p53R2 expression as a prognostic biomarker in early stage non-small cell lung cancer

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Abstract. p53R2 is a small subunit of ribonucleotide reductase (RR) which has 80% homology to hRRM2 and metastasis-suppressing potential. Previous reports suggested that the expression of p53R2 is used as a prognostic factor and chemotherapy response indicator in several types of cancer. This study aimed to elucidate the association of p53R2 expression and the clinicopathological characteristics of early stage non-small cell lung cancer (NSCLC). Immunohistochemistry was conducted on a tissue array including 92 early stage NSCLC samples. Correlations between p53R2 and clinicopathological factors, recurrence/metastasis and outcomes were analyzed. The analyses showed that there was no correlation between p53R2 expression and the clinicopathological factors. Among disease-free patients during follow-up, patients with p53R2(+) had a better outcome than those with p53R2(-) (P=0.022). By using Cox multivariate regression analysis, p53R2 (risk factor 3.801; 95% CI 1.004-9.454; P=0.044) served as a prognostic biomarker in the prediction of the survival rate for NSCLC patients. Detection of the RR subunit p53R2 may therefore be a useful prognostic marker in early stage NSCLC.

Introduction

Tumor stage is the most potent and widely accepted parameter predictive of survival for patients with stage I-IV non-small cell lung cancer (NSCLC) (1). Although locoregional control of NSCLC can be achieved by curative resection, more than 70% of patients with stage I disease suffer either a locoregional relapse or distant metastasis. A number of prognostic molecular markers have been described for patients with NSCLC, but none are currently in use in treatment decision making (2).

Ribonucleotide reductase (RR) is a highly regulated rate-limiting enzyme in the conversion of ribonucleoside diphosphate to 2'-deoxyribonucleoside diphosphate, which is essential for DNA synthesis (3). In humans, one large subunit (M1) and two small subunits (hRRM2 and p53R2) of RR have been identified (4). Inhibition of RR activity has been tested as a potential treatment modality in anticancer settings (5). The two RR small subunits, p53R2 and hRRM2, have an 80% similarity in the protein sequence (4). An *in vitro* assay showed that recombinant p53R2 protein, as well as hRRM2, interacts with hRRM1 to form a holoenzyme with the ability to convert CDP to dCDP (6,7). Piao *et al* demonstrated that p53R2 negatively modulates serum-induced MEK-ERK activity and inhibits the MEK-ERK-mediated malignancy potential of human cancer cells (8). Overexpression of p53R2 is associated with clinical response in myelodysplatic syndrome/acute myelogenous leukemia (9). It has been suggested that the opposite regulation of hRRM2 and p53R2 in the invasion potential may play a critical role in determining the invasion and metastasis phenotype in cancer cells (10). The implications of p53R2 expression, a regulatory subunit of RR, have yet to be determined and deserve further investigation. Subsequently, an immunohistochemical method was evaluated to determine the association between p53R2 expression and the clinical outcome of early stage NSCLC.

Materials and methods

Patients and samples. From January 2000 to December 2006, a total of 92 consecutive patients underwent surgical treatment for NSCLC at the China Medical University Hospital in Taichung, Taiwan. Patients who had pre-operative chemotherapy or radiotherapy were excluded from this study. Prior to surgery, written informed consent for the use of paraffin-embedded

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tissues and for information regarding sociodemographic characteristics was obtained from each patient. The study protocol was approved by the Institutional Review Board of the China Medical University Hospital. All of the available paraffin blocks were reviewed by a thoracic pathologist.

The study population consisted of 69 men and 23 women (mean age 65.3 years; range 36-83). The procedures included sampling of hilar and mediastinal lymph nodes, and the pathology of the specimens confirmed stage I (T1-2N0M0) in 66 patients and stage II (T1N1, T2N1 and T3N0) in 26 patients. Histological classification and grade were assessed by light microscopy according to the World Health Organization criteria (1). Clinical data including gender, age (≤65 vs. >65 years), smoking habits, histopathology (squamous cell carcinoma vs. adenocarcinoma vs. others), tumor stage by TNM (T1 vs. T2), lymphovascular invasion and tumor differentiation were collected from the patient charts and are shown in Table I.

Postoperative follow-up visits were scheduled 1 and 2 months after surgery, every 3 months during the first 2 years and every 6 months following that, or more frequently if required. The median duration of follow-up after a curative resection was 4.8 years.

Tissue microarray and immunohistochemical staining. Formalin-fixed and paraffin-embedded specimens were sectioned at 3 μm. The sections were then deparaffinized in xylene, rehydrated through serial dilutions of alcohol and washed in phosphate-buffered saline (PBS) (pH 7.2), which was the buffer used for all subsequent washes. For p53R2 detection, sections were heated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) and then incubated with a poly-clonal anti-p53R2 (Santa Cruz, CA, USA) for 90 min at 25°C. The conventional streptavidin peroxidase method (LSAB kit K675; Dako, Copenhagen, Denmark) was performed to develop signals, and the cells were counterstained with hematoxylin. Negative controls were obtained by omitting the primary antibody. The signal intensities were evaluated independently by three observers. Cases with 0-10% posi-tive nuclei were defined as having negative immunostaining, and cases with >10% positive nuclei were exhibited positive immunostaining. The antibody dilution buffer was used to replace antibodies to serve as a negative control.

Statistical analysis. Data were collected using an MS-Excel spreadsheet. The data were analyzed using the JMP Statistical Discovery Software version 6.0 (SAS Institute, Cary, NC, USA). For categorical data, the Fisher's exact test or the bino-mial test of proportions was employed. Survival rates were estimated using the Kaplan-Meier method, and statistical analysis was carried out using the log-rank test for equality of the survival curves. Multivariate survival analysis was carried out on variables that were found to be significant with univariate analysis using the Cox proportional hazards model. Results from this model are reported as relative risk with 95% CI. Statistical significance was set at P<0.05.

Results

Relationship of p53R2 expression with clinicopathological parameters. To elucidate the role of p53R2 in tumor progres-

Table I. Clinicopathological characteristics of the patients with early stage lung cancer.

Parameters	Patient no. (%)
Age (years)	
≤65	40 (43.5)
>65	52 (56.5)
Gender	
Female	23 (25.0)
Male	69 (75.0)
Smoking status	
Negative	46 (50.0)
Positive	46 (50.0)
Tumor type	
AD	52 (56.5)
SCC	40 (43.5)
Tumor stage	
I	66 (71.7)
II	26 (28.3)
Lymphovascular invasion	
Negative	76 (82.6)
Positive	16 (17.4)
Recurrence/metastasis	
Negative	59 (64.1)
Positive	32 (35.9)
Differentiation	
Well	12 (13.0)
Moderate	55 (59.8)
Poor	25 (27.2)

AD, adenocarcinoma; SCC, squamous cell carcinoma.

sion, 92 patients with early stage lung cancer, including 66 with stage I and 26 with stage II, were enrolled in this study. p53R2 protein expression in lung tumors was analyzed by immunohistochemistry in a tissue array section. p53R2 was expressed in 32 (34.8%) patients. The p53R2 proteins were expressed in the cytoplasm of the lung tumor cells as shown in Fig. 1. Table II shows that no correlation occurred between p53R2 and the clinicopathological parameters. However, p53R2 expression in the well-differentiated tumor cells had a higher trend of expression than in the poorly differentiated tumor cells (P=0.051).

p53R2 is an unfavorable prognostic factor in early stage NSCLC. The effects of clinical characteristics on the clinical outcome of patients with early stage NSCLC were calculated by univariate analysis. Among the characteristics, p53R2 protein expression, tumor stage and tumor recurrence/metas-tasis were significant prognostic factors (Table III; P=0.022 for p53R2 protein, P<0.0001 for tumor stage, P<0.0001 for lymphovascular invasion, P=0.0003 for tumor recurrence/metastasis and P<0.0001 for tumor differentiation). Patients with stage I lung cancer (median survival 1,151 days) had longer survival rates than those with stage II of the disease
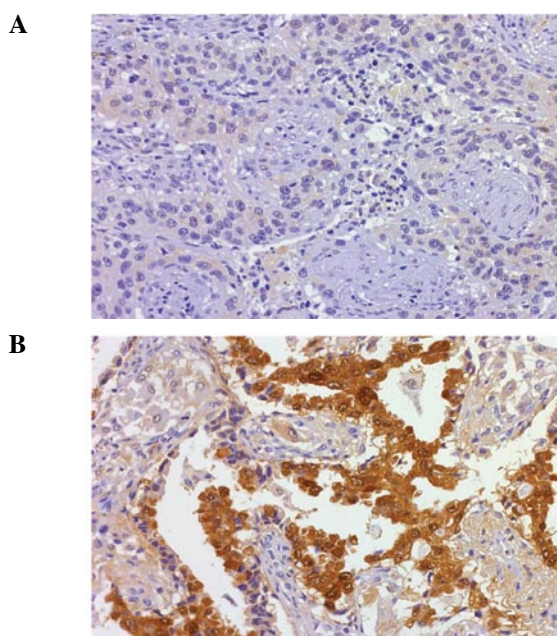
A

B

Figure 1. Immunohistochemical analysis of p53R2 protein in the lung tumors. (A) A negative result of immunostaining in the tumor cells (x100). (B) p53R2 protein expressed in the tumors (x400).
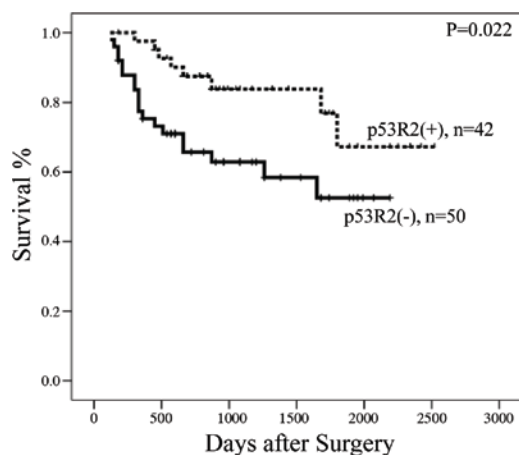

Figure 2. Kaplan-Meier survival curves in early stage NSCLC patients with or without p53R2 protein.

Table II. Association between p53R2 protein expression and clinical characteristics in patients with early stage lung cancer.

Parameters	p53R2 protein expression		P-value
	Negative	Positive	
Age (years)			
≤65	25	15	
>65	25	17	0.207
Gender			
Female	11	12	
Male	39	30	0.481
Smoking status			
Negative	24	22	
Positive	26	20	0.834
Tumor type			
AD	26	26	
SCC	24	16	0.401
Tumor stage			
I	35	31	
II	15	11	0.817
Lymphovascular invasion			
Negative	41	35	
Positive	9	7	1.000
Recurrence/metastasis			
Negative	32	28	
Positive	18	14	0.829
Differentiation			
Well	4	8[a]	
Moderate	29	26	
Poor	17	8[a]	0.121

AD, adenocarcinoma; SCC, squamous cell carcinoma. The Chi-square test was used for statistical analysis. [a]p53R2 expression in well-differentiated tumor cells had a higher trend of expression than in the poorly differentiated cells (P=0.051).

(median survival 642 days). Patients with no lymphovascular invasion (median survival 1,103 days) had longer survival rates than those with lymphovascular invasion (median survival 555 days). Patients with no tumor recurrence/metastasis (median survival 1,082 days) had longer survival rates than those with recurrence/metastasis (median survival 868 days). Patients with p53R2 protein expression (median survival 900 days) also had longer survival rates than those without p53R2 expression (median survival 660 days) (Fig. 2). Moreover, Cox's regression analysis showed that p53R2, tumor stage and recurrence/metastasis acted as significant independent prognostic factors (Table IV; P=0.044, 95% CI 1.004-9.454 for p53R2 protein; P=0.025, 95% CI 0.036-0.801 for tumor stage and P=0.020, 95% CI 0.094-0.816 for tumor recurrence/metastasis). Patients without p53R2 protein had a 3.483-fold higher risk than patients with p53R2. The results suggest that the presence of p53R2, tumor stage and recurrence/metastasis are similarly significant as unfavorable prognostic factors in early stage NSCLC.

Discussion

Numerous reports discussed p53R2 expression in response to chemotherapy (9,11). The present study analyzed the correlation of p53R2 and RRM2 protein and their clinical significance in early stage lung cancer. A recent study concluded that the overexpression of RRM2 and p53R2, but not RRM1, in mice specifically induces lung neoplasms (12). p53R2 was also found to contain a malignancy-suppressing activity (10). The role of p53R2 expression in tumor formation has yet to be determined. Uramato et al reported that p53R2 was detected in 46.2% of lung cancer patients and was higher in patients with stage II-III, pathological T3-4 and N1-3 NSCLC (13). However, p53R2 expression could not be used as an inde-

Table III. Univariate analysis of the influences of clinical characteristics on the overall survival of patients with early stage NSCLC.

Prognostic factor	No.	Median survival (days)	3-year survival (%)	Log-rank P-value
P53R2				
Negative	50	660	36.0	
Positive	42	900	40.1	0.0220
Age (years)				
≤65	40	1,074	36.0	
>65	52	957	30.8	0.5750
Gender				
Female	23	1,033	30.5	
Male	69	999	36.3	0.6020
Tumor type				
AD	52	1,020	30.8	
SCC	40	991	40.0	0.8700
Tumor stage				
I	66	1,151	43.9	
II	29	642	11.5	<0.0001
Lymphovascular invasion				
Negative	76	1,103	59.2	
Positive	16	555	6.25	<0.0001
Recurrence/metastasis				
Negative	59	1,082	37.3	
Positive	32	868	31.2	0.0030
Differentiation				
Well	12	1,105	33.0	
Moderate	55	1,140	40.0	
Poorly	25	670	24.0	<0.0001

AD, adenocarcinoma; SCC, squamous cell carcinoma. The Kaplan-Meier log-rank test was used for statistical analysis.

Table IV. Cox-regression analysis of various potential prognostic factors in patients with early stage NSCLC with different p53R2 protein expression.

Variable	RR	Unfavorable/favorable	95% CI	P-value
P53R protein	3.801	Negative/positive	1.004-9.454	0.044
Tumor stage	5.920	II/I	0.036-0.801	0.025
Lymphovascular invasion	1.360	Positive/negative	0.124-4.345	0.734
Recurrence/metastasis	3.610	Positive/negative	0.094-0.816	0.020

RR, ribonucleotide reductase. Cox-regression analysis was used for statistical analysis.

pendent prognostic marker in NSCLC (13). Additionally, previous reports showed that p53R2 expression is correlated with tumor invasion, lymph node metastasis and tumor size in esophageal and oral cancer (14,15). p53R2 expression in patients with late stage cancer was higher than that in patients with early stage esophageal cancer (14). The present study analyzed p53R2 protein expression in early stage lung cancer and found that only 45.6% (42 of 92) of the patients were positive. p53R2 protein expression was similar to previous reports (13). Additionally, we found that p53R2 protein expression

was negatively correlated with tumor cell differentiation in early stage NSCLC (Table II). These findings appear to be inconsistent with a previous report (13). Therefore, we suggest that p53R2 expression in early and advanced stages of lung cancer plays a different role.

Previous reports showed that p53R2 protein expression correlates with tumorigenesis (15). However, it was found that p53R2 expression was negatively related to the metastasis of colon adenocarcinoma (16). The invasion-suppressing ability of p53R2 was also found in oropharyngeal, prostate, pancreatic

and colon cancer cell lines (15,16). In addition, these authors determined that the invasion-suppressing ability of p53R2 was not related to RR enzymatic activity (10). Plao *et al* concluded that p53R2 negatively modulates serum-induced MEK-ERK activity and inhibits the MEK-ERK-mediated malignancy potential to suppress invasion of human lung cancer cells (8). The balance of R1 and R2 expression significantly modifies the transformation, tumorigenicity and metastatic potential (17,18). Altering the RR level may change the tumorigenicity (19). In this study, patients with p53R2(+) had a significantly higher median disease-free survival rate (900 days) compared to those with p53R2(-) (660 days) (P=0.022) (Fig. 2). Our previous study found that the disease-free survival rate was significantly higher for patients with RRM1(-) than that for patients with RRM1(+). Additionally, patients with p53R2(+)/ hRRM2(-) had a significantly higher median survival rate (960 days) than that of the other three groups of patients: p53R2(-)/hRRM2(-), 810 days; p53R2(-)/hRRM2(+), 600 days and p53R2(+)/hRRM2(+), 840 days. Thus, the expression of p53R2 and RRM2 may correlate with patient survival. Further studies should be conducted to verify the RRM1 expression in the same patient group in order to understand the role of RR in lung cancer progression.

In conclusion, we showed that the p53R2 expression is negatively correlated with tumor cell differentiation, and the presence of p53R2 protein is a favorable prognostic factor in early stage lung cancer. Therefore, we propose that the expression of p53R2 is, not only a chemotherapy indicator in late stage lung cancer, but also a tumor progression marker for early stage lung cancer.

Acknowledgements

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