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Original Article

Prognostic values of EphB1/B2 and p-EphB1/B2 expression in non-small cell lung cancer

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Abstract: Erythropoietin-producing hepatocellular carcinoma (Eph) Receptor, as a family member of receptor tyrosine kinases (RTK), plays a critical role in modulating different cell behaviors. It is also closely related to tumorigenesis. However, little has been known about its prognostic values in non-small cell lung cancer (NSCLC). Thus, we studied the expression levels of EphB1/2 and p-EphB1/2 in both NSCLC tissue and normal lung tissue, and analyzed their correlations with clinicopathological characteristics as well as NSCLC patients' survival. In the present study, 156 NSCLC tissue samples and 28 distal normal lung tissue samples were collected from 156 NSCLC patients. Afterwards, the protein levels of EphB1/2 and p-EphB1/2 were detected by immunohistochemistry. Their prognostic values were also evaluated using both univariate and multivariate survival analysis. According to the results, 44.87% (70/156) NSCLC samples were detected with positive EphB1/2 expression, significantly higher than that in distal normal lung tissue (16%, 4/25); but no difference was found regarding to p-EphB1/2 expression. With respect to the clinicopathological characteristics, there was no significant correlation between protein levels and age, gender, histological type, differentiation status as well as TNM stage. Intriguingly, it showed a clear trend of increased EphB1/2-positive rate when tumor differentiation grade developed. In the survival analysis, a positive correlation was found between positive p-EphB1/2 expression and poor survival in female (P=0.001). Then N stage (P=0.001) and TNM stage (P<0.001) were found significantly related to patients' survival in multivariate analysis. Therefore, p-EphB1/2 may serve as a prognostic predictor in female NSCLC patients.

Keywords: NSCLC, prognosis, Eph, immunohistochemistry

Introduction

Lung cancer is the leading cause of cancer related mortality on a global scale. Non-small cell lung cancer (NSCLC) is the most common type, accounting for 85% of all primary cases [1]. Despite the recent developments in treatment of NSCLC, its prognosis remains unfavorable, with a 5-year overall survival rate only 15% [2, 3]. Several biomarkers have been found related to the poor prognosis of NSCLC in the previous studies, including mammalian target of rapamycin (mTOR) [4], protein kinase B (AKT) [5], extracellular-regulated kinase 1/2 (ERK1/2) [6], epidermal growth factor receptor (EGFR) [7] and programmed cell death-ligand 1 (PD-L1) [8]. However, consistent results cannot be concluded with an expanded sample size or in other identical studies. Therefore, a more effective prognostic biomarker for NSCLC is necessary.

Erythropoietin-producing hepatocellular carcinoma (Eph) Receptor has the potential to be an ideal prognostic indicator for NSCLC [9-12]. It is a family member of receptor tyrosine kinases (RTK), consisting of an intracellular, a transmembranic and an extracellular region [13]. Eph receptor includes 2 classes, namely EphA and EphB. EphA has 9 members promiscuously binding 5 ephrin-A ligands, while EphB has 5 members binding 3 ephrin-B ligands [9]. The combination of Eph receptor and ephrin stimulates bidirectional signals (forward signal and reverse signal) in cells, which triggers the phosphorylation of Eph receptors [11]. Afterwards,

the phosphorylated Eph receptor activates downstream signaling proteins, and modulates different cellular behaviors, including cell survival, proliferation, differentiation, migration and morphological changes [14].

Some previous studies have proved the role of Eph receptor in both promoting and suppressing tumorigenicity. It influences cancer metastasis, angiogenesis and invasion, and correlated with patients' clinicopathological characteristics in several cancers [15-17]. For example, a decreased expression level of EphB1 was detected in various types of cancers, including gastric cancer [18], colorectal cancer [19], ovary carcinoma [20] and renal cell carcinoma [21]. In another study, the expression level of EphB2 was found decreased in colorectal cancer patients with liver metastasis [22]. In lung cancer, some Eph receptors were also found unregulated. It was advocated that overexpression of EphA2 predicted a shorter survival and brain metastasis in lung cancer [23]. Afterwards, it was further demonstrated that a lower expression level of EphB6 was correlated with metastatic lung cancer [24]. Moreover, the expression level of EphB3 was found significantly upregulated in NSCLC samples, and also associated with patients' clinical characteristics, including tumor size, differentiation and metastasis [25]. However, the prognostic role of EphB1/2 and p-EphB1/2 remains elusive at present.

The immunohistochemical staining assay was explored in this study. It was undertaken to determine the prognostic value of EphB1/2 and p-EphB1/2 in NSCLC, and analyze the relationships between their expression levels and clinicopathological characteristics in NSCLC patients.

Material and methods

Patients and tissue collection

In this study, 263 NSCLC patients were subsequently enrolled in West China Hospital, Sichuan University of China, from January 2008 to December 2013. All of the patients underwent a complete resection for primary NSCLC. Subsequent standard treatments were given according to the Clinical Oncology Information Network guidelines if necessary, without preoperative chemotherapy or radiotherapy. Data on age, gender, tumor size, lymph node metastasis and distant metastasis were collected by two physicians independently, according to the medical records. The TNM stage of each tumor was determined according to tumor-node-metastasis system of the International Union Against Cancer, and differentiation and histological type were estimated according to the World Health Organization's classification for NSCLC. The median follow-up time was 40.40 months (range, 2-60 months). Only 156 lung cancer tissue samples and 28 distal normal lung tissue samples from 156 NSCLC patients were finally included because of inadequate tissue and losing contact.

Immunohistochemistry

Tissues were fixed in 10% neutral formalin and embedded with paraffin within 12-24 hours after surgery. The 3- to 5-μm sections were deparaffinized in xylene and graded ethanol in distilled water, then blocked for endogenous peroxides in 3% H_2O_2 for 15 minutes. Antigen retrieval was performed in water bath by Tris/ethylenediaminetetraacetic acid solution. Subsequently, the slides were incubated with the corresponding primary antibody at 4°C overnight, then incubated with secondary antibody, goat anti-rabbit IgG (Dako, Shanghai, China) for 30 minutes. At last, Harris hematoxylin was used in counter staining. The primary antibodies used were as follows: EphB1/B2 (ab61765, ABcam), p-EphB1/B2 (ab61791, Y594, ABcam).

Immunohistochemical scoring

Results of immunohistochemical staining were assessed according to a semiquantitative scoring system, which take both the fraction and the intensity of immunohistochemical staining into consideration. The fraction score was defined as follows: 3 (>50% cells stained), 2 (20-50% cells stained), 1 (10-20% cells stained) and 0 (<10% cells stained). The intensity score was defined as follows: 3 (dark brown staining), 2 (obviously appreciable brown staining), 1 (barely detectable staining) and 0 (no appreciable staining). The total score was calculated by multiplying the fraction score and intensity score, with a range from 0 to 9. Slides with a score of 0 were defined as negative; while 1-3 and 4-9 were moderate and positive. Two pathologists independently accomplish this pro-

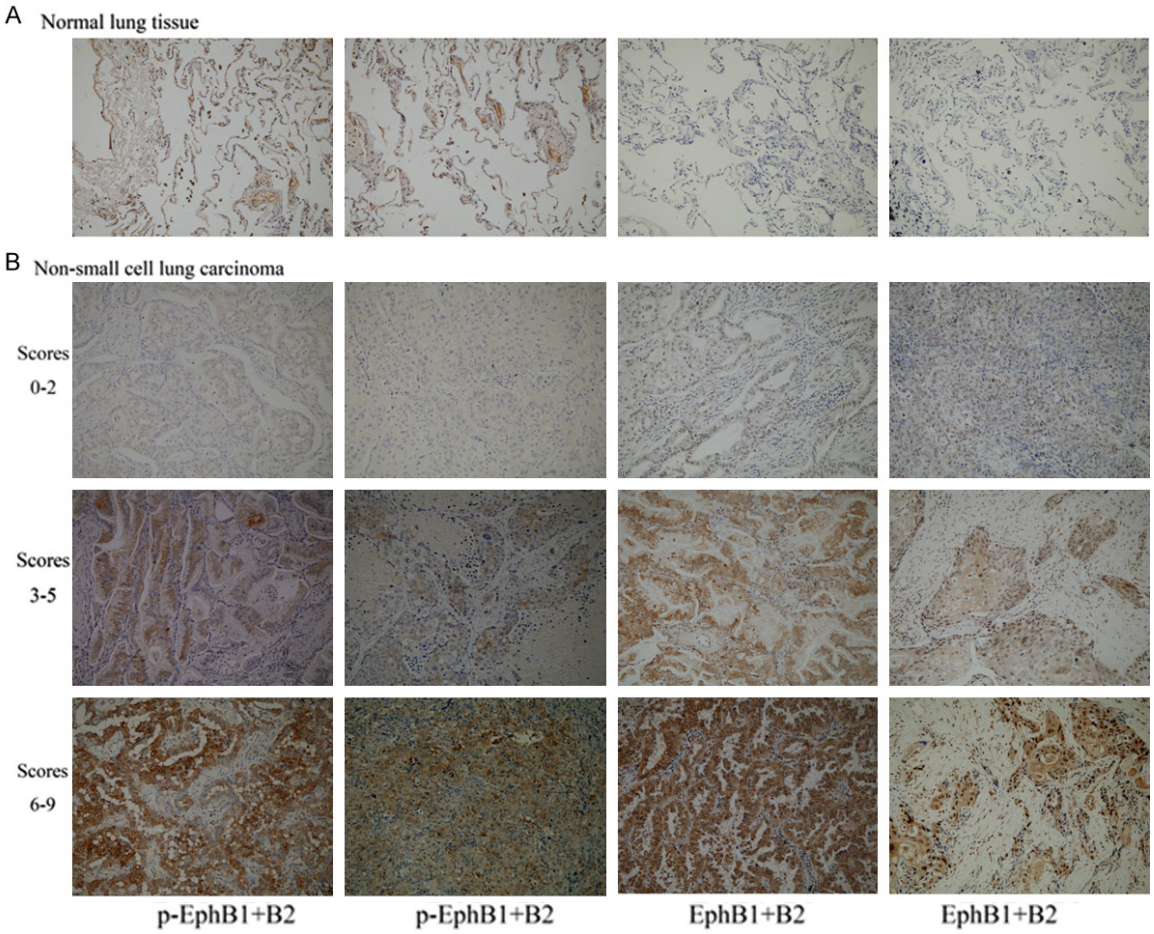

Figure 1. Expression of EphB1/2 and p-EphB1/2 in normal lung tissue (A) and non-small cell lung carcinoma (B) specimens are shown. (B) From the first to the third line, immunohistochemical staining of scores of 0-2, 3-5 and 6-9 are shown for each protein. Original magnification, ×400.

Table 1. Expression levels of EphB1/2 and p-EphB1/2 in lung cancer tissue and normal lung tissue

Protein	Expression level	Lung cancer tissue No. (%)	Normal lung tissue No. (%)	*P* value
EphB1/2	N	36 (23.08)	12 (48.00)	0.002*
	M	50 (32.05)	9 (36.00)	
	P	70 (44.87)	4 (16.00)	
p-EphB1/2	N	121 (77.56)	17 (60.71)	0.087
	M	29 (18.59)	11 (39.29)	
	P	6 (3.85)	0 (0.00)	

N, negative; M, moderate; P, positive. *P<0.05.

cedure without the knowledge of patients' clinical information.

Statistical analysis

The statistical analysis was accomplished using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Pearson Chi-squared test was used to estimate the relationship between clinicopathological characteristics and protein expression status. The Kaplan-Meier method was performed to draw survival curves and log-rank test was used to determine the significance. Subsequently, the multivariate Cox regression analysis was conducted to identify the independent prognostic factors of overall survival. Results were considered of statistical significance when P<0.05.

Results

The expression of EphB1/2 and p-EphB1/2 in lung cancer tissue and normal lung tissue

The expression patterns of EphB1/2 and p-EphB1/2 were shown in **Figure 1**. EphB1/2

Table 2. Associations between the expressions of EphB1/2 and p-EphB1/2 and clinical features of 156 patients

Variables		EphB1/2			*P* value	p-EphB1/2			*P* value
		Negative (n=36)	Moderate (n=50)	Positive (n=70)		Negative (n=121)	Moderate (n=29)	Positive (n=6)	
Age	<40 (n=9)	4 (44.4)	3 (33.3)	2 (22.2)	0.193	7 (77.8)	2 (22.2)	0 (0.0)	0.538
	40-65 (n=111)	27 (24.3)	32 (28.2)	52 (46.8)		87 (78.4)	21 (18.9)	3 (2.7)	
	>65 (n=31)	5 (16.1)	14 (45.2)	12 (38.7)		24 (77.4)	5 (16.1)	2 (6.5)	
	Missing (n=5)	0 (0.0)	1 (20.0)	4 (80.0)		3 (60.0)	1 (20.0)	1 (20.0)	
Gender	Male (n=104)	24 (23.1)	37 (35.6)	43 (41.3)	0.359	82 (78.8)	19 (18.3)	3 (2.9)	0.419
	Female (n=47)	12 (25.2)	12 (25.2)	23 (48.9)		36 (76.6)	9 (19.1)	2 (4.3)	
	Missing (n=5)	0 (0.0)	1 (20.0)	4 (80.0)		3 (60.0)	1 (20.0)	1 (20.0)	
Histological type	ADC (n=78)	17 (21.8)	26 (33.3)	35 (44.9)	0.541	60 (76.9)	15 (19.2)	3 (3.8)	0.058
	SCC (n=66)	19 (28.8)	19 (28.8)	28 (42.2)		52 (78.8)	12 (18.2)	2 (3.0)	
	Others (n=10)	0 (0.0)	4 (40.0)	6 (60.0)		8 (80.0)	2 (20.0)	0 (0.0)	
	Missing (n=2)	0 (0.0)	1 (50.0)	1 (50.0)		1 (50.0)	0 (0.0)	1 (50.0)	
Differentiation	Low (n=44)	13 (29.5)	14 (31.8)	17 (38.6)	0.467	38 (86.4)	5 (11.4)	1 (2.3)	0.318
	Moderate (n=69)	18 (26.1)	22 (31.9)	29 (42.0)		54 (78.3)	11 (15.9)	4 (5.8)	
	High (n=4)	1 (25.0)	1 (25.0)	2 (50.0)		3 (75.0)	1 (25.0)	0 (0.0)	
	Missing (n=39)	4 (10.3)	13 (33.3)	22 (56.4)		26 (66.7)	12 (30.8)	1 (2.6)	
pT stage	1 (n=22)	10 (45.5)	6 (27.3)	6 (27.3)	0.11	19 (86.4)	2 (9.1)	1 (4.5)	0.365
	2 (n=80)	17 (21.3)	26 (32.5)	37 (46.3)		61 (76.3)	15 (18.8)	4 (5.0)	
	3 (n=18)	1 (5.6)	8 (44.4)	9 (50.0)		15 (83.3)	3 (16.7)	0 (0.0)	
	4 (n=24)	7 (29.2)	7 (29.2)	10 (41.7)		20 (83.3)	4 (16.7)	0 (0.0)	
	Missing (n=12)	3 (25.0)	3 (25.0)	8 (66.7)		6 (50.0)	5 (41.7)	1 (8.3)	
pN stage	0 (n=78)	21 (26.9)	30 (38.5)	27 (34.6)	0.099	62 (79.5)	14 (17.9)	2 (2.6)	0.185
	1, 2, 3 (n=66)	14 (21.2)	17 (25.8)	35 (53.0)		53 (80.3)	10 (15.2)	3 (4.5)	
	Missing (n=12)	1 (8.3)	3 (25.0)	8 (66.7)		6 (50.0)	5 (41.7)	1 (8.3)	
pM stage	0 (n=137)	35 (25.5)	45 (32.8)	57 (41.6)	0.196	110 (80.3)	23 (16.8)	4 (2.9)	0.091
	1 (n=7)	0 (0.0)	2 (28.6)	5 (71.4)		5 (71.4)	1 (14.3)	1 (14.3)	
	Missing (n=12)	1 (8.3)	3 (25.0)	8 (66.7)		6 (50.0)	5 (41.7)	1 (8.3)	
Stage	1 (n=49)	16 (32.7)	17 (34.7)	16 (32.7)	0.144	38 (77.6)	10 (20.4)	1 (2.0)	0.125
	2 (n=44)	6 (13.6)	17 (38.6)	21 (47.7)		36 (81.8)	5 (11.4)	3 (6.8)	
	3 (n=45)	13 (28.9)	11 (24.4)	21 (46.7)		37 (82.2)	8 (17.8)	0 (0.0)	
	4 (n=6)	0 (0.0)	2 (33.3)	4 (66.7)		4 (66.7)	1 (16.7)	1 (16.7)	
	Missing (n=12)	1 (8.3)	3 (25.0)	8 (66.7)		6 (50.0)	5 (41.7)	1 (8.3)	

expression was detected in both nucleus and cytoplasm, while p-EphB1/2 was detected in cytoplasm. Of the 156 specimens, there were 36 (23.08%) specimens with EphB1/2-negative expression, 50 (32.05%) specimens with EphB1/2-moderate expression and 70 (44.87%) specimens with EphB1/2-positive expression; while 121 (77.56%) specimens with p-EphB1/2-negative expression, 29 (18.59%) specimens with p-EphB1/2-moderate expression and 6 (3.85%) specimens with p-EphB1/2-positive expression.

Comparisons of EphB1/2 and p-EphB1/2 in lung cancer tissue with normal lung tissue are shown in **Table 1**. Expression level of EphB1/2 was significantly increased in lung cancer tissue (P=0.002). Nevertheless, no significant difference was found in p-EphB1/2 expression between lung cancer tissue and normal lung tissue (P=0.087).

Relationship between the expression of EphB1/2 and p-EphB1/2 (two proteins) and clinical features

The main clinical features of patients were summarized in **Table 2**. No significant relationships were found between the EphB1/2 expression, p-EphB1/2 expression and clinical characteristics, including age, gender, histological type, differentiation status and TNM stage.

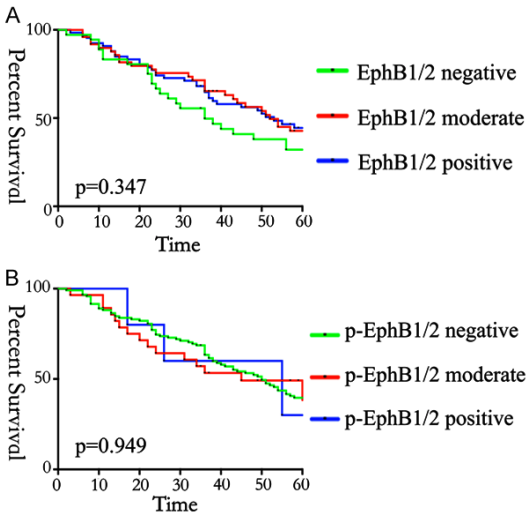

Figure 2. Correlation between EphB1/2 or p-EphB1/2 expression and overall survival in non-small cell lung cancer (NSCLC). A. Survival of EphB1/2 negative, moderate and positive expression; B. Survival of p-EphB1/2 negative, moderate and positive expression.

There was a clear trend of increased EphB1/2-positive rate when tumor differentiation grade developed. The EphB1/2-positive rate was 38.6% in poor-differentiated tumors, 42.0% in moderate-differentiated tumors and 50.0% in well-differentiated tumors. However, no significant difference was found (P=0.476). Similarly, the EphB1/2-positive rate also increased with a progressed pN stage and pM stage, but still no significant difference (P=0.099 and P=0.196, respectively).

The association of the expression of EphB1/2 and p-EphB1/2 (two proteins) with overall survival of NSCLC patients

The Kaplan-Meier method was used to calculate the relationships between the expression levels of EphB1/2, p-EphB1/2 and patients' 5-year median survival rate (**Figure 2**). There was a trend that negative expression of EphB1/2 was correlated with poor survival in NSCLC patients. However, no significant relationship was found (P=0.224, **Figure 2A**). Similarly, there was also no significant relationship between p-EphB1/2 expression and overall survivals of NSCLC patients (P=0.747, **Figure 2B**).

Afterwards, we performed subgroup analysis in patients with negative/moderate/positive expressions of EphB1/2 (**Figure 3**). The results indicated that patients with negative EphB1/2 expression had a shortened survival time in the subgroups of male (P=0.348, **Figure 3B**), adenocarcinoma (P=0.180, **Figure 3C**), moderate/well differentiation (P=0.307, **Figure 3F**), N0 (P=0.102, **Figure 3G**), M0 (P=0.359, **Figure 3K**). Nevertheless, no significant correlations were found. Moreover, no trends or significant differences were found in subgroups of female (P=0.148, **Figure 3A**), squamous cell carcinoma (P=0.709, **Figure 3D**), poor differentiation (P=0.977, **Figure 3E**), N1/2/3 (P=0.452, **Figure 3H**), stage I/II (P=0.938, **Figure 3I**) or stage III/IV (P=0.181, **Figure 3J**).

Meanwhile, we also performed subgroup analysis in patients with negative/moderate/positive expressions of p-EphB1/2 (**Figure 4**). A positive correlation was found between positive p-EphB1/2 expression and poor survival in female (P=0.001, **Figure 4A**). Furthermore, it was found that positive p-EphB1/2 expression was correlated with shortened survival time in subgroups of N1/2/3 (P=0.413, **Figure 4H**) and stage III/IV (P=0.367, **Figure 4J**), but no significant relationship. In subgroups of male (**Figure 4B**), adenocarcinoma (**Figure 4C**), squamous cell carcinoma (**Figure 4D**), poor differentiation (**Figure 4E**), moderate/well differentiation (**Figure 4F**), N0 (**Figure 4G**) and stage I/II (**Figure 4I**), no significant differences were found.

Interestingly, we didn't draw subgroup analysis in patients with distant metastasis because of the small sample size. There were only 7 patients in M1 subgroup, among which 2 patients with moderate EphB1/2 expression and 5 patients with positive EphB1/2 expression. No patients with negative EphB1/2 expression were found in M1 subgroup. Similarly, subgroup analysis was not conducted according to differentiation status, because no patients with positive p-EphB1/2 expression were found in subgroup of high differentiation (**Figure 4E**).

Multivariate analysis

The multivariate Cox regression analysis was used to further evaluate the correlation of EphB1/2 and p-EphB1/2 expressions and clinical features with survival rate. As shown in **Table 3**, lymph node metastasis (P=0.001), TNM stage (P=0.000) and EphB1/2 expression (P=0.038) were independently associated with

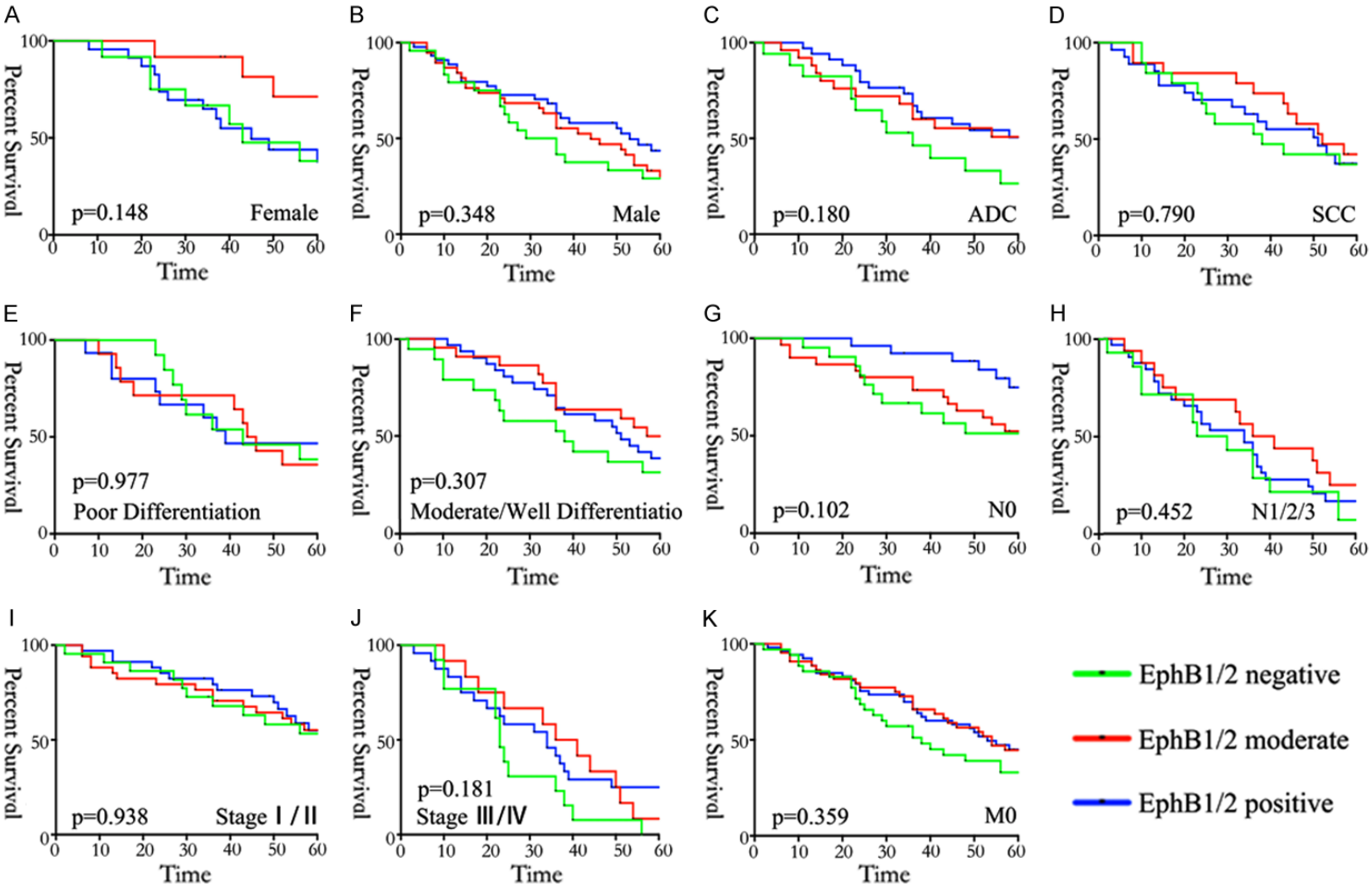

Figure 3. Kaplan-Meier curves for patients' survival according to EphB1/2 expression. The survival analysis are stratified by EphB1/2-negative, EphB1/2-moderate and EphB1/2-positive expression in female (A), male (B), ADC (C), SCC (D), poor differentiation (E), moderate/well differentiation (F), N0 (G), N1/2/3 (H), Stage I/II (I), stage III/IV (J), M0 (K), respectively.

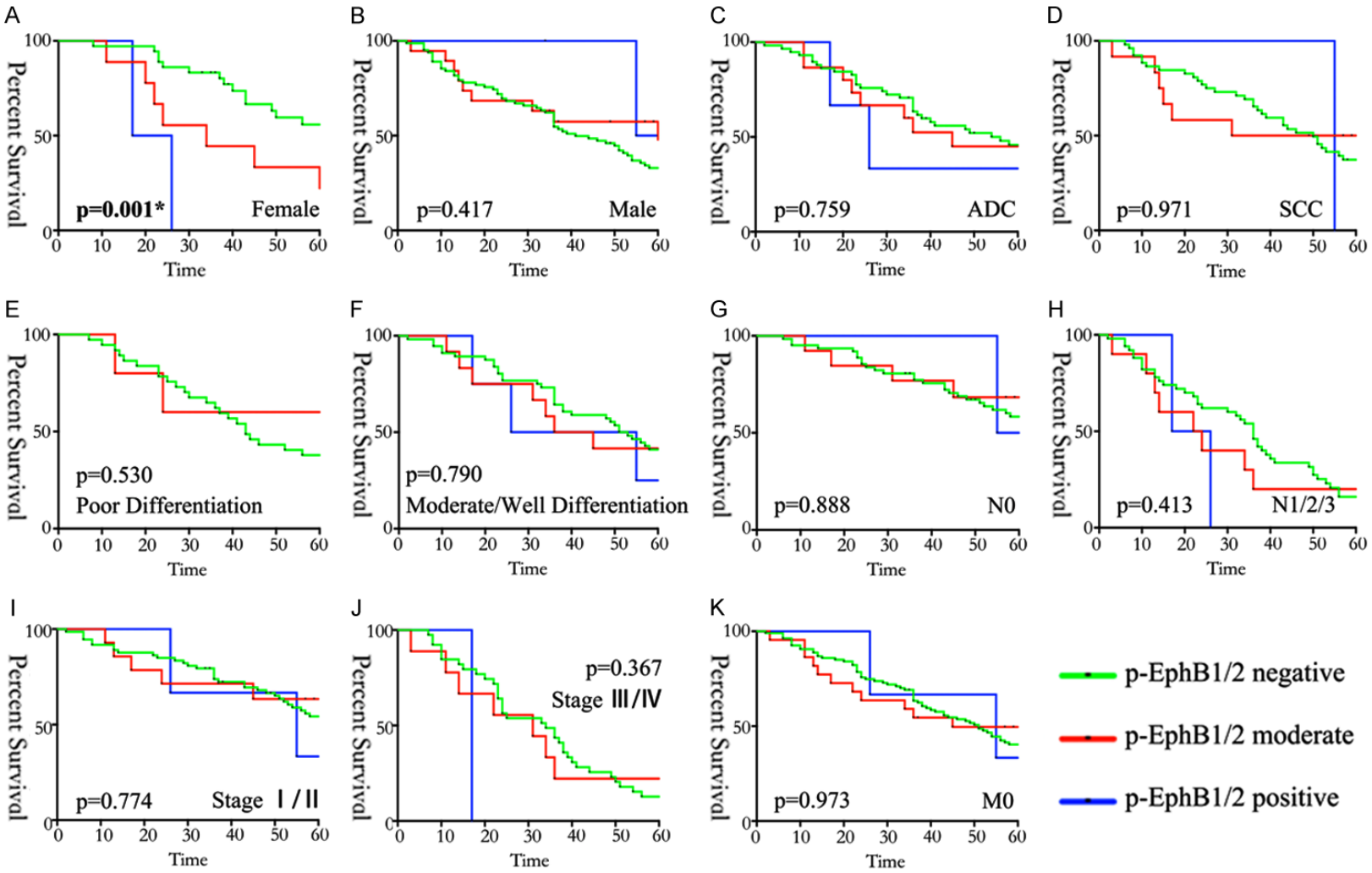

Figure 4. Kaplan-Meier curves for patients' survival according to p-EphB1/2 expression. The survival analysis are stratified by p-EphB1/2-negative, EphB1/2-moderate and EphB1/2-positive expression in female (A), male (B), ADC (C), SCC (D), poor differentiation (E), moderate/well differentiation (F), N0 (G), N1/2/3 (H), Stage I/II (I), stage III/IV (J), M0 (K), respectively.

Table 3. Multivariate Cox regression analysis of overall survival

Variables	SE	Exp (B)	p	95% CI for exp (B)
Gender	0.345	0.546	0.080	0.278-1.074
Age (<40/40-65/≥65)	0.287	0.754	0.324	0.430-1.322
Histological types (SCC/ADC/Others)	0.311	0.913	0.771	0.496-1.682
Differentiation status (low/moderate to well)	0.301	1.354	0.314	0.750-2.445
N stage (N0/N1, N2, N3)	0.285	0.382	0.001**	0.218-0.668
M stage (M0/M1)	0.558	1.937	0.236	0.649-5.779
TNM stage (1+2/3+4)	0.268	3.458	0.000**	2.047-5.842
EphB1/2 (negative/moderate/positive)	0.172	0.7	0.038*	0.499-0.980
p-EphB1/2 (negative/moderate/positive)	0.283	1.557	0.118	0.894-2.711

SCC, squamous cell carcinoma; ADC, adenocarcinoma; *P<0.05; **P<0.01.

overall survival, but p-EphB1/2 expression had no significant relationship with survival (P= 0.118).

Discussion

In this study, immunohistochemistry was used to detect the expression status of EphB1/2 and p-EphB1/2 in 156 NSCLC specimens and 28 normal lung tissue samples. Protein level of EphB1/2 was significantly increased in lung cancer tissue, but no difference was found in p-EphB1/2 expression. The correlations between their expression levels and patients' clinicopathological characteristics as well as survival status were also analyzed. In these specimens, 32.05% and 18.59% were detected moderate expression for EphB1/2 and p-EphB1/2; while 44.87% and 3.85% were detected positive expression. Based on our results, statistically significant difference was not found between EphB1/2, p-EphB1/2 expression and clinicopathological characteristics. Neither did it be detected between their expression and overall survival. In subgroup analysis, only p-EphB1/2 expression was found correlated with poor survival in female lung cancer patients. Nevertheless, we found a clear trend that EphB1/2 positive rate increased when tumor differentiation level, pN or pM stage developed. Furthermore, we also noticed a poor survival in patients with negative EphB1/2 expression.

To our knowledge, various studies have emphasized the importance of EphB1 and EphB2 in organism function development. Both EphB1 and B2 played critical roles in the formation of ipsilateral projection in optic chiasm [26, 27]. Besides, EphB1 was related to bone cancer pain [28, 29]. Meanwhile, they also participated in tumorigenesis. In EphB1-methylated acute myelogenous leukemia (AML) cell, the reintroduction of EphB1 expression enhanced the cascade of p53, p21, ATR and CDK1, then enforced the programmed cell death [30]. In another study using a recellularized human colon model, EphB1 and EphB2 were implicated in colorectal cancer progression and identified as invasion-driver genes [31].

According to the present study, EphB1/2 was associated with a favorable survival. This conclusion has been demonstrated in previous studies. In serous ovarian carcinoma, loss of EphB1 expression was associated with a significantly worse overall survival [20]. It was also demonstrated that EphB1 was significantly depressed in pediatric AML patients with poor overall survival [30]. In lung cancer, q-PCR was further used to identify EphB1 gene's role in predicting progression [32]. However, no effective evidence has been found to support EphB2 as a survival predictor. The correlation between EphB1/2 and survival might be due to EphB1/2 gene mutation. In Satu's study, EphB1 and EphB2 were detected frequently mutated in lung tumor sample. Furthermore, these mutations were often together with other therapeutic targets of lung cancer, including EGFR and KRAS [33]. Identical results were also concluded in NSCLC, which demonstrated that genomic alteration was found commonly in EphB1 gene [34].

The present study has also demonstrated that p-EphB1/2 was correlated to poor survival in NSCLC. In addition, we found an interesting phenomenon that p-EphB1/2 positive sample was infrequent in lung cancer, and no

p-EphB1/2 positive sample was detected in patients with low differentiation level. Therefore, we postulated that EphB1/2 phosphorylation played a critical role in NSCLC differentiation. However, this hypothesis couldn't be properly justified for limited sample size in the present study. Furthermore, we didn't find related researches but a study in AML. It found that EphB1 protein phosphorylation was significantly depressed in pediatric AML samples [30].

Meanwhile, several limitations existed in the present study. Firstly, the sample size was relatively small, especially in the subgroup of p-EphB1/2 positive expression. Then, the type, dilution of primary antibody and the score system of immunohistochemistry weren't standardized, leading to the disagreements between different studies. Therefore, an optimized cohort study with a larger sample size and a unified methodology is needed to further demonstrate the relationships of EphB1/2, p-EphB1/2 expression and the survival of lung cancer patients.

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Disclosure of conflict of interest

None.

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