Original Article

Impact of RBM10 and PD-L1 expression on the prognosis of pathologic N1–N2 epidermal growth factor receptor mutant lung adenocarcinoma

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Background: Impact of RNA-binding motif protein 10 (RBM10) and programmed death-ligand 1 (PD-L1) on the postoperative prognosis of patients with epidermal growth factor receptor gene mutation (*EGFR*-Mt) lung adenocarcinoma with pathological lymph node metastasis is still unclear.

Methods: Patients who underwent curative surgery for pN1–N2 *EGFR*-Mt lung adenocarcinoma (n=129) harboring the *EGFR* exon 19 deletion mutation (Ex19) (n=66) or *EGFR* exon 21 L858R mutation (Ex21) (n=63) between January 2010 and December 2020 were included in this retrospective study. The prognoses of patients with low/high cytoplasmic RBM10 expression and PD-L1 negativity/positivity based on immunohistochemistry (IHC) of resected specimens were compared using the log-rank test. The effects of RBM10 and PD-L1 expression on overall survival (OS) were examined via multivariable analysis using the Cox proportional hazards regression model. The effects of RBM10 and PD-L1 expression on progression-free survival (PFS) of EGFR-tyrosine kinase inhibitors (TKIs) therapy among patients with recurrent pN1–N2 *EGFR*-Mt lung adenocarcinoma (n=67) were examined using log-rank tests.

Results: The RBM10 low expression group showed significantly better 5-year OS than the RBM10 high expression group (89.4% *vs.* 71.5%, P=0.020), and the PD-L1 negative group tended to have longer 5-year OS than the PD-L1 positive group (86.4% *vs.* 68.4%, P=0.050). Multivariable analysis showed that high RBM10 expression [hazard ratio (HR), 3.12; 95% confidence interval (CI): 1.19–8.17; P=0.021] and PD-L1 positivity (HR, 3.80; 95% CI: 1.64–8.84; P=0.002) were independent poor prognostic factors for OS. PFS of patients with relapse and first-line EGFR-TKI treatment was significantly better in the PD-L1-negative group than in the PD-L1-positive group (34.5 *vs.* 12.1 months, P=0.045). PFS of patients with Ex21 relapse and first-line EGFR-TKI treatment was significantly better in the RBM10 low expression group than in the RBM10 high expression group (25.5 *vs.* 13.0 months, P=0.025).

Conclusions: High RBM10 expression and PD-L1 positivity are poor prognostic factors for OS in patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma after curative surgery. In patients with recurrent pN1–N2 *EGFR*-Mt lung adenocarcinoma, PD-L1 and RBM10 expression may influence response to EGFR-TKIs.

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Keywords: Epidermal growth factor receptor gene mutation (*EGFR*-Mt); overall survival (OS); programmed death-ligand 1 (PD-L1); primary lung adenocarcinoma; RNA-binding motif protein 10 (RBM10)

Submitted Jun 01, 2023. Accepted for publication Aug 29, 2023. Published online Sep 25, 2023.
doi: 10.21037/tlcr-23-355
View this article at: https://dx.doi.org/10.21037/tlcr-23-355

Introduction

Epidermal growth factor receptor gene mutations (*EGFR*-Mt) account for approximately half of the driver gene mutations in lung adenocarcinoma in Asian populations, with exon 19 deletion mutation (Ex19) and exon 21 L858R mutation (Ex21) accounting for 85–90% of *EGFR*-Mt (1-4). Lung adenocarcinomas harboring *EGFR*-Mt recur at a high rate even after complete resection (5), with a poor 5-year disease-free survival rate of 26.2% (5,6). Although EGFR-tyrosine kinase inhibitors (TKIs) are effective after recurrence (7,8), the prognosis of patients with *EGFR*-Mt lung adenocarcinoma with pathological lymph node metastasis (stage pN1–pN2) after curative surgery is poor, with 5-year overall survival (OS) rates of 65% for patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma and 48% for patients with pN1–N2 Ex21 lung adenocarcinoma (6,9). Even with EGFR-TKI treatment, 20–30% of patients with advanced or recurrent *EGFR*-Mt lung adenocarcinoma have a poor prognosis owing to the low initial response (10). Overcoming the low response to EGFR-TKIs will improve OS of patients with *EGFR*-Mt lung adenocarcinoma after resection. New therapeutic targets that improve the prognosis of patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma after surgery need to be elucidated.

RNA-binding motif protein 10 (RBM10) is involved in the regulation of mRNA splicing, apoptosis induction, angiogenesis, and cell growth inhibition (11,12). Several studies have reported that RBM10 expression was correlated with various factors related to prognosis in solid tumors, including lung cancer (13,14) and breast cancer (15). Recently, Nanjo *et al.* (16) reported that genetic inactivation of *RBM10* in *EGFR*-Mt cells diminished EGFR-TKI-mediated apoptosis. However, the functional roles of RBM10 in *EGFR*-Mt lung adenocarcinoma remain unknown and require further elucidation.

Programmed death-ligand 1 (PD-L1) expression in tumors is associated with the response to immune checkpoint inhibitors, and higher PD-L1 expression is associated with higher therapeutic efficacy (17,18). Recent clinical and preclinical studies have shown that high PD-L1 expression was associated with a reduced response to EGFR-TKIs in patients with unresectable advanced *EGFR*-Mt lung adenocarcinoma (19-21). However, the impact of PD-L1 expression on prognosis in patients with *EGFR*-Mt lung adenocarcinoma after surgery remains unknown.

This study examined the relationship between RBM10/PD-L1 expression, as measured using immunohistochemistry (IHC), and both the prognosis of pN1–N2 *EGFR*-Mt lung adenocarcinoma after resection and the efficacy of EGFR-TKI treatment after recurrence. We present this article in accordance with the STROBE reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-355/rc).

Highlight box

Key findings

- High cytoplasmic RNA-binding motif protein 10 (RBM10) expression and programmed death-ligand 1 (PD-L1) positivity were poor prognostic factors for overall survival in patients with pN1–N2 epidermal growth factor receptor gene mutation (*EGFR*-Mt) lung adenocarcinoma after curative surgery. In patients with recurrent pN1–N2 *EGFR*-Mt, PD-L1 and RBM10 expression may influence response to EGFR-tyrosine kinase inhibitors (TKIs).

What is known and what is new?

- RBM10 participates in the regulation of mRNA splicing, apoptosis induction, angiogenesis, and cell growth inhibition. The expression of PD-L1 in tumors is associated with the response to immune checkpoint inhibitor therapy.
- This study examined the relationship between RBM10/PD-L1 expression (measured via immunohistochemistry) and both the prognosis of pN1–N2 *EGFR*-Mt after resection and the efficacy of EGFR-TKI therapy after recurrence.

What is the implication, and what should change now?

- Evaluation of RBM10 and PD-L1 expression could be useful in considering postoperative and post-relapse treatment of patients with pN1–N2 *EGFR*-Mt. Large prospective studies are necessary to evaluate the usefulness of RBM10 and PD-L1 expression in predicting prognosis in patients with pN1–N2 *EGFR*-Mt, and determining the efficacy of EGFR-TKI after relapse.

Methods

Patients

This retrospective study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional review board of Kanagawa Cancer Center (2019 Eki-174) and individual consent for this retrospective analysis was waived.

Of the 321 patients with stage pN1–N2 lung adenocarcinoma who underwent lobectomy or a greater extent of lung resection with lymph node dissection at Kanagawa Cancer Center between January 2010 and December 2020, 129 patients (40.2%) who harbored *EGFR* mutations in either Ex19 (n=66) or Ex21 (n=63) were included in this study. Patients who received neoadjuvant therapy were excluded. Patients who received adjuvant EGFR-TKI therapy were also excluded.

Pathological diagnosis and detection of EGFR mutation

Pathological diagnosis was based on hematoxylin and eosin (HE) staining of formalin-fixed paraffin-embedded tissue sections. To confirm the diagnosis of adenocarcinoma, IHC for thyroid transcription factor 1 and Alcian blue staining were performed. Synaptophysin, CD56, chromogranin A, p63, and p40 staining procedures were also performed as needed. Blood vessel and pleural invasion were evaluated using EVG staining, and lymphatic vessel invasion was evaluated using D2-40, if necessary. For the *EGFR* mutation analysis of lung adenocarcinoma, the loop-hybrid mobility shift assay method (22), Cycleave/fragment method (23), or the Cobas® *EGFR* mutation test v2 (24) was performed on surgically resected specimens.

IHC analyses

Tissue microarray (TMA)

The most representative tumor areas containing tumor cells and excluding necrotic areas were marked on the HE-stained slides. Tumor samples were obtained using a 2-mm-diameter core, embedded in a paraffin acceptor block, and placed in a block to produce a TMA block.

Evaluation of RBM10 expression in the TMA

TMA thin sections were deparaffinized, immersed in 10 mM sodium citrate buffer for 20 min at 121 ℃, and incubated with rabbit polyclonal anti-RBM10 antibody (1:300, cat no. HPA034972; Sigma) for 1 h at room temperature. After confirmation of tumor cells in the HE-stained TMA, RBM10 expression was evaluated by assessing the staining intensity of the tumor cytoplasm and classified as low, moderate, or high (*Figure 1*). The percentage of stained tumor cells among all tumor cells in the TMA was evaluated in 5% increments. RBM10 expression was considered high if tumor cells in the TMA were stained as high intensity or ≥75% of the tumor cells in the TMA were stained as moderate intensity. RBM10 expression was considered low if tumor cells in the TMA were stained as low intensity or <75% of the tumor cells in the TMA were stained moderate intensity (*Figure 1*).

In situ hybridization (ISH) of RBM10

ISH to detect *RBM10* mRNA sequences in the cytoplasm (25) was performed on the representative sections of pN1–N2 *EGFR*-Mt lung adenocarcinomas using the RNAscope Probe-Hs-RBM10 (cat no. 419881; Advanced Cell Diagnostics, Hayward, CA, USA) and visualized using the RNAscope 2.5 HD Reagent Kit—RED (cat no. 322350; Advanced Cell Diagnostics) according to the manufacturer's protocol.

Evaluation of PD-L1 expression

The TMA sections were incubated with a rabbit monoclonal anti-PD-L1 antibody (clone E1L3N, 1:300, cat no. 13684; Cell Signaling Technology) for 1 h at room temperature. The tumor was considered PD-L1 positive if more than 1% of tumor cells in the TMA were stained and was considered negative if less than 1% of tumor cells were stained.

Primary/secondary outcomes of this study and word definitions

The primary outcome of this study was to compare the OS of patients with *EGFR*-Mt lung adenocarcinoma in RBM10 low/high and PD-L1 negative/positive expression groups. The second outcome was to compare the progression-free survival (PFS) of patients with recurrent *EGFR*-Mt lung adenocarcinoma in the RBM10 low/high and PD-L1 negative/positive expression groups.

Intrathoracic recurrence included cervicothoracic lymph node recurrence, lung recurrence, and pleural dissemination. Distant recurrence included central nervous system, abdominal organ, and bone metastases. The EGFR-TKIs administered included first-generation (gefitinib or erlotinib), second-generation (afatinib), and third-generation (osimertinib) EGFR-TKIs and administration

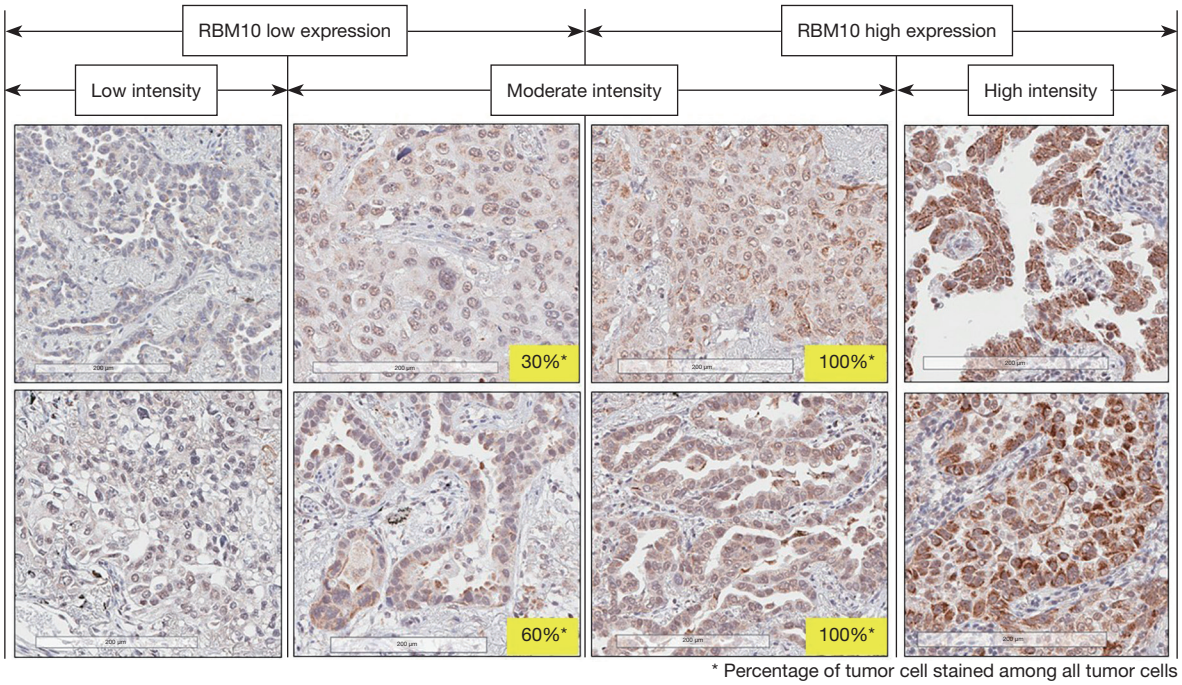

Figure 1 RBM10 expression on immunohistochemistry (original magnification ×400). RBM10 expression was considered high if tumor cells in the TMA were stained as high intensity or ≥75% of the tumor cells in the TMA were stained as moderate intensity. RBM10 expression was considered low if tumor cells in the TMA were stained as low intensity or <75% of the tumor cells in the TMA were stained moderate intensity. RBM10, RNA-binding motif protein 10; TMA, tissue microarray.

of EGFR-TKIs after relapse was based on the decision of respiratory physician. EGFR-TKIs were administered unless the patient did not wish to receive the treatment or was intolerant to the treatment due to a systemic condition. The time interval from surgery to all-cause death was defined as OS, and it was censored if the patients were event-free at the last follow-up. The time interval from the administration of the first-line EGFR-TKI to progression or death was defined as PFS.

Statistical analysis

Continuous variables between the two groups were compared using the Mann-Whitney *U* test or Student's *t*-test based on the results of normality testing. Categorical variables between the two groups were compared using Fisher's exact test. The OS curve of patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma and the PFS of patients with recurrent pN1–N2 *EGFR*-Mt lung adenocarcinoma with low/high RBM10 expression and PD-L1 negativity/positivity were created using the Kaplan-Meier method. The OS curves of the two groups were compared using the log-rank test. To estimate the median follow-up period, the reverse Kaplan-Meier method was performed. The Cox proportional hazards regression model was applied for the univariable and multivariable analyses for the following variables: age (<65/≥65 years), sex (male/female), carcinoembryonic antigen (CEA) level (≤10/>10 ng/mL), tumor size by computed tomography (CT) (≤3.0/>3.0 cm), blood vessel invasion (–/+), lymphatic vessel invasion (–/+), pleural invasion (–/+), pathological lymph node metastasis (–/+), EGFR-TKI administration (–/+), *EGFR* mutation subtype (Ex19/Ex21), RBM10 expression (low/high), and PD-L1 expression (negative/positive). Multivariable analysis was performed with the significant variables (P<0.10) identified in the univariable analysis. P value <0.05 was considered statistical significance. EZR on R commander version 1.61 was used for all statistical analyses (26).

Results

RBM10 mRNA expression using RNAscope

RBM10 mRNA expression, as evaluated using RNAscope in

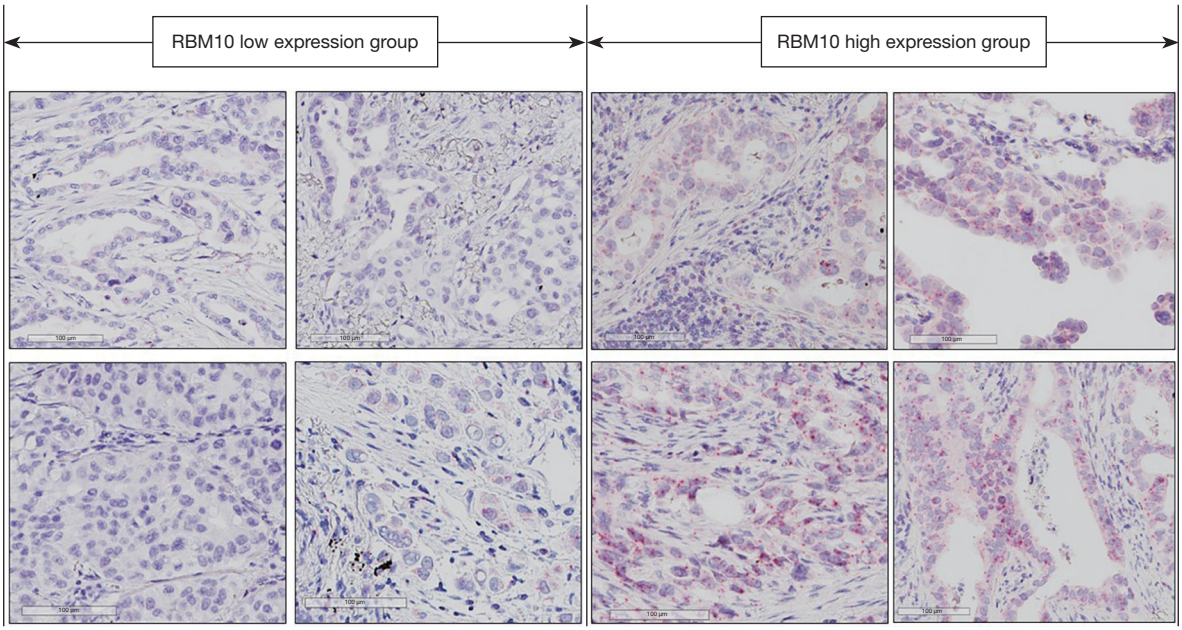

Figure 2 *RBM10* mRNA expression was evaluated by RNA in situ hybridization using RNAscope® technology on the semiserial sections used for the immunohistochemistry of RBM10 protein that demonstrated representative cytoplasmic or nuclear staining. Hybridization probe, Probe-Hs-RBM10 and HybEZ™ Hybridization System was used according to the manufacturer's protocol (see Methods). *RBM10* mRNA was visualized as cytoplasmic red dots (original magnification, ×400). RBM10, RNA-binding motif protein 10.

representative RBM10 cytoplasmic high and low expression tumors, was prominent in the RBM10 high expression group as measured by using IHC, while *RBM10* mRNA expression was low in the RBM10 low expression group (*Figure 2*).

Comparison of the clinicopathological features of pN1–N2 EGFR-Mt lung adenocarcinoma with low/high RBM10 expression and negative/positive PD-L1 expression

Among the 129 patients with *EGFR*-Mt lung adenocarcinoma, 44 (34.1%) and 21 (16.3%) showed high RBM10 expression and positive PD-L1 expression, respectively (*Table 1*). Ex21 lung adenocarcinoma was observed in 44 patients (51.8%) in the low RBM10 expression group and 19 patients (43.2%) in the high RBM10 expression group (P=0.458) (*Table 1*). Pathological ipsilateral mediastinal lymph node involvement (pN2) was more frequent in patients with low RBM10 expression group than in patients with high RBM10 expression group (70.6% *vs.* 40.9%, P=0.001; *Table 1*). Ex21 lung adenocarcinoma was observed in 51 patients (47.2%) in the PD-L1-negative group and in 12 patients (57.1%) in the PD-L1-positive group (P=0.478) (*Table 1*). The solid adenocarcinoma subtype was more frequent in the PD-L1 positive group than in the PD-L1 negative group (38.1% *vs.* 8.3%, P=0.007; *Table 1*). Moreover, the PD-L1 negative group had a lower maximum standardized uptake value than the PD-L1 positive group (7.1 *vs.* 11.2, P=0.005; *Table 1*).

Impact of RBM10 and PD-L1 expression on the OS of patients with pN1–N2 EGFR-Mt lung adenocarcinoma

The median follow-up period was 54.7 months. The 5-year OS rate was significantly lower in the RBM10 high expression group than in the RBM10 low expression group (89.4% *vs.* 71.5%, P=0.020; *Figure 3A*) and tended to be worse in the PD-L1 positive group than in the PD-L1 negative group (86.4% *vs.* 68.4%; P=0.050; *Figure 3B*).

In the multivariable analysis, high RBM10 expression [hazard ratio (HR), 3.12; 95% confidence interval (CI), 1.19–8.17; P=0.021], PD-L1 positivity (HR, 3.80; 95% CI: 1.64–8.84; P=0.002), and pN2 (HR, 4.19; 95% CI: 1.57–11.2; P=0.004) were independent prognostic factors for OS (*Table 2*).

Table 1 Comparison of the clinicopathological features of pN1–N2 *EGFR* mutant lung adenocarcinomas (n=129) with low/high RBM10 expression and negative/positive PD-L1 expression

Variables	RBM10			PD-L1		
	Low[†1] (n=85)	High[†2] (n=44)	P value[§]	Negative[‡1] (n=108)	Positive[‡2] (n=21)	P value[§]
Age (years), mean (SD)	67.0 (11.1)	65.0 (12.3)	0.344[¶]	65.6 (11.2)	69.7 (12.8)	0.144[¶]
Male, n (%)	42 (49.4)	23 (52.3)	0.853	53 (49.1)	12 (57.1)	0.634
Brinkman index, mean (SD)	239 (375.3)	272 (301.6)	0.174[//]	254 (363.1)	231 (287.5)	0.834[//]
CEA (ng/mL), mean (SD)	8.4 (12.9)	7.6 (10.1)	0.779[//]	7.5 (10.0)	11.2 (19.3)	0.655[//]
CT tumor size (cm), mean (SD)	3.4 (1.4)	3.7 (1.7)	0.300[¶]	3.5 (1.4)	3.6 (1.9)	0.784[¶]
PET maxSUV, mean (SD)	7.7 (4.8)	7.9 (5.1)	0.795[//]	7.1 (4.4)	11.2 (5.8)	0.005[//]
Lymphatic vessel invasion +, n (%)	50 (58.8)	24 (54.5)	0.709	63 (58.3)	11 (52.4)	0.637
Blood vessel invasion +, n (%)	65 (76.5)	32 (72.7)	0.671	84 (77.8)	13 (61.9)	0.166
Pleural invasion +, n (%)	41 (48.2)	23 (52.3)	0.713	58 (53.7)	6 (28.6)	0.055
pN2 +, n (%)	60 (70.6)	18 (40.9)	0.001	67 (62.0)	11 (52.4)	0.468
Subtype of adenocarcinoma, n (%)						
Lepidic	2 (2.4)	2 (4.5)		3 (2.8)	1 (4.8)	
Papillary	30 (35.3)	20 (45.5)		43 (39.8)	7 (33.3)	
Acinar	35 (41.1)	16 (36.4)		47 (43.5)	4 (19.0)	
Micropapillary	4 (4.7)	3 (6.8)		6 (5.6)	1 (4.8)	
Solid	14 (16.5)	3 (6.8)	0.417	9 (8.3)	8 (38.1)	0.007
Recurrence +, n (%)	58 (68.2)	33 (75.0)	0.542	77 (71.3)	14 (66.7)	0.794
Initial site of recurrence, n (%*)						
Intrathoracic only	29 (50.0*)	13 (39.4*)		39 (50.6*)	3 (21.4*)	
Distant	29 (50.0*)	20 (60.6*)	0.385	38 (49.4*)	11 (78.6*)	0.078
1st line EGFR-TKI, n (%*)	44 (75.9*)	23 (69.7*)	0.622	57 (74.0*)	10 (71.4*)	1.000
First-generation	17 (29.3*)	9 (27.3*)		21 (27.3*)	5 (35.7*)	
Second-generation	2 (3.5*)	3 (9.1*)		4 (5.2*)	1 (7.1*)	
Third-generation	25 (43.1*)	11 (33.3*)	0.447	32 (41.5*)	4 (28.6*)	0.479
EGFR mutation, n (%)						
Exon 19 deletion mutation	41 (48.2)	25 (56.8)		57 (52.8)	9 (42.9)	
Exon 21 L858R point mutation	44 (51.8)	19 (43.2)	0.458	51 (47.2)	12 (57.1)	0.478

[†1], RBM10 low expression: tumor cells stained as low intensity or <75% of the tumor cells stained moderate intensity; [†2], RBM10 high expression: tumor cells stained as high intensity or ≥75% of the tumor cells stained as moderate intensity; [‡1], PD-L1 negative expression: less than 1% of tumor cells stained; [‡2], PD-L1 positive expression: more than 1% of tumor cells stained; *, percentage in recurrent cases; [§], Fisher's exact test; [¶], Student's *t*-test; [//], Mann-Whitney *U* test. *EGFR*, epidermal growth factor receptor; RBM10, RNA-binding motif protein 10; PD-L1, programmed death-ligand 1; SD, standard deviation; CEA, carcinoembryonic antigen; CT, computed tomography; PET, positron emission tomography; SUV, standardized uptake value; TKI, tyrosine kinase inhibitor.

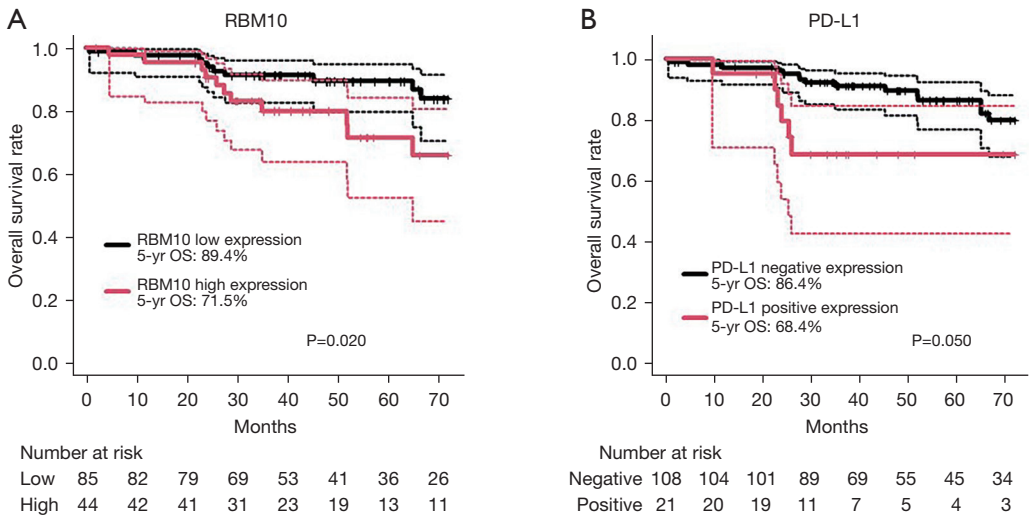

Figure 3 Comparison of overall survival between the low and high RBM10 expression groups (A) and PD-L1 negative/positive groups (B) among patients with stage pN1–N2 *EGFR* mutant lung adenocarcinoma. The solid line describes the probability of survival, and the dotted lines represent the 95% confidence interval. RBM10, RNA-binding motif protein 10; OS, overall survival; PD-L1, programmed death-ligand 1; *EGFR*, epidermal growth factor receptor.

Table 2 Univariable and multivariable analyses of the overall survival of patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma (n=129)

Variables	Univariable analysis			Multivariable analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age (≥65 years)	1.68	0.69–4.05	0.250	–	–	–
Sex (male)	1.64	0.72–3.76	0.243	–	–	–
CEA (>10 ng/mL)	0.40	0.09–1.70	0.214	–	–	–
CT tumor size (>3.0 cm)	1.69	0.72–3.95	0.226	–	–	–
Lymphatic vessel invasion (+)	0.84	0.38–1.89	0.679	–	–	–
Blood vessel invasion (+)	1.11	0.44–2.80	0.830	–	–	–
Pleural invasion (+)	0.87	0.39–1.95	0.741	–	–	–
pN2 (*vs.* pN1)	2.29	0.91–5.78	0.079	4.19	1.57–11.2	0.004
EGFR-TKI administration	0.76	0.34–1.71	0.513	–	–	–
EGFR exon 21 L858R	1.93	0.85–4.37	0.114	–	–	–
RBM10 high expression	2.51	1.12–5.60	0.025	3.12	1.19–8.17	0.021
PD-L1 positive expression	2.46	0.97–6.23	0.058	3.80	1.64–8.84	0.002

EGFR-Mt, epidermal growth factor receptor gene mutation; HR, hazards ratio; CI, confidence interval; CEA, carcinoembryonic antigen; CT, computed tomography; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; RBM10, RNA-binding motif protein 10; PD-L1, programmed death-ligand 1.

Impact of RBM10 and PD-L1 expression on the OS of patients with Ex21 and Ex19 lung adenocarcinoma

Figure 4 shows an analysis of OS according to *EGFR* subtype. Among patients with Ex21, those in the RBM10 high expression group had poorer OS than those in the RBM10 low expression group (83.9% *vs.* 60.0%, P=0.012; *Figure 4A*). There was no significant difference between the RBM10 high and low expression groups among patients

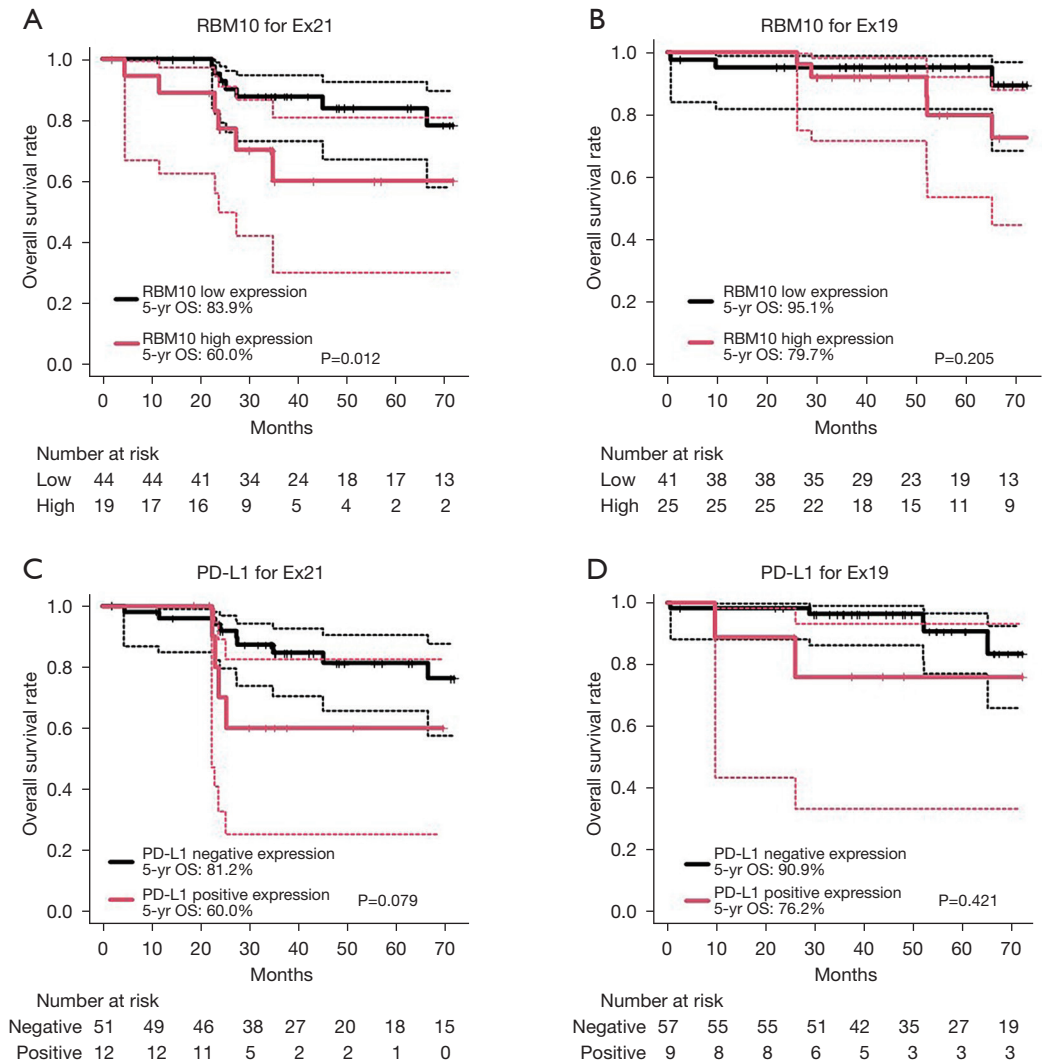

Figure 4 Comparison of overall survival among patients with low and high RBM10 expressing Ex21 lung adenocarcinoma (A) and Ex19 lung adenocarcinoma (B). Comparison of overall survival in patients with PD-L1-positive and -negative Ex21 (C) and Ex19 lung adenocarcinoma (D). The solid line describes the probability of survival, and the dotted lines represent the 95% confidence interval. RBM10, RNA-binding motif protein 10; OS, overall survival; PD-L1, programmed death-ligand 1; Ex21, exon 21 L858R mutation; Ex19; exon 19 deletion mutation.

with Ex19 lung adenocarcinoma (*Figure 4B*). There was no difference in the OS of patients with the different *EGFR* mutation subtypes according to PD-L1 positivity (*Figure 4C,4D*).

Impact of RBM10 and PD-L1 expression on the PFS of patients with recurrent EGFR-Mt lung adenocarcinoma who were treated with first-line EGFR-TKIs

Among the patients treated with first-line EGFR-TKIs after recurrence (n=67), PFS was not significantly different between the RBM10 high and low expression groups (*Figure 5A*). PFS was better in the PD-L1 negative group than in the PD-L1 positive group (median, 34.5 *vs.* 12.1 months, P=0.045; *Figure 5B*). Among the patients with Ex21 who were treated with first-line EGFR-TKIs, the RBM10 low expression group had significantly better PFS than the high expression group (median PFS, 25.5 *vs.* 13.0 months, P=0.025; *Figure 5C*). In the multivariable analysis, high RBM10 expression in Ex21 (HR, 3.16; 95% CI: 1.17–8.53; P=0.023) were independent

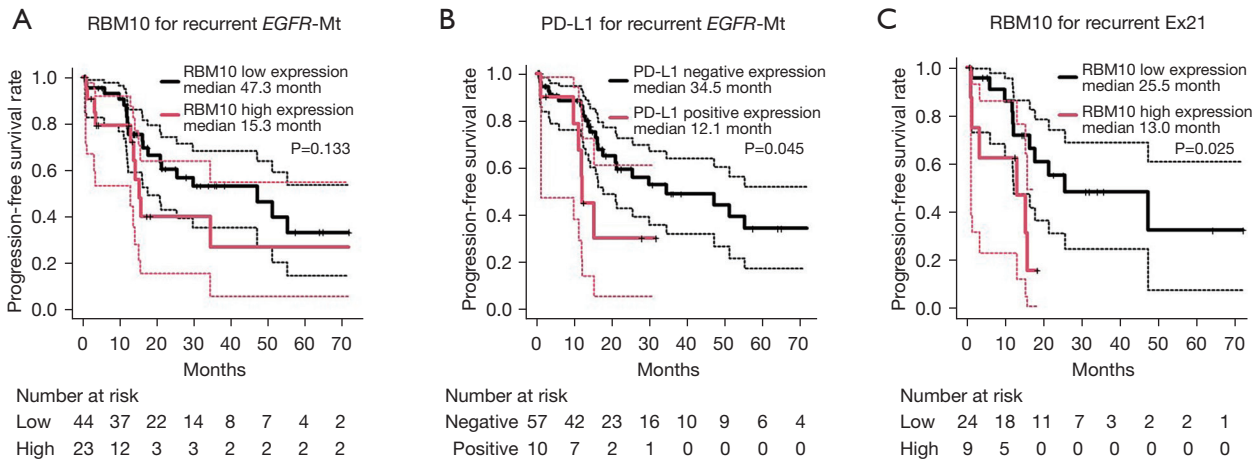

Figure 5 Comparison of PFS of patients with recurrent *EGFR* mutant lung adenocarcinoma who were treated with first-line EGFR-TKIs between the RBM10 low/high expression (A) and PD-L1 negative/positive groups (B). Comparison of PFS among patients with recurrent Ex21 lung adenocarcinoma who were treated with first-line EGFR-TKIs in the RBM10 low/high expression groups (C). The solid line describes the probability of survival, and the dotted lines represent the 95% confidence interval. RBM10, RNA-binding motif protein 10; *EGFR*-Mt, epidermal growth factor receptor gene mutation; PD-L1, programmed death-ligand 1; Ex21, exon 21 L858R mutation; PFS, progression-free survival; TKIs, tyrosine kinase inhibitors.

prognostic factors for PFS (Table S1).

Discussion

This is the first study to demonstrate that high RBM10 protein expression in the cytoplasm and PD-L1 positivity, as measured by IHC, are independent poor prognostic factors in patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma after curative surgery. Among patients with recurrent *EGFR*-Mt lung adenocarcinoma, the PD-L1-positive group showed a poor response to first-line EGFR-TKI treatment. In addition, among patients with recurrent Ex21 lung adenocarcinoma, the RBM10 high expression group also showed a poor response to first-line EGFR-TKI treatment. Thus, the response to first-line EGFR-TKIs in patients with recurrent *EGFR*-Mt lung adenocarcinoma could be predicted based on cytoplasmic RBM10 and PD-L1 expression. RBM10 and PD-L1 may be key biomolecular targets that could improve the prognosis of patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma. RBM10 shows particular promise for pN1–N2 Ex21 lung adenocarcinoma after surgery.

Little is known about the relationship between RBM10 expression and prognosis in patients with solid tumors. Sun *et al.* (13) compared the prognosis of 90 patients with pN0–N2 primary lung adenocarcinomas based on nuclear and cytoplasmic RBM10 expression and reported that pN1–N2 was more frequent in patients with high RBM10 expression and that OS was poorer in the RBM10 high expression group than in the RBM10 low expression group. In contrast, Guan *et al.* (14) reported that low RBM10 expression was associated with advanced stage in 41 primary lung adenocarcinomas. Rintala-Maki *et al.* (15) reported that RBM10 v2, a variant of RBM10, was associated with poor prognosis in 61 patients with breast cancer. The present study demonstrated that pN2 was more frequent in the RBM10 low expression group, indicating that low RBM10 expression was associated with advanced stage in pN1–N2 *EGFR*-Mt lung adenocarcinoma, similar to the findings of Guan *et al.* (14). However, the RBM10 low expression group showed better OS due to the higher efficacy of EGFR-TKI treatment in patients with low RBM10 expression than in those with high RBM10 expression.

As shown in *Table 1*, high RBM10 expression was observed in 44 (34.1%) patients. To date, few studies have evaluated the IHC expression of RBM10 in primary lung cancer tissues using the RBM10 antibody No. HPA034972 (Sigma); therefore, there are no criteria for its cut-off value for RBM10 expression. Zhang *et al.* classified RBM10 staining intensity into four levels (strong, moderate, faint, and no staining) and defined RBM10-positive as 10% of tumor cells or more being stained at a strong or moderate

intensity, while the rest were defined as RBM10-negative; 63 out of 87 lung cancer patients (72.4%) were RBM10-positive (27). In the present study, 34.1% of pN1–N2 *EGFR*-Mt lung adenocarcinomas showed high RBM10 expression, which was lower than that reported by Zhang *et al.* This may be attributable to the fact that our study population consisted of patients with pN1–N2 or advanced lung cancer with a higher RBM10 expression threshold than that reported by Zhang *et al.*

A previous study showed that RBM10 overexpression inhibited various malignant behaviors of lung adenocarcinoma, including cell cycle progression, cell viability and proliferation, and colony formation (11,14,28,29). RBM10 suppressed lung adenocarcinoma cell proliferation via the RAP1/AKT/CREB signaling pathway, independent of the MAPK/ERK and P38/MAPK signaling pathways (28). RBM10 promoted apoptosis via the AKT signaling pathway and by activating p53 (28,30,31). These functional roles of RBM10 support the finding of a higher frequency of pN2 in the RBM10 low expression group than in the high RBM10 expression group. Moreover, our findings were consistent with those of a study by Lengel *et al.* that showed that mutation of the *RBM10* gene, which is considered a tumor suppressor gene, is often associated with non-metastatic early stage lung adenocarcinoma (32). However, other studies have shown contradictory results: upregulation of RBM10 expression stimulated proliferative signaling pathways in lung adenocarcinoma cells (12,13) and inhibited the stimulation of apoptotic signaling pathways in lung adenocarcinoma cells (13,33). Therefore, further elucidation of the molecular role of RBM10 in primary lung adenocarcinoma is required.

Little is known about the role of RBM10 in *EGFR*-Mt lung adenocarcinoma. A previous preclinical study reported that Src family kinases downstream of EGFR directly phosphorylate the tyrosine residues of RBM10 and promote transfer of RBM10 from the nucleus to the cytoplasm to regulate FilGAP, which is associated with cancer cell invasion and metastasis (34,35). Recently, Nanjo *et al.* (16) reported that a *RBM10* mutation resulting in the loss of RBM10 expression limited the initial response to EGFR-TKI due to suppression of mitochondria-mediated apoptosis in response to EGFR-TKI in *EGFR*-Mt tumor cells caused by a decreased ratio of Bcl-xS to Bcl-xL. They also compared the PFS of 70 patients with stage IIIB/IV advanced *EGFR*-Mt lung adenocarcinoma and showed that the *RBM10* mutation-positive group (n=13) had a lower response to EGFR-TKI treatment than the *RBM10* mutation-negative group (n=57) (median PFS, 5.7 *vs.* 13.4 months; P<0.0001) (16). However, the present study showed that high RBM10 expression in the cytoplasm was associated with lower efficacy of EGFR-TKI treatment. One possible reason for this paradoxical result was that the polyclonal anti-RBM10 antibodies used in our study (cat no., HPA034972) recognize the N-terminus of RBM10, whiles the ones used in Nanjo's study (cat no., A301-006A) recognize the C-terminus of RMB10. These two polyclonal antibodies probably did not completely recognize the same molecular variants of RBM10. A second possible reason was that we evaluated cytoplasmic staining of RBM10, while Nanjo *et al.* evaluated nuclear staining of RBM10 using IHC. The present study findings indicated high *RBM10* mRNA expression in tumors with high cytoplasmic RBM10 IHC staining (*Figure 2*), possibly indicating that cytoplasmic RBM10 staining was specific. The abundance of nuclear and cytoplasmic RBM10 could have different functional meanings. A third possibility is that we assessed surgically resected specimens of pN1–N2 *EGFR*-Mt, whereas Nanjo *et al.* assessed unresectable *EGFR*-Mt lung adenocarcinoma. Considering these reasons, further IHC analyses using a highly specific monoclonal antibody against RBM10 are necessary.

The present study showed that RBM10 expression affected the prognosis of patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma after curative resection because of the low response to first-line EGFR-TKIs, especially in patients with Ex21 lung adenocarcinoma. The response rate to EGFR-TKIs has been reported to be 75–80% (36,37). However, in some patients, the duration of response to EGFR-TKIs is short, and the prognosis is poor (10,38). Previous studies have reported a lower response to EGFR-TKIs and shorter PFS in patients with Ex21 lung adenocarcinoma than in patients with Ex19 lung adenocarcinoma (39,40). The higher frequency of compound mutations and lower frequency of *EGFR* variant alleles in Ex21 lung adenocarcinoma than in Ex19 lung adenocarcinoma were reported to be associated with the lower response to EGFR-TKIs in Ex21 lung adenocarcinoma (41,42). Nanjo *et al.* (16) reported a lower response to EGFR-TKIs in patients with Ex21 lung adenocarcinoma, which was associated with the higher frequency of *RBM10* co-mutations in Ex21 than in Ex19 (3% *vs.* 15%) (16). The results of the present study suggest that RBM10 may be a novel target associated with EGFR-TKI sensitivity and prognosis after curative surgery in patients with pN1–N2 Ex21 lung adenocarcinoma. However, further

biomolecular studies aimed at elucidating the functional role of RBM10 in pN1–N2 Ex21 lung adenocarcinoma are needed.

In the present study, PD-L1 expression was an independent prognostic factor for OS in patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma after surgery. PFS was worse in the PD-L1 positive group than in PD-L1 negative group in patients with recurrent disease treated with first-line EGFR-TKIs, which was consistent with previous reports showing that PD-L1 positivity was associated with worse PFS and a lower objective response rate among patients with stage IV unresectable *EGFR*-Mt lung adenocarcinoma (19-21,43). A previous *in vitro* study reported that PD-L1 contributes to primary resistance to EGFR-TKIs in *EGFR*-Mt cells by inducing epithelial-mesenchymal transition via activation of the TGF-β/Smad pathway (20).

In a large-scale meta-analysis, PD-L1 was shown to be associated with worse OS in patients with resectable lung cancer (44); however, there are only a few reports on the association between prognosis and PD-L1 expression in *EGFR*-Mt lung adenocarcinoma after resection. Bai *et al.* (45) analyzed PD-L1 expression in 73 patients with p-stage I–IV *EGFR*-Mt lung adenocarcinoma using PD-L1 antibody clone E1L3N and reported that 19 patients were positive for PD-L1 (26.0%) and that PD-L1 was a poor prognostic factor for OS after resection. Takamochi *et al.* (46) analyzed PD-L1 expression in 438 patients with p-stage I–IV *EGFR*-Mt lung adenocarcinoma using PD-L1 antibody 22C3 and reported that 89 patients who were PD-L1 positive (20.8%) had poor RFS, although there was no difference in OS after surgery. The present study was the first to analyze PD-L1 expression in pN1–N2 *EGFR*-Mt lung adenocarcinoma and show that PD-L1 positivity is a poor prognostic factor for OS in patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma.

This study has several limitations. First, this is a single-center retrospective study with a small sample size, and a large prospective study is warranted to draw firm conclusions. Second, because RBM10 and PD-L1 expression was analyzed in TMAs, errors may have been caused due to tumor heterogeneity. Third, as this study included patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma, it is unclear whether the results apply to pathological lymph node-negative or unresectable stage IV *EGFR*-Mt lung adenocarcinoma. Fourth, this study did not analyze the correlation between RBM10 expression and *RBM10* mutations. *RBM10* mutations, including missense and frame shift mutations, were identified in 8–22% of lung adenocarcinomas (47-49). Thus, further studies are necessary to evaluate the correlation between cytoplasmic expression of RBM10 and *RBM10* mutations in *EGFR*-Mt lung adenocarcinoma and the biomolecular function of cytoplasmic RBM10, especially in Ex21 lung adenocarcinoma.

Conclusions

High expression of RBM10 and PD-L1 positivity are poor prognostic factors for OS in patients with pN1–N2 *EGFR*-Mt lung adenocarcinoma after curative surgery. The response to EGFR-TKIs can be predicted based on PD-L1 expression in patients with recurrent *EGFR*-Mt lung adenocarcinoma and RBM10 expression in patients with recurrent Ex21 lung adenocarcinoma.

Acknowledgments

Mr. Nakamura Yoshiyasu and Ms. Yoshihara Mitsuyo, Kanagawa Cancer Center Research Institute are acknowledged for their excellent technical assistance.

Funding: This study was funded by a grant from Kanagawa Cancer Foundation and JSPS KAKENHI (Grant Number JP21K16519).

Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-355/rc

Data Sharing Statement: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-355/dss

Peer Review File: Available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-355/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-23-355/coif). TI reports receiving Grant from Kanagawa Cancer Foundation and JSPS KAKENHI (Grant Number JP21K16519). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional review board of Kanagawa Cancer Center (2019 Eki-174) and individual consent for this retrospective analysis was waived.

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Cite this article as: Isaka T, Miyagi Y, Yokose T, Saito H, Kasajima R, Watabe K, Shigeta N, Kikunishi N, Shigefuku S, Murakami K, Adachi H, Nagashima T, Ito H. Impact of RBM10 and PD-L1 expression on the prognosis of pathologic N1–N2 epidermal growth factor receptor mutant lung adenocarcinoma. Transl Lung Cancer Res 2023;12(10):2001-2014. doi: 10.21037/tlcr-23-355