

ORIGINAL ARTICLE

Prognostic Significance of Combinations of RNA-Dependent Protein Kinase and EphA2 Biomarkers for NSCLC

Chengcheng Guo, MD, PhD,*† Ruping Shao, MD,* Arlene M. Correa, PhD,* Carmen Behrens, MD,‡ Faye M. Johnson, MD,‡ Maria G. Raso, MD, PhD,§ Ludmila Prudkin, MD, PhD,§ Luisa M. Solis, PhD,§ Maria I. Nunez, MD, PhD,§ Bingliang Fang, MD, PhD,* Jack A. Roth, MD,* Ignacio I. Wistuba, MD,‡§ Stephen G. Swisher, MD,* Tongyu Lin, MD, PhD,|| and Apar Pataer, MD, PhD*

Introduction: RNA-dependent protein kinase (PKR) is an independent prognostic variable in patients with non-small-cell lung cancer (NSCLC). In the current study, we investigated the correlation between PKR and 25 other biomarkers for NSCLC, identified the markers that could further improve the prognostic significance of PKR and elucidated the mechanisms of interaction between these markers and PKR. **Methods:** Tissue microarray samples obtained from 218 patients with lung cancer were stained with an anti-PKR antibody and antibodies against 25 biomarkers. Immunohistochemical expression was scored and used for Kaplan–Meier survival analysis. The interaction between PKR and EphA2 in NSCLC cell lines was examined.

Results: We found that PKR was associated with EphA2 and that the prognostic information regarding NSCLC provided by the combination of PKR and EphA2 (P/E) was significantly more accurate than that provided by either marker alone. The 5-year overall survival rate in patients with PKR^{low}/EphA2^{high} (20%) was significantly lower than that of patients with PKR^{high}/EphA2^{low} (74%), patients with

PKR^{high}/EphA2^{high} (55%), and patients with PKR^{low}/EphA2^{low} (55%) ($p < 0.0001$). We also found that the PKR:EphA2 (P/E) ratio was significantly associated with prognosis ($p < 0.0001$). Univariate and multivariate Cox analyses revealed that this P/E combination or ratio was an independent predictor of overall survival. In addition, induction of PKR expression reduced EphA2 protein expression levels in NSCLC cell lines.

Conclusions: PKR/EphA2 is a significant predictor of prognosis for NSCLC. PKR/EphA2 may be a promising approach to improving screening efficiency and predicting prognosis in patients with NSCLC.

Key Words: PKR, EphA2, Biomarker, Lung cancer.

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The progression of non-small-cell lung cancer (NSCLC) is driven by a variety of cell signaling pathways. NSCLC is molecularly heterogeneous, leading to differences in clinical outcome and requires the identification of multiple biomarkers of NSCLC to accurately predict outcome.^{1,2} One potential biomarker of NSCLC is RNA-dependent protein kinase (PKR), a serine/threonine kinase initially identified as an innate immune antiviral protein that has a role in other cellular functions, including apoptosis, growth regulation, cell proliferation, and inflammation.³⁻⁷ We previously demonstrated that a PKR pathway is necessary for induction of cell death in various cancer cells after different treatments.⁸⁻¹¹ In addition, autophosphorylated PKR can catalyze the phosphorylation of target substrates of eIF2 α , thereby dramatically inhibiting protein synthesis, inducing apoptosis, and increasing the expression of proapoptotic factors such as Fas.^{3,4} PKR may have a role as a tumor suppressor in patients with leukemia or other hematologic malignancies.¹²⁻¹⁴ PKR is a prognostic marker of several human cancers, including lung, liver, colon, and head and neck cancers.^{15,16} What's more, high PKR expression predicts favorable outcomes in patients with lung cancer.^{17,18} New biomarkers that can further improve the prognostic significance of PKR must, however, be identified.

*Department of Thoracic and Cardiovascular Surgery, The University of Texas MD Anderson Cancer Center, Houston, TX; †Department of Neuro-Oncology, Sun Yat-Sen University Cancer Center, Guangzhou, Guangdong, People's Republic of China; ‡Department of Thoracic Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX; §Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas; and ||Department of Medical Oncology, Sun Yat-Sen University Cancer Center, Guangzhou, Guangdong, People's Republic of China.

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Address for correspondence: Apar Pataer, MD, PhD, Department of Thoracic and Cardiovascular Surgery, Unit 1489, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030. E-mail: apataer@mdanderson.org; Tongyu Lin, MD, PhD, Department of Medical Oncology, Sun Yat-Sen University Cancer Center, Guangzhou, Guangdong, People's Republic of China. E-mail: tongyulin@hotmail.com

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In the current study, we used immunohistochemical analysis to investigate the association between PKR and 25 biomarkers and found that the combination of PKR and EphA2 is a significantly more accurate prognostic indicator for NSCLC than either marker alone. EphA2 expression is frequently elevated in several different types of cancer cells, including lung, breast, colon, esophageal, ovarian, pancreatic, and prostate cancer cells.^{19,20} High EphA2 protein expression predicts poor survival and disease recurrence in patients with these cancers.^{21,22} These opposing functions of PKR and EphA2 led us to investigate the mechanisms of these two biomarkers' interaction in NSCLC cells.

PATIENTS AND METHODS

Patients and Tissue Samples

A total of 218 patients with NSCLC (119 adenocarcinomas and 99 squamous cell carcinomas) with stage I through IV disease who were undergoing resection of their primary cancer at The University of Texas MD Anderson Cancer Center between 1997 and 2001 were used for this study. Patients were excluded from the study if they had neoadjuvant or adjuvant therapy. The specimens of which were obtained from Lung Cancer Specialized Program of Research Excellence Tissue Bank at The University of Texas MD Anderson Cancer Center under a protocol approved by the MD Anderson Institutional Review Board.^{17,21,23} Detailed clinical and pathological information, including demographic data, smoking history (never and ever smokers), pathological tumor, node, metastasis stage, and overall survival data, were available for all patients. The tissue samples were selected for tissue microarray (TMA) construction. TMAs were constructed using triplicate 1-mm-diameter cores from each specimen.

Histopathologic Evaluation

Immunohistochemical staining for PKR and the other 25 biomarkers was performed as described previously.¹⁷ Briefly, formalin-fixed and paraffin-embedded tissue histology sections (5 μ m thick) were deparaffinized, hydrated, and heated in a steamer for 10 minutes with 10 mmol/liter of sodium citrate (pH 6.0) for antigen retrieval. Peroxide blocking was performed with 3% H₂O₂ in methanol at room temperature for 15 minutes, followed by 10% bovine serum albumin in TBS-t for 30 minutes. The slides were incubated with primary antibody at 1:400 dilution for 65 minutes at room temperature. After washing with phosphate-buffered saline, incubation with biotin-labeled secondary antibody for 30 minutes followed. Finally, the samples were incubated with a 1:40 solution of streptavidin-peroxidase for 30 minutes. The staining was then developed with 0.05% 3'-diaminobenzidine tetrahydrochloride prepared in 0.05 mol/liter of Tris buffer at pH 7.6 containing 0.024% H₂O₂ and then counterstained with hematoxylin. Formalin-fixed and paraffin-embedded lung tissues with normal bronchial epithelia were used as a positive control. For a negative control, we used the same specimens used for the positive controls, replacing the primary antibody with phosphate-buffered saline.

Immunohistochemical expression was quantified by two independent pathologists (Drs Pataer and Raso) blinded

to the patient treatment and outcome. Immunohistochemical expression for TMA was quantified using a 4-value intensity score (0 for negative, 1 for weak, 2 for moderate, and 3 for strong), and the percentage of tumor cells within each category was estimated. A final score was obtained by multiplying both intensity and extension values ($0 \times \% \text{ negative tumor cells} + 1 \times \% \text{ weakly stained tumor cells} + 2 \times \% \text{ moderately stained tumor cells} + 3 \times \% \text{ strongly stained tumor cells}$). The score ranged from a minimum of 0 to a maximum of 300. Cases with discordant scores between observers were reevaluated.

Cell Lines and Reagents

H1299 and H322 lung cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Both H1299 and H322 cancer cells and their treatment with various adenovirus vectors have been well standardized in our laboratory. In addition, we have extensive experience in performing *in vivo* gene transduction assays in these cell lines. We can transfect greater than 95% of these cells with vectors. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10-mM glutamine, 100-U/ml penicillin, and 100-mg/ml streptomycin (Life Technologies, Inc., Grand Island, NY) in a 5% carbon dioxide atmosphere at 37°C.

A small interfering RNA (siRNA) targeting the EphA2 receptor and a control nontargeting siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ephrin-A1 Fc and control Fc were obtained from R&D Systems (Minneapolis, MN). An anti-PKR (K-17) antibody was obtained from Santa Cruz Biotechnology. An anti-phosphorylated PKR (p-PKR) (Thr446) antibody was obtained from Epitomics (Burlingame, CA); a mouse anti-EphA2 antibody (Clone D7) was obtained from Millipore (Billerica, MA); and a mouse anti- β -actin antibody, which was used as the control antibody, was obtained from Sigma (St. Louis, MO).

SiRNA Transfection

H1299 and H322 lung cancer cell lines were transfected with EphA2 siRNA or control nontargeting siRNA. Briefly, lung cancer cells were plated at a density of 2.0×10^5 cells per well in RPMI 1640 medium. Cells were treated with RPMI 1640 medium 24 hours after plating. Transfected cells were incubated for a total of 96 hours at 37°C before western blot analysis.

Treatment of Ephrin-A1 for the Deregulation of EphA2 Expression

The cells were seeded in six-well plates (2×10^5 cells per well) in serum-deprived RPMI 1640 medium overnight and then stimulated with 1 μ g/ml ephrin-A1 Fc or control Fc for 6 or 24 hours. Cells were collected at the end of stimulation for western blot analysis.

Adenoviral Vector Transfection

For adenoviral vector transfection experiments, cancer cells were cultured at 70% confluence, transfected with

adenoviral luciferase or adenoviral wild-type PKR vectors for 72 hours and then evaluated for PKR protein expression through western blot analysis. Western blot analysis was performed as described previously.⁹

Statistical Analysis

Biomarkers were assigned to either low- or high-level groups based on the median score (cutoff point for biomarkers). In the univariate analysis, continuous and categorical variables were analyzed using the independent samples *t* test and χ^2 test, respectively. The Kaplan–Meier method was used to estimate survival probability as a function of time for the study patients. The log-rank test was used to measure between-group differences in patient survival time. The influence of biomarker expression on survival time was calculated using the multivariate Cox proportional hazards model with adjustment for clinical and histopathologic parameters (age, sex, smoking status, and tumor histologic subgroup). The two-sided *t* test was used to test equal proportion between groups in two-way contingency tables. The generalized estimating equation approach was used to estimate differences in the means for the data between groups. Statistical significance was set at a *p* value of less than 0.05.

RESULTS

The Association between PKR and Protein Markers

Using TMAs and immunohistochemical analysis, we previously found that PKR expression was significantly associated with overall survival in patients with stage I through IV NSCLC who had not received neoadjuvant or adjuvant therapy.¹⁷ Wistuba and colleagues performed expression studies of 25 different protein markers according to their interest by using the same TMAs (Table 1).^{17,21,23} We next attempt to explore the correlation between PKR and these markers. We analyzed associations between PKR and known NSCLC markers and found that PKR protein expression was significantly associated with DNA nucleotide methyltransferase 1 (DNMT1), thyroid transcription factor 1 (TTF-1), and EphA2 (Table 1).

Complementary Prognostic Value of PKR and Markers

We sought to determine whether DNMT1, TTF-1, and EphA2 markers provided prognostic information for NSCLC in addition to that provided by PKR. We analyzed the combinations of PKR and DNMT1, PKR and TTF-1, and PKR and EphA2 in patients with NSCLC. We combined PKR and each marker to stratify patients into four groups: high expression of both PKR and the biomarker (high/high); high PKR expression and low marker expression (high/low); low PKR expression and high marker expression (low/high); and low expression of both PKR and the biomarker (low/low). The survival curves of patients with NSCLC based on the expression of PKR and DNMT1 (Fig. 1A), TTF-1 (Fig. 1B), or EphA2 (Fig. 1C) are shown in Figure 1. As PKR/EphA2 was associated with the poorest survival durations in patients with

NSCLC at stage I specifically (data not shown) and at all stages (Fig. 1C), we focus on the relationship and mechanism between PKR and EphA2.

We also stratified patients into three groups based on the combined PKR/EphA2 expression data: high PKR expression and low EphA2 expression (high/low), low PKR expression and high EphA2 expression (low/high), and high or low expression of both proteins (others). Among patients with all stage of NSCLC, the 5-year overall survival rate in patients (20%) with PKR^{low}/EphA2^{high} was significantly lower than that of patients (74%) with PKR^{high}/EphA2^{low}, patients (55%) with PKR^{high}/EphA2^{high}, and patients (55%) with PKR^{low}/EphA2^{low} (Fig. 1D). Among patients with stage I NSCLC, the 5-year overall survival rate in patients (20%) with PKR^{low}/EphA2^{high} was significantly lower than that in patients (81%) with PKR^{high}/EphA2^{low}, patients (61%) with PKR^{high}/EphA2^{high}, and patients (61%) with PKR^{low}/EphA2^{low} (Fig. 1E). Our results also revealed that the PKR:EphA2 ratio was significantly associated with prognosis and was an independent indicator of survival duration in patients with NSCLC at all stages (Fig. 1F, *p* < 0.0001) and at stage 1 (Fig. 1G, *p* < 0.0001). Immunohistochemical assays showed that both PKR and EphA2 proteins were primarily localized in the cytoplasm of tumor cells. Representative images of PKR and EphA2

TABLE 1. Correlation between PKR and Biomarkers on Patients with NSCLC

Marker	Rho	<i>p</i> Value
5T4 mem	−0.05	0.55
BEK nuc	−0.01	0.93
CAV-a cyt	−0.03	0.79
Amphiregulin cyt	−0.15	0.17
SOX2 cyt	0.04	0.68
CD24 cyt	−0.03	0.79
CD44 cyt	−0.06	0.56
EZH2 nuc	0.09	0.30
FEN1 nuc	0.16	.015
FLG nuc	−0.07	0.52
HER3 cyt	0.14	0.09
HEY1 cyt	−0.04	0.72
IL11R cyt	0.03	0.71
JNK mem	0.10	0.29
IRAK-1 nuc	−0.07	0.51
Jagged1 cyt	−0.02	0.85
NFKB nuc	−0.09	0.26
SDC1	0.15	0.17
Her2/Neu nuc	0.13	0.12
RFC1 cyt	0.04	0.67
GLUT4 cyt	0.14	0.10
Ki67 nuc	0.09	0.27
DNMT1 cyt	0.41	<0.0001
TTF1 cyt	0.28	<0.0001
EphA2 cyt	0.45	<0.0001

Cyt, cytosol staining; mem, membrane staining; nuc, nuclear staining.

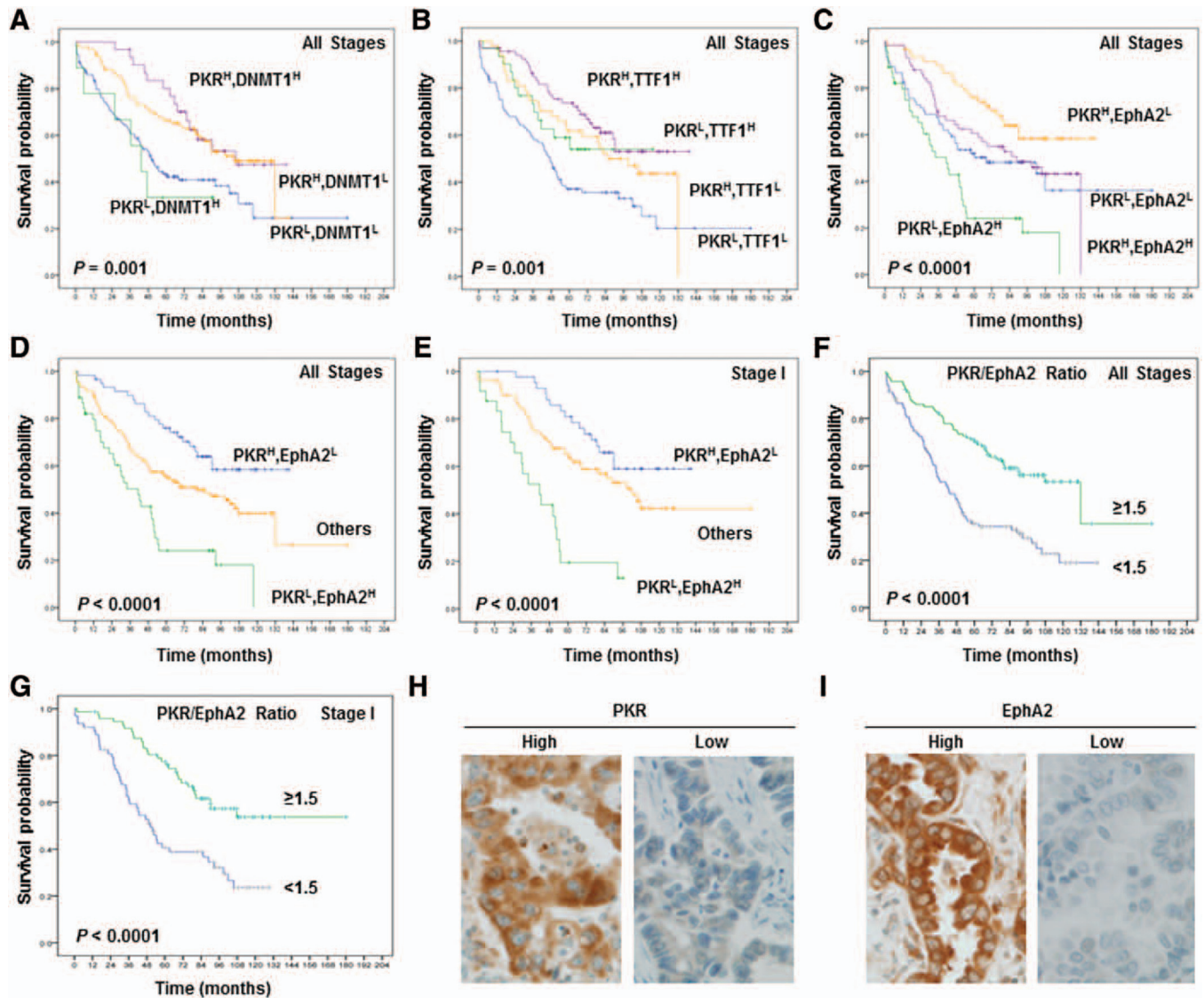


FIGURE 1. Prognostic significance of markers as assessed using Kaplan–Meier survival estimates and the log-rank test. Kaplan–Meier survival curves showing the differences in survival duration using PKR combined with DNMT1 (A), TTF-1 (B), and EphA2 (C) in patients with all stages of NSCLC. The survival rate in patients with PKR^{low}/EphA2^{high} was significantly lower than that in patients with PKR^{high}/EphA2^{low}, patients with PKR^{high}/EphA2^{high}, and patients with PKR^{low}/EphA2^{low} at all NSCLC stages (D) and at stage I (E). The PKR:EphA2 ratio was significantly associated with prognosis for NSCLC at all stages (F) and at stage I (G). The survival rate in the patients with a PKR:EphA2 ratio of 1.5 or less was significantly lower than that in patients with a PKR:EphA2 ratio of 1.5 or more than 1.5 ($p < 0.0001$). Immunohistochemical staining examples for the expressions of PKR (H) and EphA2 (I) in the NSCLC cells cytoplasm (original magnification $\times 400$). NSCLC, non–small-cell lung cancer; PKR, RNA-dependent protein kinase.

expression in the cytoplasm of NSCLC cells are shown in Figure 1H and I.

Univariate Cox proportional hazards regression analysis revealed that age, pathologic tumor, node, metastasis stage, pathologic T classification, pathologic N classification, PKR expression, EphA2 expression, and PKR/EphA2 expression significantly affected overall survival (Table 2). Multivariate Cox proportional hazards regression analysis revealed that the expression of PKR or EphA2 was significantly associated with overall survival rate after accounting for the effects of age and pathologic T and N classification (data not shown)

and that PKR/EphA2 was an independent prognostic predictor of overall survival rate ($p < 0.0001$) (Table 3). Univariate and multivariate Cox proportional hazards regression analysis revealed that the PKR:EphA2 ratio was an independent prognostic predictor of overall survival rate (data not shown). We next investigated the relationships between PKR:EphA2 ratio and other clinicopathologic features. We did not detect any statistically significant correlations between PKR:EphA2 ratio and patient's gender ($p = 0.2$) and pathological stage ($p = 0.39$) (Table 4). We observed that higher PKR:EphA2 ratio was associated with the adenocarcinomas subtype ($p = 0.003$)

TABLE 2. Univariate Cox Hazards Analysis Results for Overall Survival in Patients with NSCLC

Characteristic	No. of patients	HR (95% CI)	p Value
Age (continuous)	218	104 (1.02–1.06)	<0.0001
Gender			
Female (Reference)	113	1.00	
Male	105	1.27 (0.89–1.81)	0.20
Histology			
Adenocarcinoma (Reference)	119	1.00	
Squamous cell carcinoma	99	1.50 (1.04–2.14)	0.03
Tobacco history			
No (Reference)	38	1.00	
Yes	180	1.56 (0.91–2.69)	0.11
Pathological stage			0.04
IA/IB (Reference)	135	1.00	
IIA/IIB	47	1.20 (0.75–1.90)	0.45
IIIA/IIIB	30	1.97 (1.20–3.26)	0.01
IV	6	2.22 (0.81–6.1)	0.12
PKR			
Low (Reference)	108	1.00	
High	110	0.51 (0.36–0.74)	<0.0001
EphA2			
Low (Reference)	116	1.00	
High	102	1.50 (1.05–2.15)	0.02
PKR/EphA2			<0.0001
PKR ^H , EphA2 ^L (Reference)	53	1.00	
PKR ^L , EphA2 ^H	45	3.47 (1.99–6.03)	<0.0001
Others	120	1.69 (1.03–2.75)	0.03

CI, confidence interval; HR, hazard ratio.

and tobacco history (nonsmoker, $p = 0.001$). In previous report, we have demonstrated that PKR is closely associated with its phosphorylation (p-PKR), and patients with high expression of both PKR and p-PKR had significantly longer survival than did those with other combinations of expression levels.¹⁸ In the current study, we observed that higher PKR:EphA2 ratio was associated with the high p-PKR (phospho-PKR) expression (Table 4), and EphA2 protein expression negatively correlate with p-PKR protein expression ($\rho = -0.41$, $p < 0.0001$). We found that patients with high expression of both PKR and p-PKR had lower EphA2 expression and patients with high expression of PKR and low expression of p-PKR had higher EphA2 expression. Our data suggest that p-PKR and EphA2 may negatively regulate each other in lung tumor.

PKR Induction Inhibits EphA2 Protein Expression

As PKR has the strongest association with EphA2 (Table 1), and PKR/EphA2 combination was associated with the poorest survival durations in patients with NSCLC (Fig. 1C), we further investigated the mechanism of interaction between PKR and EphA2. We first determined whether knockdown (depletion) of EphA2 by EphA2 siRNA in human lung cancer cells (H1299 and H322) causes induction or activation

TABLE 3. Multivariate Cox Hazards Analysis Results for Overall Survival in Patients with NSCLC

Characteristic	No. of patients	HR (95% CI)	p Value
Age (Continuous)	218	1.05 (1.03–1.07)	<0.0001
Pathological stage			0.01
IA/IB (Reference)	135	1.00	
IIA/IIB	47	1.24 (0.77–1.99)	0.37
IIIA/IIIB	30	1.84 (1.11–3.05)	0.01
IV	6	3.97 (1.41–11.21)	0.009
PKR/EphA2			<0.0001
PKR ^H , EphA2 ^L (Reference)	53	1.00	
PKR ^L , EphA2 ^H	45	3.16 (1.8–5.55)	<0.0001
Others	120	1.84 (1.12–3.02)	0.01

CI, confidence interval; HR, hazard ratio.

of PKR. Our results showed that knockdown of EphA2 expression by EphA2 siRNA in H1299 and H322 human lung cancer cells did not affect PKR or p-PKR protein levels (Fig. 2A).

We next determined whether inhibition of the Ras pathway by ephrin-A1 in NSCLC cells caused induction of PKR or p-PKR. Ephrin-dependent EphA2 activation inhibits Ras-Raf-MEK-ERK signaling by activating the RasGTPase-activating protein, p120RasGAP, and down-regulates EphA2 expression by causing receptor internalization and degradation.²⁴ Ephrin-A1, which is a major ligand for EphA2, is anchored to the plasma membrane by a glycosylphosphatidylinositol linkage, but soluble forms of this ligand released

TABLE 4. Relationships between the PKR:EphA2 Ratio and Clinicopathologic Characteristics and Phospho-PKR Expression in 218 Patients with NSCLC

Characteristics	Total No. (%)	PKR:EphA2 Ratio		p Value
		<1.5 (%)	≥1.5 (%)	
Gender				
Female	113 (52)	47 (45)	66 (58)	0.06
Male	105 (48)	57 (55)	42 (42)	
Histology				
Adenocarcinoma	119 (55)	46 (44)	73 (64)	0.003
Squamous carcinoma	99 (45)	58 (56)	41 (36)	
Tobacco history				
No	38 (17)	9 (9)	29 (25)	0.001
Yes	180 (83)	95 (91)	85 (75)	
Pathological stage				
IA/IB	135 (62)	63 (61)	72 (63)	0.39 ^a
IIA/IIB	47 (22)	24 (23)	23 (20)	
IIIA/IIIB	30 (14)	16 (15)	14 (12)	
IV	6 (2)	1 (1)	5 (5)	
Phospho-PKR (p-PKR)				
High	78 (47)	27 (33)	51 (61)	<0.0001 ^b
Low	87 (53)	54 (67)	33 (39)	

^aThe p value was calculated between pathologic stage I and II–IV.

^bTotal 165 patients have all three markers data (PKR, EphA2, and p-PKR).

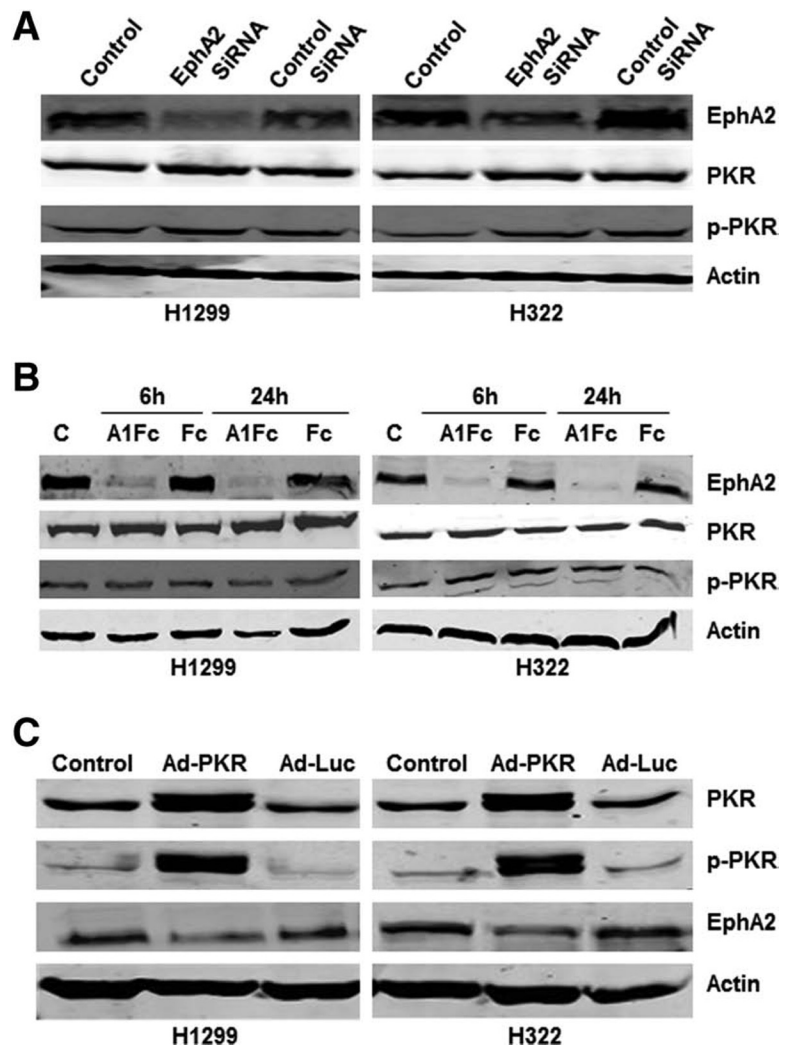


FIGURE 2. Induction of PKR expression inhibited EphA2. *A and B*, Western blot analysis of PKR, p-PKR, and EphA2 protein expression in H1299 and H322 cancer cells after knockdown of EphA2 expression by siRNA and ephrin A1Fc. Treatment with EphA2 targeting siRNA (*A*) or 1 μ g/ml ephrin A1Fc or Fc for 6 and 24 hours (*B*) did not affect PKR or p-PKR protein expression levels. C, control; A1Fc, ephrin A1Fc. Fc used as control. (*C*) Western blot analysis of PKR, p-PKR, and EphA2 protein expression in H1299 and H322 cancer cells 72 hours after treatment with Ad-Luc (2500 viral particles/cell) and Ad-PKR (2500 viral particles/cell). Ad-Luc, adenoviral luciferase; Ad-PKR, adenoviral PKR; PKR, RNA-dependent protein kinase.

from the cell surface can also activate EphA2.^{24–26} Our experiment revealed that degradation of EphA2 by ephrin-A1 Fc in H1299 and H322 lung cancer cells did not increase PKR or p-PKR expression (Fig. 2B). We next determined whether using expression vectors to induce PKR or p-PKR expression in lung cancer cells reduced EphA2 protein expression levels. Compared with control and adenoviral luciferase, adenoviral PKR inhibited EphA2 protein expression in H1299 and H322 cancer cell lines 72 hours after treatment (Fig. 2C).

DISCUSSION

In a previous study, Wistuba and colleagues used TMAs to measure the expression of 25 protein markers in NSCLC samples.^{21,23} Using the same TMAs, we found that the loss of PKR expression was correlated with an aggressive disease course and that high PKR expression predicted a subgroup of patients with a better survival.¹⁷ In the current study, we investigated the association between PKR and the 25 markers and sought to identify the markers that could provide prognostic information in addition to that provided by PKR. We found that PKR protein expression was significantly

correlated with DNMT1, TTF-1, and EphA2 expression and that PKR/EphA2 expression was a significant predictor of overall survival duration in patients with NSCLC. We also found that the PKR:EphA2 ratio was an independent predictor of survival duration in patients with NSCLC.

The findings of the current study not only identify PKR/EphA2 as a novel predictor of prognosis but also suggest that targeting PKR and/or EphA2 has therapeutic potential in patients with NSCLC. PKR's phosphorylation of eIF2 α inhibits protein synthesis in host NSCLC cells, thereby causing apoptosis.^{7,27,28} High PKR expression indicates a favorable prognosis for a variety of malignancies, suggesting that PKR plays an important role effecting apoptosis in cancer cells and suppressing tumor progression.²⁸ We previously found that high PKR or p-PKR expression in NSCLC cells correlated with a favorable prognosis, which is consistent with previous observations that PKR activation is associated with apoptosis induction.^{8,9,11,17,29} Several compounds can induce PKR-dependent apoptosis in cancer cells,^{30,31} indicating that induction of expression or activation of PKR is one potential novel approach to treating cancer. EphA2 is detectable in many cancer cells, including ovarian, prostate, colon, and brain

cancer cells.³² In recent one study, Ishikawa M et al. reported that higher expression of EphA2 and *ephrin-A1* is related to favorable clinicopathological features in pathological stage I non-small-cell lung carcinoma.³³ However, presence of EphA2 has been associated with poor prognosis in many patients with cancer.^{19–23,34} We anticipate that the PKR/EphA2 biomarker can be used to identify patients with NSCLC who may benefit from antitumor therapy with EphA2 pathway inhibitors.

Recently, studies showed that MEDI-547, an EphA2 targeted fully human monoclonal antibody (1C1) conjugate, had a significant role in inhibiting tumor growth in mouse xenograft and rat syngeneic models of prostate and endometrial cancers.^{35,36} Furthermore, some peptides can selectively bind to the ligand-binding domain of EphA2 and an *ephrin A1*–*Pseudomonas aeruginosa* exotoxin A conjugate have shown promise in delivering drugs to tumors.³⁷ Use of the combination of a PKR activator and an EphA2 inhibitor may be a promising therapy for NSCLC. In the future, we will investigate the use of combination therapy for NSCLC targeting both PKR and EphA2.

An understanding of PKR/EphA2 protein interactions is vital to the study of lung cancer and clarifying their contribution to prognosis. In the current study, we observed no significant changes in the expression of PKR or p-PKR in the NSCLC cells in which EphA2 expression was down-regulated, which indicates that EphA2 may not inhibit expression of PKR directly. One potential route of indirect inhibition of PKR is the Ras-Raf-MEK-ERK pathway, which negatively regulates PKR and positively regulates the EphA2 pathway.^{38–41} In the current study, however, treating NSCLC cells with *ephrin-A1*, which down-regulates both the EphA2 and Ras-Raf-MEK-ERK pathways,³⁹ did not affect the expression of PKR or p-PKR, suggesting that the Ras pathway does not regulate PKR or EphA2 in these cells. We found that induction of expression of PKR by adenoviral expression of PKR inhibited expression of EphA2 protein in lung cancer cells. We observed that EphA2 protein expression positively correlate with PKR and negatively correlate with phospho-PKR (p-PKR) proteins. The possible explanation for this association is that the high expression of EphA2 may stimulate negative feedback (PKR induction), and activation of PKR (p-PKR) by others inhibit EphA2 protein expression. We also suspect that PKR and EphA2 regulate several common downstream targets and that cancer cell growth depends on the balance of PKR/EphA2 protein expression. Further study is needed to identify common targets of PKR and EphA2 pathways in NSCLC tumors.

In conclusion, PKR/EphA2 is a significant predictor of prognosis in patients with NSCLC. Further studies of multiple NSCLC markers may yield additional information about the prognostic significance of PKR/EphA2. PKR/EphA2 may be a promising approach to improving screening efficiency and predicting prognosis in patients with NSCLC. We observed that induction of PKR expression in lung cancer cells inhibited EphA2 protein expression in vitro. The possible explanation is that a high PKR:p-PKR ratio turns off protein synthesis, decreases EphA2 expression, and inhibits tumor growth.

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