
Adenocarcinoma	38	13 (34.2)	25 (65.8)	21 (55.3)	17 (44.7)	21 (55.3)	17 (44.7)	21 (55.3)	17 (44.7)
Non-adenocarcinoma	34	15 (44.1)	19 (55.9)	19 (55.9)	15 (44.1)	13 (38.2)	21 (61.8)	24 (70.6)	10 (29.4)
Differentiation									
High-median	40	15 (37.5)	25 (62.5)	20 (50)	20 (50)	11 (27.5)	29 (72.5)	19 (47.5)	21 (52.5)
Low	32	13 (40.6)	19 (59.4)	20 (62.5)	12 (37.5)	23 (71.9)	9 (28.1)	26 (81.3)	6 (18.7)
Staging									
I/II	47	16 (34.0)	31 (66.0)	23 (48.9)	24 (51.1)	22 (46.8)	25 (53.2)	30 (63.8)	17 (36.2)
III	20	10 (50.0)	10 (50)	14 (70.0)	6 (30.0)	11 (55.0)	9 (45.0)	12 (60.0)	8 (40.0)
IV	5	2 (40.0)	3 (60)	3 (60.0)	2 (40.0)	1 (20.0)	4 (80.0)	3 (60.0)	2 (40.0)
Node metastasis									
Yes	25	12 (48.0)	13 (52.0)	17 (68.0)	8 (32.0)	12 (48)	13 (52.0)	15 (60.0)	10 (40.0)
No	47	16 (34.0)	31 (66.0)	23 (48.9)	24 (51.1)	22 (46.8)	25 (53.2)	30 (63.8)	17 (36.2)

ERCC1, excision repair cross-complementing 1; *RRM1*, ribonucleotide reductase subunit M1; *TUBB3*, class III β-tubulin; *TYMS*, thymidylate synthase.

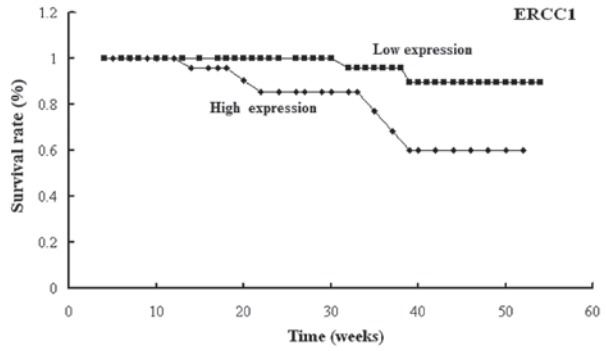

Figure 1. Survival curve following surgery in 72 patients with non-small cell lung cancer based on the mRNA expression of the *ERCC1* gene. The one-year survival rates of the patients with high *ERCC1* expression and low *ERCC1* expression were 78.6 and 95.4%, respectively. P<0.001. *ERCC1*, excision repair cross-complementing 1.

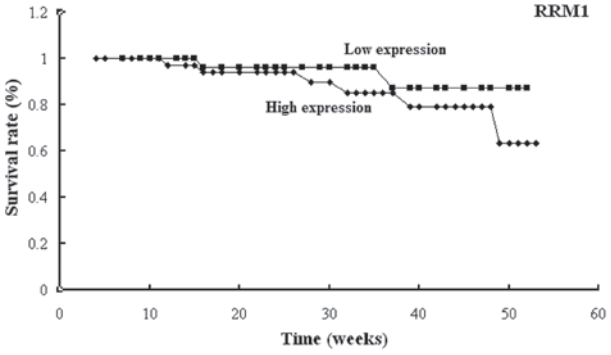

Figure 2. Survival curve following surgery in 72 patients with non-small cell lung cancer based on the mRNA expression of the *RRM1* gene. The one-year survival rates of the patients with high *RRM1* expression and low *RRM1* expression were 85.0 and 93.7%, respectively. P=0.001. *RRM1*, ribonucleotide reductase subunit M1.

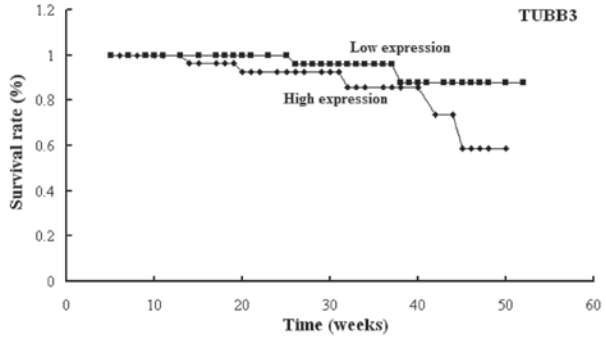

Figure 3. Survival curve following surgery in 72 patients with non-small cell lung cancer based on the mRNA expression of the *TUBB3* gene. The one-year survival rates of the patients with high *TUBB3* expression and low *TUBB3* expression were 85.3 and 94.7%, respectively. P=0.001. *TUBB3*, class III β-tubulin.

expression of the *ERCC1* gene in adenocarcinoma (34.2%) was found to be significantly lower than that in non-adenocarcinoma (44.1%; P<0.05). Furthermore, the incidence rates of *TYMS* and *TUBB3* expression in high-median differentiation tissue samples were observed to be significantly lower than those in low differentiation tissue samples (P<0.05). However, no correlation was observed between *ERCC1*, *RRM1*, *TUBB3*

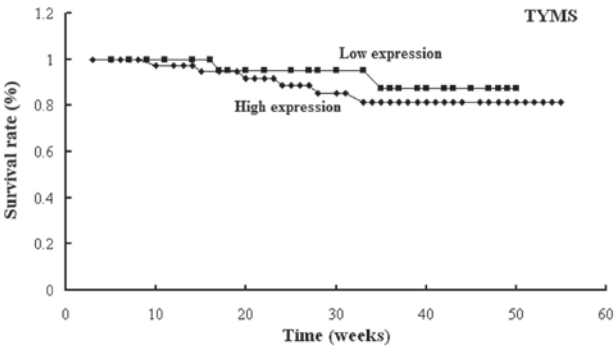

Figure 4. Survival curve following surgery in 72 patients with non-small cell lung cancer based on the mRNA expression of the *TYMS* gene. The one-year survival rates of the patients with high *TYMS* expression and low *TYMS* expression were 86.7 and 92.6%, respectively. P=0.001. *TYMS*, thymidylate synthase.

or *TYMS* expression and age, gender, smoking status, TNM stage or lymph node metastasis.

Correlation between ERCC1, RRM1, TUBB3 or TYMS mRNA expression and patient survival. Following surgery, all 72 patients with NSCLC were treated with platinum-based doublet chemotherapy. The median number of chemotherapy cycles was 4.5. The follow-up duration was 379 days. There were eight cases of mortality. The overall survival rate was found to be 88.9%. The results indicated that high expression of *ERCC1* was associated with poor prognosis (P<0.001) and the one-year survival rates of the patients with high *ERCC1* expression and low *ERCC1* expression were 78.6 and 95.4%, respectively (Fig. 1). Similar results were found for the *RRM1*, *TUBB3* and *TYMS* genes (Figs. 2-4). High expression levels of *RRM1*, *TUBB3* or *TYMS* were observed to be significantly associated with poor prognosis (P=0.001, P=0.001 and P=0.001, respectively). The one-year survival rates of the patients were: 85 and 93.7% for those with high and low *RRM1* expression, respectively; 85.3 and 94.7% for those with high and low *TUBB3* expression, respectively; and 86.7 and 92.6% in those with high and low *TYMS* expression, respectively.

Discussion

The combination of two cytotoxic drugs, for example platinum agents combined with non-platinum agents, including gemcitabine, has become the standard first-line treatment for patients with NSCLC (10). However, drug resistance is a major problem in NSCLC. Not all patients benefit from chemotherapy; thus, it is important to identify predictive markers for response to chemotherapy.

In the present study, the *ERCC1, RRM1, TUBB3* and *TYMS* genes were investigated. ERCC1 is a key factor involved in nuclear excision repair for platinum-induced adducts. There is increasing evidence that reduced DNA repair capacity is correlated with enhanced survival with platinum-based chemotherapy (11,12). However, certain studies have reported that the overexpression of *ERCC1* is associated with improved prognosis (13,14). These contradictory reports may be due to regional disparity or differences in experimental procedures. The present study found that

patients with NSCLC with high (positive) *ERCC1* expression had a poor prognosis and that the incidence rate of *ERCC1* expression in non-adenocarcinoma was higher than that in adenocarcinoma (P<0.05). Like ERCC1, RRM1 also has a role in DNA repair processes. RRM1 is the predominant cellular determinant of the efficacy of the nucleoside analog gemcitabine, and high expression of *RRM1* is associated with chemoresistance to gemcitabine-based therapy (15). Clinical studies have shown that *RRM1* expression is correlated with the expression of *ERCC1* (11,16). The present study indicated that *RRM1* expression may be correlated with *ERCC1* expression, but not *TUBB3* and *TYMS* expression; however, no statistical significance was found (r=0.193; P>0.05; data not shown). Statistical significance may increase with expansion of the sample size. These results indicate that the patients with NSCLC with high *RRM1* expression had a poor prognosis. TUBB3 is a structural protein and it has been reported that high expression of *TUBB3* is correlated with low response rates in patients with NSCLC treated with chemotherapy regimens using an anti-tubulin drug (17). However, studies have shown conflicting results, possibly due to variation in trial design or tumor stage analysis (18). The present study suggested that overexpression of *TUBB3* was associated with poor prognosis in patients with NSCLC. Furthermore, the incidence rate of *TUBB3* expression in high-median differentiation tissue samples was found to be significantly lower than that in the low differentiation tissue samples (P<0.05). TYMS is important for maintaining the dTMP pool for DNA synthesis and repair. Various studies have reported that high expression of *TYMS* is an unfavorable prognostic factor (19-21); however, it has also been reported to be a favorable prognostic factor (22). The findings of the present study indicate that patients with NSCLC with high *TYMS* expression had a poor prognosis and that the incidence rate of *TYMS* expression in high-median differentiation tissue samples was significantly lower than that in the low differentiation tissue samples (P<0.05).

In conclusion, although the sample size used in the present study was small, the findings indicate that ERCC1, RRM1, TUBB3 and TYMS are prognostic factors for survival and may be predictive biomarkers for platinum-based chemotherapy in patients with NSCLC. Detecting the mRNA expression of these four genes may be useful for predicting the efficacy of chemotherapy and may enhance survival in NSCLC.

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