Expression Status of Ribonucleotide Reductase Small Subunits hRRM2/p53R2 as Prognostic Biomarkers in Stage I and II Non-small Cell Lung Cancer

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Abstract. *Overexpression of ribonucleotide reductase M2 (hRRM2) and p53-dependent RR small subunit (p53R2) has been correlated with tumor malignancy and progression in several types of cancer. The aim of this study was to determine the association of p53R2/hRRM2 expression with clinicopathological characteristics of stage I and II non-small cell lung cancer (NSCLC). Immunohistochemistry was conducted on a tissue array that included 92 samples. Correlations between hRRM2 and p53R2 expression and clinicopathological factors, recurrence/metastasis, and outcomes were analyzed. The analyses revealed that there was no correlation between p53R2 expression and clinicopathological factors; hRRM2 was only positively related to poor tumor differentiation (p=0.006). Regarding overall survival during the follow-up period, patients with p53R2+/hRRM2- tumors had the best outcomes (p<0.01). Multivariant Cox analysis revealed that p53R2 (risk=0.232, 95% CI=0.086-0.626, p=0.004) not only served as a prognostic biomarker to predict survival, but also as an independent biomarker to predict disease-free survival (risk=0.545, 95% CI=0.301-0.987, p=0.045) of patients with NSCLC. Therefore, we consider that the expression of p53R2 can be used not only as a biomarker for overall survival, but also as an indicator for tumor recurrence. Based on our finding, p53R2 expression seems more important than that of hRRM2 in prognosis of early-stage lung cancer.*

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Tumor stage is the most powerful and widely accepted parameter predictive of survival for patients with non-small cell lung cancer (NSCLC) within the stages I to IV (1). Although locoregional control of NSCLC can be achieved by curative resection, more than 30% of patients with stage I disease experience relapse within 5 years. Many prognostic molecular markers have been described for patients with NSCLC, but none are currently being used in treatment decision making (2).

Ribonucleotide reductase (RR) is a highly regulated rate-limiting enzyme, which is essential for DNA synthesis as it converts ribonucleoside diphosphate to 2'-deoxyribonucleoside diphosphate (3). Mammalian RR is a multimeric enzyme comprised of the large R1 subunit and the small R2 subunit. In humans, one large subunit (M1) and two small subunits (hRRM2 and p53R2) of RR have been identified (3, 4). The two small RR subunits p53R2 and hRRM2 have an 80% similarity in protein sequence (4). RRM1 reduced the development of tumors and metastasis and it has therefore been suggested that *RRM1* is a tumor suppressor gene (5, 6). hRRM2, which contains a tyrosine free radical and a non-heme iron for enzyme activity (7) exhibits significant proliferative activity in cancer cells of both humans and other species (mice, rats, monkeys) (8). A high level of RRM2 expression correlates with cellular invasiveness (9), tumor angiogenesis (10), metastasis (11), and poor patient outcome (12). Thus, RRM2 is considered a crucial protein both in malignant progression and in gemcitabine chemoresistance. An *in vitro* assay showed

that recombinant p53R2 protein, as well as hRRM2, interact with hRRM1 to form a holoenzyme with the ability to convert cytosine diphosphate (CDP) to dCDP (13, 14). p53R2 is a direct target of the tumor suppressor gene *p53*, and its induction in response to DNA damage assists in G 2 arrest and provides DNA precursors for DNA repair (15, 16). The dysfunction of p53R2 could result in failure of DNA damage repair and thus lead to gene mutation or cellular apoptotic activation (17). Interestingly, it has been investigated that opposing regulation of hRRM2 and p53R2 in noted potential might play a critical role in determining the invasion and metastatic phenotype in cancer cells (18, 19).

For the RR subunit, clinical studies have indicated that lower RRM1 gene expression resulted in longer overall survival and a better response to gemcitabine-based chemotherapy for several types of cancer (5). But the expression of p53R2 and hRRM2, the regulatory subunits of RR, is largely unknown and warrants further investigation. The aims of this study were to examine the expressions of p53R2 and hRRM2 in surgical specimens of early-stage NSCLC and to evaluate whether such expressions are useful for predicting clinical outcomes.

Patients and Methods

Patients and samples. From January 2000 to December 2006, a total of 92 consecutive patients underwent surgical treatment for NSCLC at China Medical University Hospital in Taichung, Taiwan. Patients who had preoperative chemotherapy or radiotherapy were excluded from this study. Written informed consent for the use of the paraffin-embedded tissues and information regarding sociodemographic characteristics, as approved by the Institutional Review Board at the above hospital, was obtained from each patient before surgery. A thoracic pathologist reviewed all of the available paraffin blocks.

Patients were diagnosed with lung cancer based on the pathologic assessment of cytologic or tissue specimens under microscopic examination by a pathologist. A medical history was taken and a physical examination was performed to evaluate the general condition and cancer symptoms of each patient. A series of images, including a chest x-ray, chest computed tomography, and a whole-body bone scan were performed. A complete blood count, blood biochemistry tests, a pulmonary function test and bronchoscopy were also performed. The study population consisted of 69 men and 23 women (mean age, 64.3 years; age range, 38-78 years). All procedures, including sampling of hilar and mediastinal lymph nodes and the pathology of all specimens, confirmed stage I disease (T1-2N0M0) in 66 patients and stage II disease (T1N1, T2N1, T3N0) in 26 patients. Histological classification and grade were assessed by light microscopy according to WHO criteria (UICC 7th edition). Clinical data including gender, age (≤65 years *vs.* >65 years), smoking habit, histopathology (squamous cell carcinoma, SCC *vs.* adenocarcinoma, AD), tumor stage by TNM (T1 *vs.* T2), lymphovascular invasion, and tumor differentiation were collected from patient charts.

Postoperative follow-up was scheduled at 1, 2, and then every 3 months during the first 2 years after surgery and every 6 months thereafter, or more frequently if needed. The median duration of follow-up after curative resection was 4.8 years.

Tissue microarray and immunohistochemical staining. The tissue microarrays of resectable early stage NSCLC were obtained from the Department of Pathology at China Medical University Hospital in Taichung, Taiwan. We constructed a tissue microarray using triplicate 2.5 mm cores from formalin-fixed and paraffin-embedded specimens of the primary tumor to analyze the p53R2 and RRM2 protein expression. Briefly, after deparaffinization, the endogenous peroxidase activity was blocked with 3% H_2O_2. The array slides were incubated with normal goat serum for 20 min, and then the primary antibody was applied to the slides for 20 min at room temperature. After 7 minutes of hydrogen peroxide treatment, the array slides were incubated with horseradish peroxidase labeled polymer conjugated with corresponding antibodies for 30 minutes. Then 3,3'-diaminobenzidine [0.05 g of 3,3'-diaminobenzidine and 100 ml of 30% H_2O_2 in 100 mL of PBS] was applied for 5 and 10 min, respectively. Each slide was counterstained with hematoxylin (DAKO, Carpinteria, CA, USA). PBS was used as a negative control. Two independent observers evaluated the staining intensity to maintain consistency. Negative controls were obtained by leaving out the primary antibody. A mouse polyclonal antibody against hRRM2, which is commercially produced by Convance (Princeton, NJ, USA) using recombinant hRRM2 peptide, was used for immunohistochemical staining. Rabbit antibody against p53R2 was purchased from Alexis BioChemical Company (Lausen, Switzerland) and was applied for immunohistochemical staining (1:200 dilution). After immunohistochemical staining with anti-p53R2 and anti-hRRM2 antibodies, two pathologists examined each sample for consistency. Three observers independently evaluated the signal intensities. Negative immunostaining was defined as 0-10% positive nuclei and cases with more than 10% positive nuclei were classified as positive immunostaining.

Statistical analysis. Statistical analysis was performed using the SPSS statistical software program (Version 17.0 SPSS Inc., Chicago, IL, USA). For categorical data, we employed Fisher's exact test, or χ^2 test of proportions. Survival rates were estimated using the Kaplan and Meier method, and statistical analysis was carried out using the log-rank test for equality of the survival curves. Multivariate survival analysis, using Cox's proportional hazard model, was carried out for the variables which were significant in the univariate analysis. Results from this model are reported as relative risks with 95% confidence intervals. Statistical significance was set at $p<0.05$.

Results

Relationship of hRRM2, p53R2 expression with clinicopathological parameters. To elucidate the role of p53R2 and hRRM2 in tumor progression, 92 patients with early-stage lung cancer, 66 with stage I and 26 with stage II cancer, were collected in this study. The expression of p53R2 and hRRM2 protein in lung tumors was analyzed by immunohistochemistry in a tissue array section. There was hRRM2 expression in 25 (27.2%) patients and p53R2 was expressed in 42 (45.6%) patients. Both proteins were predominantly expressed in the cytoplasm of the lung tumor cells as shown in Figure 1B and

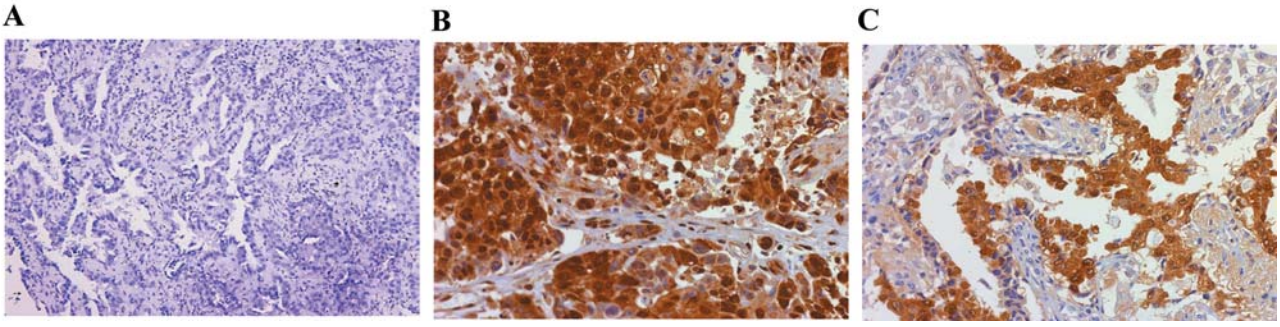

Figure 1. *Immunohistochemical analysis of hRRM2 and p53R2 protein in lung tumors. A: A negative immunostaining result in tumor cells ×100; B: hRRM2 protein expressed in tumors ×400; and C: p53R2 protein expressed in tumors ×400.*

C. As shown in Table I, positive hRRM2 expression in poorly differentiated tumor was significantly more frequently than in well and moderately differentiated tumor (p=0.006). No other correlation was found between hRRM2 expression and other clinical parameters (Table I). In addition, based on our previous report (20) there was no correlation between p53R2 and the clinical pathological parameters. We only found that p53R2 expression in well-differentiated tumor cells had a trend higher for expression than in poorly differentiated tumor cells (p=0.121).

The influence of hRRM2 and p53R2 expression on overall survival (OS) and disease free survival (DFS) of early stage lung cancer patients. We hypothesized that the expression status of p53R2 and hRRM2 contributed to tumor progression and metastasis. Therefore, we expected that p53R2 and hRRM2 expression would be associated with OS and DFS patients with early-stage lung cancer. For the 92 patients enrolled, the median follow-up was 57.6 months. Disease recurred in 32 patients (10, local recurrence; 21, distant metastasis; 1, local recurrence and distant metastasis) and 27 patients (27 of 92) died from their disease. None of the patients received adjuvant treatment before surgical therapy. Kaplan-Meier analysis showed that patients with negative p53R2 expression had a lower median OS than those with positive p53R2 expression (660 *vs*. 900 days, p=0.022; Figure 2A) (20). In addition, we observed that hRRM2-positive patients had a lower median OS than hRRM2-negative patients (834 *vs*. 1072 days, p=0.044; Figure 2B). After combining the expression status of hRRM2 and p53R2, we found that the patients with p53R2-positive and hRRM2- positive expression had the best outcome (p=0.003) compared with the other three groups (Figure 2C).

Among the parameters, no significant correlations were found between DFS and hRRM2 protein expression (p=0.399; Figure 2D). Additionally, there was also no correlation between the patients' DFS and p53R2 protein expression (p=0.901; Figure 2E). After combining the expression status of hRRM2 and p53R2, no correlations were found between the expression of these two protein and the patient's DFS (p=0.362; Figure 2F).

Table I. *The association between RRM2 and p53R2 protein expression and clinical characteristics in early-stage lung cancer patients.*

Parameter	RRM2 protein			P53R2 protein		
	–	+	*p*-Value	–	+	*p*-Value
Age (years)						
≤65	30	10	0.443	25	15	0.207
>65	37	15		25	27	
Gender						
Female	14	9	0.177	11	12	0.481
Male	53	16		39	30	
Smoking status						
Negative	32	14	0.640	24	22	0.834
Positive	35	11		26	20	
Tumor type						
AD	34	18	0.098	26	26	0.401
SCC	33	7		24	16	
Tumor stage						
I	50	16	0.314	35	31	0.817
II	17	9		15	11	
Lymphovascular invasion						
Negative	57	19	0.358	41	35	1.000
Positive	10	6		9	7	
Recurrence/Metastasis						
Negative	43	17	0.809	32	28	0.829
Positive	24	8		18	14	
Differentiation						
Well	12	0	0.006	4	8	0.121
Moderate	42	13		29	26	
Poorly	13	12		17	8	

AD: Adenocarcinoma; SCC: squamous cell carcinoma. χ^2 test was used for statistical analysis. p53R2 data analyzed in this study are based on prior lung tumor samples (20).

p53R2 expression is an independent prognostic factor of OS and DFS in early-stage lung cancer. Multivariate Cox

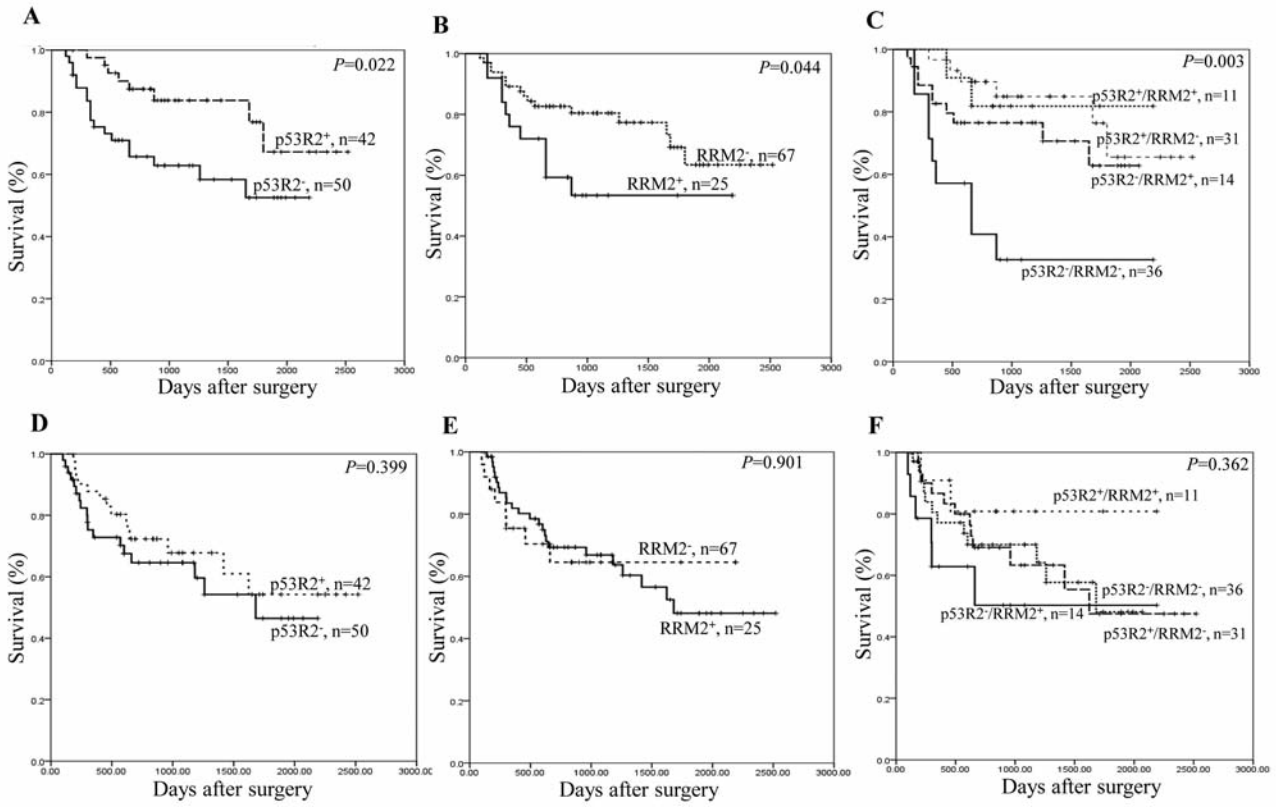

Figure 2. *Overall survival (OS) and disease-free survival (DFS) curves for all studied patients with hRRM2 (A and D), p53R2 expression (B and E), and combined hRRM2 and p53R2 protein expression (C and F). A to C are OS and D to F are DFS.*

regression analysis, conducted after the parameters of age, gender, lymphovascular invasion tumor type, and tumor stage were adjusted, showed that the relative risk (RRs) for OS and DFS in patients with positive p53R2 expression were 0.232 and 0.545, respectively (95% CI=0.086-0.626, p=0.004 for OS; 95% CI=0.301-0.987, p=0.045 for DFS; Table II). However, no statistical significance of hRRM2 expression was found. These results suggest that the loss of p53R2 may result in the inability to inhibit tumor malignancy in patients and leads to poor OS and DFS. p53R2 protein expression in malignant tumors in patients with early-stage lung cancer seems more important than that of hRRM2.

The influence of p53R2 and RRM2 expression on clinical outcome of early-stage lung cancer. The influence of p53R2 and RRM2 protein expression on the outcome of early-stage lung cancer patients was assessed by a log-rank test. Among the parameters, the patients' survival rate was significantly associated with p53R2 and/or RRM2 protein expression (Table III). Patients with positive expression (40.1%) had a significantly higher survival rate than those with negative expression (36.0%), respectively. Similarly, patients with negative RRM2 protein expression (41.8%) had a significantly higher survival rate than those with positive expression (16.0%), respectively. More interestingly, patients with the p53R2–/RRM2– or p53R2+/RRM2+ had a markedly higher survival rate than those with the p53R2+/RRM2– or p53R2–/RRM2+ (p=0.003, log-rank test; Table III).

Discussion

As is known from previous studies, patients with lower *RRM1* and *RRM2* gene expression live longer and exhibit a better response to chemotherapy for several types of cancer (5, 21-25). Zheng *et al*. indicated that analyzing RRM1 protein in NSCLC specimens is difficult because of technical limitations (26). Therefore, most previous reports discuss the correlation between RRM1 mRNA expression and gemcitabine/cisplatin therapy response. In addition, Månsson *et al*. 2003 indicated that p53R2 may have a splice variant (27). Therefore, most studies discuss the correlation between p53R2 and clinicopathological characteristics with the focus on protein expression (28). In this study, we focused on p53R2 and RRM2 protein expression and the clinical outcome.

Most reports have discussed RRM2 expression with response to chemotherapy in lung cancer (6, 29). Studies have

Table II. *Cox regression analysis of various potential prognostic factors in early stage NSCLC patients with different RRM2 and p53R2 protein expression.*

		Overall survival			Disease-free survival		
Variable	Unfavorable/favorable	RR	95% CI	*p*-Value	RR	95% CI	*p*-Value
RRM2 protein	Positive/negative	1.693	0.677-4.233	0.260	1.523	0.815-2.847	0.187
p53R2 protein	Negative/positive	0.232	0.086-0.626	0.004	0.545	0.301-0.987	0.045
Age, years	>65/≤65	6.142	2.103-17.942	0.001	1.702	0.940-3.084	0.079
Gender	Male/female	1.932	0.687-5.436	0.212	1.169	0.583-2.344	0.660
Tumor stage	II/I	9.330	2.790-31.198	<0.0001	1.267	0.337-4.754	0.726
Tumor type	SCC/AD	1.032	0.394-2.703	0.949	1.305	0.715-2.381	0.386
Lymphovascular invasion	Positive/negative	7.986	0.650-6.069	0.229	3.959	0.969-1.176	0.055
Recurrence/metastasis	Positive/negative	2.321	0.962-5.596	0.061	-	-	-

AD: Adenocarcinoma; SCC: squamous cell carcinoma; RR: relative risk; CI: confidence interval. p53R2 data analyzed in this study are based on prior lung tumor samples (20).

shown that alterations in the balance of R1 and R2 expression can significantly modify transformation, tumorigenicity, and metastatic potential (30). A recent report indicated that overexpression of RRM2 and p53R2, but not RRM1, in mice specifically induces lung neoplasma (31). Only one paper indicated that the p53R2 immunocytochemical marker alone plays an important prognostic role in NSCLC, and the DNA repair pathway mediated by p53R2 may be responsible for controlling the growth of lung cancer (32). To the best of our knowledge, ours is the first report to help us understand the role of RRM2 and p53R2 protein expression in lung tumor tissues and their clinical significance in early-stage lung cancer. In our study, patients with p53R2+ or hRRM2– had a significantly higher median OS rate compared with patients with p53R2– or hRRM2+ (Figure 2; Table II). Additionally, patients with p53R2+/hRRM2– had a significantly higher median survival rate (960 days) than the other three groups of patients with (p53R2–/hRRM2–, 810 days; p53R2–/hRRM2+, 600 days; p53R2+/hRRM2+, 840 days; $p=0.003$; Table III). When adjusted with the other clinical factors, only p53R2 but not hRRM2 was used as an independent prognostic factor (Table II; $p=0.004$, 95% CI=0.086-0.626). We also found that p53R2 expression was used as an independent factor for DFS (Table II; $p=0.045$, 95% CI=0.301-0.987). These results suggest that the role of p53R2 in early stage lung tumor progression is more important than that of hRRM2. Piao *et al*. indicated that p53R2 negatively modulates serum-induced MAPK/ERK kinase/extracellular signal-regulated kinase (MEK–ERK) activity and inhibits the MEK–ERK-mediated malignant potential of human cancer cells (33). Therefore, we considered that the expression of p53R2 protein in NSCLC tumors to be not only a biomarker for chemotherapy response in late stage lung cancer but also an independent prognostic factor in patients with early-stage lung cancer.

Table III. *Univariate analysis of influence of p53R2 and RRM2 on overall survival of patients with early-stage NSCLC.*

Prognostic factor	Number of patients (days)	Median survival (%)	3-Year survival	Log-rank *p*-Value
P53R2				
Negative	50	660	36.0	0.022
Positive	42	900	40.1	
RRM2				
Negative	67	1072	41.8	0.044
Positive	25	834	16.0	
P53R2/RRM2				
–/–	36	810	44.0	0.003
–/+	14	600	14.0	
+/–	31	960	45.0	
+/+	11	840	27.0	

Kaplan-Meier log rank test was used for statistical analysis. p53R2 data analyzed in this study are based on prior lung tumor samples (20).

p53R2 has been found to have malignancy-suppressing activity (19). In our previous study, we found that p53R2 protein expression correlated negatively with tumor cell differentiation in early-stage NSCLC (20). Positive p53R2 expression confered significantly better overall survival ($p=0.022$). These findings appear to be inconsistent with a previous report. Uramato *et al*. (2006) reported that p53R2 was higher in patients with stage II/III, pathological T3-4, and N1-3 NSCLC (32). However, it was not possible to use p53R2 expression as an independent prognostic marker in NSCLC (32). Additionally, previous reports have shown that p53R2 expression correlated with tumor invasion, lymph node metastasis, and tumor size in esophageal and oral cancer (34, 35). p53R2 expression in patients with late stage cancer was higher than in patients with early-stage

esophageal cancer (34). Therefore, we suggest that p53R2 expression may play a different role in early and advanced stages of lung cancer.

Recent reports have indicated that overexpression of RRM2 and p53R2, but not RRM1 in mice, specifically induces lung neoplasms (31), which might be independent of RR enzyme activity because there is lung tumor induction in RRM2 and p53R2 but not in RRM1 transgenic mice (31). Cell model experiments have demonstrated that RRM2 interacts with a variety of oncogenes to promote cell transformation and tumorigenesis (36). Both human and mouse cell models have demonstrated that RRM1 has malignancy-suppressing activity (6), whereas hRRM2 has been proven to play a critical role in enhancing invasive potential (35, 37). In the present study, although the relationship of hRRM2 protein expression and lymphovascular invasion did not reach statistical significance (p=0.358), the frequency of hRRM2 expression in the lymphovascular invasion group (6 of 16; 37.5%) was higher than in the non-invasion group (19 of 76; 25.0%). Furthermore, the hRRM2 expression in poorly differentiated tumor was significantly more frequently expressed in well-differentiated tumor (p=0.006). Therefore, we consider that the expression of hRRM2 in patients with early-stage lung cancer may be involved in enhancing tumor cell malignancy.

In conclusion, we showed that expression of p53R2 protein is a favorable prognostic factor and biomarker of relapse in early stage lung cancer. The role of p53R2 in early-stage lung cancer seems more important than that of hRRM2. The expression of p53R2 can be used not only as a independent biomarker for OS but also as an indicator for tumor recurrence.

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Conflict of Interest

We declare that we have no proprietary, financial, professional or other personal interest of any kind in any product, service and/or company that could be constructed as influencing the position presented in, or the review of this manuscript.

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