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Tumor mutation burden as a biomarker for lung cancer patients treated with pemetrexed and cisplatin (the JIPANG-TR)

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Abstract

The JIPANG study is a randomized phase III study of pemetrexed/cisplatin (Pem/Cis) versus vinorelbine/cisplatin (Vnr/Cis) for completely resected stage II-IIIA non-squamous non-small cell lung cancer (Ns-NSCLC). This study did not meet the primary endpoint (recurrence-free survival, RFS) but Pem/Cis had a similar efficacy to Vnr/Cis with a better tolerability. Tumor mutation burden (TMB) is thought to have a predictive value of immune checkpoint inhibitors. However, the relevance of TMB to cytotoxic chemotherapy remains unknown. This exploratory study investigates the relationship between tumor mutation profiles and clinical outcome of Pem/Cis. Formalin-fixed, paraffin-embedded tumor tissues (n = 389) were obtained from the patients. Mutation status of tissue DNA was analyzed by targeted deep sequencing. Epidermal growth factor receptor (*EGFR)* mutations were detected frequently in Ns-NSCLC (139/374). Patients without any *EGFR* mutations experienced longer RFS in the Pem/Cis arm versus Vnr/Cis arms. Pem/Cis in patients with high TMB (≥12-16 mut/Mb) tended to have improved survival. In patients with wild-type *EGFR*, TMB ≥ 12 mut/Mb was significantly associated with improved RFS with Pem/Cis versus Vnr/Cis (not reached vs 52.5 months; hazard ratio (HR) 0.477). It could be proposed that TMB was predictive of RFS benefit with Pem/Cis versus Vnr/Cis in Ns-NSCLC. Further investigation is required to determine whether TMB combined with *EGFR* mutation status could be used as a predictive biomarker.

KEYWORDS
adjuvant chemotherapy, next-generation sequencing, non-squamous non-small cell lung cancer, pemetrexed, tumor mutation burden (TMB)

1 | INTRODUCTION

Patients with early stage non-small cell lung cancer (NSCLC) are operable but cure of a significant proportion is disrupted by recurrence. Adjuvant chemotherapy in early stage NSCLC patients is currently considered the standard treatment and associated with an approximately 5% survival benefit at 5 years.[1] Cisplatin plus vinorelbine is recommended as a standard adjuvant treatment for stage II-III resected NSCLC patients. Therefore, randomized controlled studies have been conducted to investigate treatments with higher therapeutic effects.

Clinical application of biomarkers is warranted for operable cancer patients to identify patients at increased risk for recurrence such as Oncotype DX and MammaPrint for breast cancer patients. Clinically relevant predictive biomarkers can identify patients most likely to benefit from adjuvant chemotherapy supporting the decision-making of postoperative adjuvant therapy and to predict its efficacy.

The JIPANG study is a randomized phase III study of pemetrexed/cisplatin (Pem/Cis) versus vinorelbine/cisplatin (Vnr/Cis) for completely resected stage II-IIIA non-squamous non-small cell lung cancer (Ns-NSCLC).[2] This phase III study did not meet the primary endpoint (recurrence-free survival, RFS) but Pem/Cis had a similar efficacy to Vnr/Cis with better tolerability. Exploratory analysis demonstrated hazard ratios in patients with and without epidermal growth factor receptor (*EGFR)* mutations were 1.38 (95% CI, 0.95-1.99) for Pem/Cis and Vnr/Cis 0.87 (95% CI, 0.69-1.09), respectively. Pemetrexed and cisplatin combination therapy is considered as one of the options for postoperative chemotherapy for stage II-IIIA Ns-NSCLC, especially *EGFR* wild type.[2] We have planned an exploratory biomarker study (JIPANG-TR) to identify the predictive biomarkers, the arm of which is beneficial for each patient through next-generation sequencing (NGS)-based mutation profiling of tumor tissues.

Amplicon deep sequencing is a powerful technology to analyze formalin-fixed, paraffin-embedded (FFPE) tumor samples. Tumor mutation burden (TMB) is reported as the total number of non-synonymous variants or single nucleotide variants per tumor genomic region.[3] Relatively large targeted deep sequencing provides TMB as well as mutation status of hundreds of genes.[4-6] Lung cancer compared with other solid cancers is characterized by a relatively high level of non-synonymous mutations resulting in the production of neo-epitopes of which TMB in lung cancer is a predictor of response to immunotherapies.[7-9] However, the relevance of TMB to cytotoxic chemotherapy is not yet fully understood. This study investigates the association of TMB and other mutation profiles with clinical

outcomes of two platinum-containing regimens to determine which patients benefited by each adjuvant chemotherapy.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens and outcome

A total of 389 (48.4%) of the 803 patients in the JIPANG study enrolled to the JIPANG-TR study between March 2012 and August 2016 at each institute. All patients provided written informed consent to participate in the study, including the collection of tumor tissue for analysis. Clinical outcome of overall survival (as a primary endpoint) and recurrence-free survival (as a secondary endpoint) was elucidated in the JIPANG study.[2] Overall survival was defined as the time from randomization to death from any cause. Recurrence-free survival was defined as time from randomization to disease recurrence, or death, whichever occurred first.

This study was designed as a prospective and exploratory study aimed at characterizing somatic mutations in tumor tissues and comparing tumor mutation status, including TMB and recurrence-free survival, to Pem/Cis or Vnr/Cis adjuvant therapy. This study was conducted in compliance with the Helsinki Declaration and the Ethical Guidelines for Medical and Health Research Involving Human Subjects by the Japanese government and has been approved by the ethics committee in each institute.

2.2 | Tissue processing

Tumor tissues were obtained at resectable operation and pathologically confirmed as non-squamous, non-small cell lung cancer. The collected FFPE tumor specimens (n = 389) underwent histological review, and only those containing sufficient tumor cells (at least 10%) as revealed by hematoxylin-eosin staining were subjected to nucleic acid extraction. DNA was isolated from the tissue with the use of an AllPrep DNA/RNA FFPE Kit (Qiagen, Valencia, CA). Quality and quantity of the nucleic acid were verified with the use of a NanoDrop 2000 device and PicoGreen dsDNA Reagent (all from Thermo Scientific, Wilmington, DE).

2.3 | Next-generation sequencing

The targeted DNA library comprising approximately 1.2 Mb coding regions of 409 genes for panel sequencing was constructed using an Ion AmpliSeq Comprehensive Cancer Panel (CCP) (Thermo Fisher Scientific) in accordance with the manufacturer's recommended protocol. Briefly, 40 ng DNA was subjected to multiplex PCR amplification with the use of an Ion AmpliSeq Library Kit 2.0 and Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific), covering all exons in 409 genes. After multiplex PCR, Ion Xpress Barcode Adapters (Thermo Fisher Scientific) were ligated to the PCR products, which were then purified with the use of Agencourt AMPure XP beads (Beckman Coulter, Brea, CA). The purified libraries were pooled and then sequenced with the use of an Ion Torrent S5 instrument and Ion 550 Chip Kit (all from Thermo Fisher Scientific). DNA sequencing data were accessed through the Torrent Suite ver. 5.10 program (Thermo Fisher Scientific). Reads were aligned against the hg19 human reference genome, and variants were called with the use of Variant Caller ver. 5.10. Raw variant calls were filtered with depth of coverage of <19 and were manually checked using the integrative genomics viewer (IGV, Broad Institute). Germline mutations were excluded with the use of the Genome Aggregation Database (gnomAD). The TMB scores were computed by the workflow of the Ion Reporter 5.10 using the Oncomine Tumor Mutation Load w2.0 workflow (Thermo Fisher Scientific).

2.4 | Statistical analysis

Patients were classified based on *EGFR* mutation status of tumor tissues. For biomarker analyses of each somatic mutation, the predictive and prognostic values were assessed by comparing the RFS of each arm (Pem/Cis and Vnr/Cis) in the JIPANG-TR study. The JMP (ver. 14.0, SAS Institute) and GraphPad Prism software (ver. 8, GraphPad Software Inc) were used for statistical analysis. Cox proportional hazards regression model was applied to perform univariate analyses. Relations between mutation status and patient characteristics were evaluated using the chi-squared (χ^2) test. Kaplan-Meier curves were used to estimate survival, and the log-rank test was used to compare times to events between groups. *P*-values of <.05 were considered statistically significant.

3 | RESULTS

3.1 | Sample availability and clinical characteristics

This study included 389 patients in 22 institutes, of whom 389 had an FFPE tumor specimen available for assessment (Figure 1). Three samples contained less than 10% tumorous regions. DNA of the other 386 tumor samples was extracted. One sample contained less DNA concentration. After excluding samples with tissue quantity or quality that was inadequate for sequencing, 385 samples were sequenced on the targeted panel of 409 genes. Sequencing quality of samples was assessed by the percentage of reads that covered targeted regions (>90%) and the amount of deamination (<100). Eleven of 385 samples were filtered out and remaining 374 samples were subjected to the analysis. The samples were obtained from 181 and 193 patients for Pem/Cis compared with Vnr/Cis, respectively. Patient characteristics of the analyzed samples are summarized in Table 1. Baseline patient's characteristics in the subgroup were generally comparable to the primary study. Two-year RFS was similar in the two treatment arms. The hazard ratios of the two treatment arms were not substantially different between the primary and subgroup

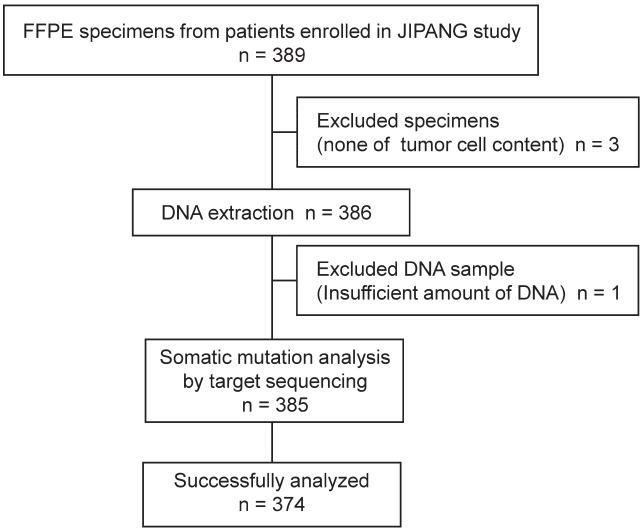

FIGURE 1 Cohort chart for the present study

studies. Thus, one may establish similarity of patient characteristics and efficacy between the primary and subgroup analysis cohorts.

3.2 | *EGFR* mutations and mutation burden

Nonsynonymous *EGFR* mutations were frequently identified in 139 samples (37.2%) by targeted sequencing (Table 2). Exon 19 deletion mutations and the single point mutation exon 21 Leu858Arg (L858R) are the most common mutations of *EGFR* with a population of 13.6 and 12.3%, respectively. High concordance (97.1%) of *EGFR* common mutation (exon 19 deletion and L858R) status was observed between the targeted panel and in vitro diagnostics (IVD) kits such as cobas *EGFR* mutation kit ver.2 and therascreen in the JIPANG study with sensitivity of 95.7% and specificity of 97.5% for the targeted panel versus IVD kits, respectively (Table S1). Additionally, we detected uncommon *EGFR* mutations in 30.2% of the *EGFR*-mutant population of the JIPANG-TR study. The uncommon mutation includes exons 3,

TABLE 2 Epidermal growth factor receptor (*EGFR*) mutations and tumor mutation burden (TMB) by targeted deep sequencing

	Vnr/Cis (n = 193)	Pem/Cis (n = 181)	*P**
EGFR mutation			
WT	115 (59.6)	120 (66.3)	0.7660
Exon 18	3 (1.6)	4 (2.2)	
Exon 19	31 (16.1)	22 (12.2)	
Exon 20	9 (4.7)	7 (3.9)	
Exon 21	30 (15.5)	23 (12.7)	
Others	5 (2.6)	5 (2.8)	
TMB number			
Median	10.1 (2.5-83.8)	9.3 (2.5-79.8)	0.2782

*The *P* value was calculated by using the chi-squared (χ^2) test.

TABLE 1 Baseline demographics and outcome for patients with subgroup study and primary study

	Subgroup study (n = 374)		Primary study (n = 784)	
	Vin/Cis	Pem/Cis	Vin/Cis	Pem/Cis
Demographic characteristics				
Patients	193 (51.6)	181 (48.4)	395 (50.4)	389 (49.6)
Median age	65 (33-75)	64 (31-75)	65 (33-75)	64 (28-75)
Gender				
Male	111 (57.5)	114 (63.0)	235 (59.5)	227 (58.4)
Female	82 (42.5)	67 (37.0)	160 (40.5)	162 (41.6)
Clinical stage				
IIA	66 (34.2)	57 (31.5)	132 (33.4)	134 (34.4)
IIB	19 (9.8)	22 (12.2)	57 (14.4)	51 (13.1)
IIIA	108 (56.0)	102 (56.3)	206 (52.2)	204 (52.4)
EGFR mutations[a]				
WT	140 (72.5)	141 (77.9)	300 (75.9)	292 (75.1)
MT	53 (27.5)	40 (22.1)	95 (24.1)	97 (24.9)
Efficacy				
2-year RFS (%)	63 (95% CI 56-69)	62 (95% CI 55-69)	61 (95% CI 56-65)	59 (95% CI 54-64)
RFS HR	0.90 (95% CI 0.67-1.21)		0.98 (95% CI 0.81-1.20)	

Note: Demographic data are n (%) or range.

Abbreviations: 95% CI, 95% confidence interval; HR, hazard ratio; MT, mutant type; RFS, recurrence-free survival; WT, wild type.

[a]Epidermal growth factor receptor (*EGFR*) mutation; common *EGFR* mutation (exon 19 deletion and L858R) status was examined by IVD kits (cobas or therascreen).

4, 6, 9, 12, 13, 15, 18, 19, 20, 21, 26, 27, and 28 point mutations and exon 20 insertion.

The tumor mutation burden (TMB) was determined by counting the non-synonymous mutations per Mb. There was no significant difference in the number of TMB between Pem/Cis and Vnr/Cis group (Table 2).

3.3 | Association of mutation profile with clinical outcome

In this JIPANG-TR study, a subgroup (n = 374) of the patients enrolled in the JIPANG study was analyzed. There was no significant difference of RFS between the Pem/Cis and Vnr/Cis arms (Figure S1A). JIPANG study demonstrated that patients without common *EGFR* mutations detected by IVD kits showed favorable outcomes by Pem/Cis compared with Vnr/Cis.[2] In the JIPANG-TR study, *EGFR* mutations were detected in 139 patients by targeted deep sequencing (Table 2). Patients with any *EGFR* mutation experienced shorter RFS in Pem/Cis (median; 18.9 months) compared with the Vnr/Cis arm (median; 30.4 months) but was not significant (log-rank $P = .3016$) (Figure S2A). Patients without any *EGFR* mutations (*EGFR* wild type) experienced longer RFS in Pem/Cis (median; not reached) compared with Vnr/Cis (52.6 months) as reported previously (log-rank $P = .1580$) (Figure S2B).

3.4 | Mutation burden and clinical outcome

To determine the preferable TMB cutoff level for predictive analyses, we conducted forest plot analyses for all cutoff levels (4-20 mutations/Mb) to assess the RFS of Pem/Cis or Vnr/Cis arms for 374 patients. Minimum hazard ratio (HR) of the high TMB group was 0.541 at 16 mutations/Mb for favorable outcomes in Pem/Cis with longer RFS (Figure 2A), whereas that of the low TMB group was 1.33 at 6 mutations/Mb for favorable outcomes in Vnr/Cis (Figure 2B). However, these were not significant.

When focusing on the subgroup with *EGFR* wild type, forest and Kaplan-Meier plots demonstrated longer RFS periods in *EGFR* wild-type patients treated with Pem/Cis than Vnr/Cis groups with high TMB but not low TMB values (Figures 3 and 4). Minimum HR of the high TMB group was 0.477 at a cut-off level of 12 mutations/Mb for favorable outcomes in Pem/Cis with significantly longer RFS ($P = .0376$) (Figure 3A), whereas no significant predictive values of TMB was observed in the subgroups with *EGFR* mutations (Figure 3B). Median RFS periods of Vnr/Cis and Pem/Cis groups were 52.5 months and not reached, respectively (log-rank $P = .0333$). The survival curve of the Pem/Cis group with TMB ≥ 12 (Figure 4) reached a plateau in the pemetrexed group at 24-36 months, which is significantly different to the whole picture. The percentage of patients with TMB ≥ 12 who will benefit from pemetrexed was 35.8% (43/120 in the Pem/Cis group). This showed

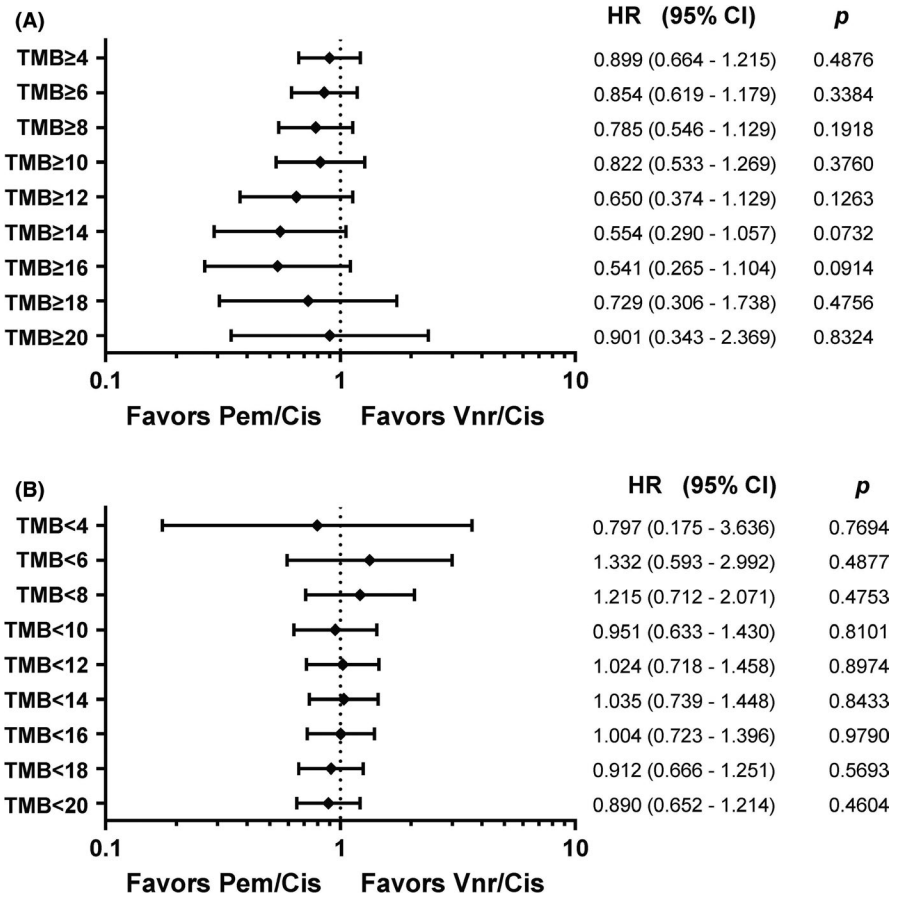

FIGURE 2 Forrest plot of recurrence-free survival (RFS) periods of all patients with high-tumor mutation burden (TMB) (A) and low-TMB (B) levels. Nonsynonymous TMB were calculated using a 409-gene targeted panel. HR, hazard ratio; 95% CI, 95% confidence interval. *significant (<.05)

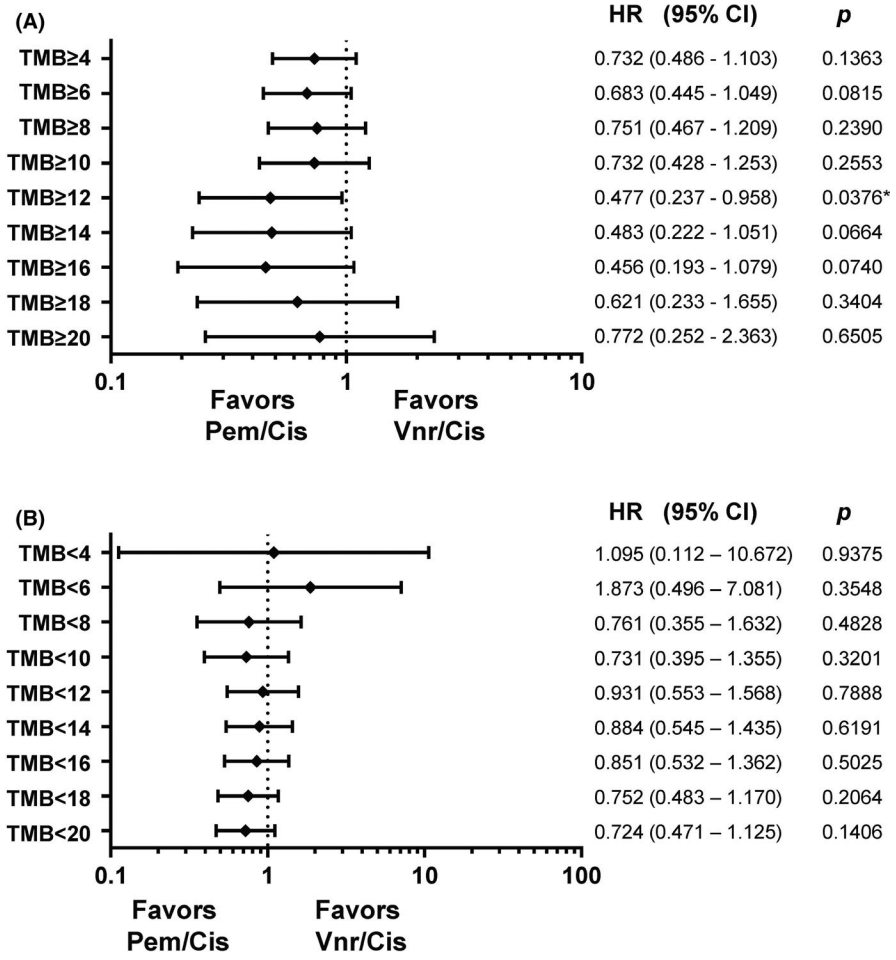

FIGURE 3 Forrest plot of recurrence-free survival (RFS) periods of high-tumor mutation burden (TMB) (A) and low-TMB (B) patients without any epidermal growth factor receptor (*EGFR*) mutations (*EGFR* wild type). HR, hazard ratio; 95% CI, 95% confidence interval. *significant (<.05)

that the 12 mutations/Mb cut-off provided a more sensitive value for the prediction of Pem/Cis in terms of RFS. However, the higher TMB groups were not significant. Characteristics of the group were investigated as to whether this subgroup had more stage IIA in the Pem/Cis arm (Table S2). No significant difference was observed between TMB-high (TMB ≥ 12) and -low (TMB < 12) subgroups in the Pem/Cis arm.

4 | DISCUSSION

Cytotoxic chemotherapy improves outcomes in patients with resected NSCLC, but the benefit might be limited.[1] Predictive biomarkers that can identify patients most likely to benefit from adjuvant chemotherapy are yet to be developed. Here, we have shown that high TMB patients without any *EGFR* mutations could achieve benefit from adjuvant chemotherapy of Pem/Cis. TMB is a predictive biomarker for immune-checkpoint inhibitors.[9,10] In contrast, clinical relevance of TMB to cytotoxic chemotherapy is not yet fully understood. Recently Devarakonda et al reported the clinical significance of TMB in resected NSCLC patients.[11] They demonstrated high nonsynonymous TMB was prognostic for favorable outcomes in patients with resected NSCLC. Lung cancer-specific survival benefit with adjuvant chemotherapy was more pronounced in patients with low nonsynonymous TMB (≤4 mutations/Mb). However, no specific chemotherapeutic regimen was focused upon. In our study, two doublet chemotherapy regimens were compared in the randomized phase III study. Our results then proposed the more precise medicine for the treatment of choice of the specific regimen based on TMB. In addition, they showed that benefit with adjuvant chemotherapy was more pronounced in patients with low nonsynonymous TMB. In contrast, Pem/Cis was more pronounced in patients with high TMB (≥12 mutations/Mb) in our study. Although the reason for the discrepancy remains unclear, it might be dependent on the mode of action of the regimen. Several factors influence high TMB status.[12,13] Genomic-unstable tumors tend to harbor higher TMB. An antimetabolite pemetrexed inhibits DNA synthesis and therefore it is likely that pemetrexed shows a higher antitumor effect on high TMB tumors. In addition, combination of pemetrexed with cisplatin might act synergistically because of increased S phase population of cell cycle distribution.[14]

In the subgroup analysis of patients with Ns-NSCLC without *EGFR* mutations, recurrence-free survival tended to be better in the pemetrexed plus cisplatin group. Pemetrexed acts as a multitarget antifolate agent, inhibiting three enzymes in the folate metabolic pathway. A previous study showed that the *EGFR* mutation status influenced the clinical benefit of adjuvant chemotherapy with tegafur-uracil, an antimetabolite that combines a fluorouracil prodrug

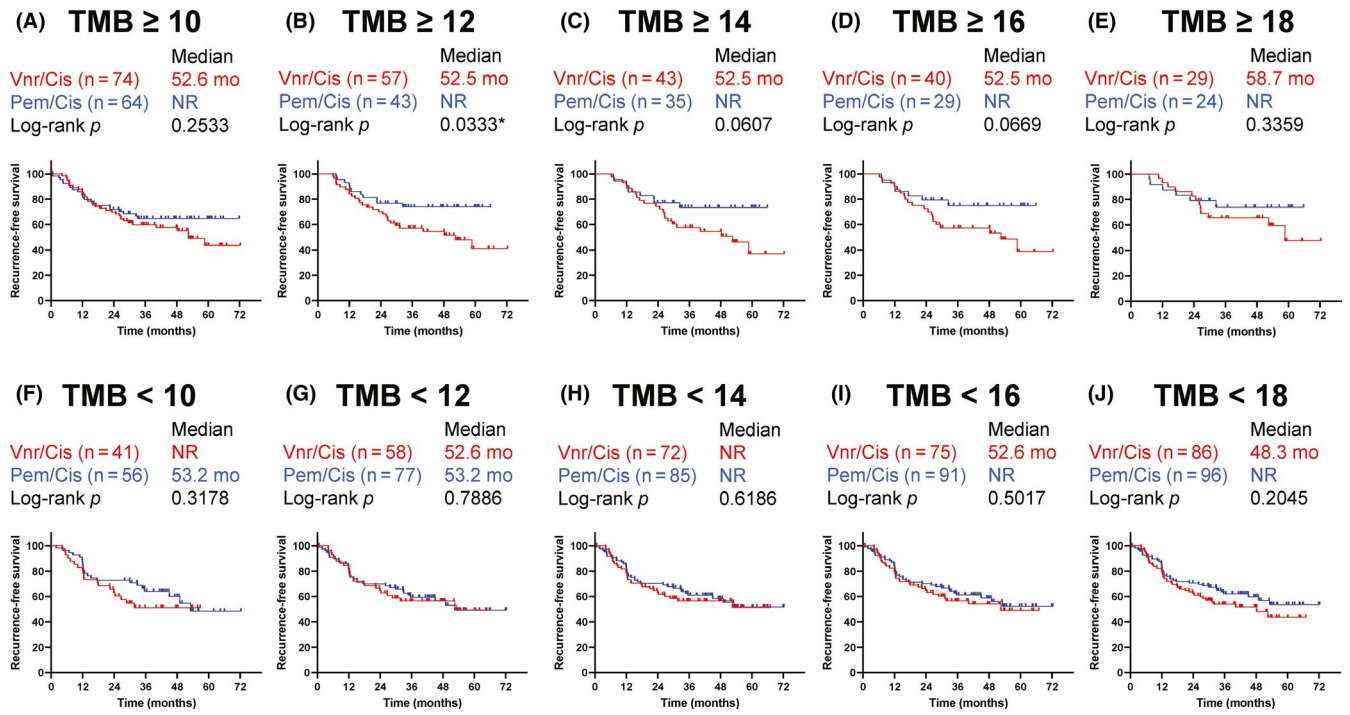

FIGURE 4 Kaplan-Meier curves of recurrence-free survival (RFS) by tumor mutation burden (TMB) status for patients without any epidermal growth factor receptor (*EGFR*) mutations. (A) patients with TMB ≥ 10 Mb; (B) patients with TMB ≥ 12 Mb; (C) patients with TMB ≥ 14 Mb; (D) patients with TMB ≥ 16 Mb; (E) patients with TMB ≥ 18 Mb; (F) patients with TMB < 10 Mb; (G) patients with TMB < 12 Mb; (H) patients with TMB < 14 Mb; (I) patients with TMB < 16 Mb; (J) patients with TMB < 18 Mb. *significant (<.05). Red line, Vnr/Cis arm; blue line, Pem/Cis arm

and uracil, in patients with resected lung adenocarcinoma.[15] In an in vitro study, *EGFR* mutant cells were less sensitive to fluorouracil (FU) compared with *EGFR* wild-type cells.[15] In patients with *EGFR* wild-type NSCLC, pemetrexed may produce a higher response rate than that observed in patients with *EGFR* mutations.[16] These findings of the current study indicate that *EGFR* mutation status might influence the efficacy of adjuvant chemotherapy among patients with Ns-NSCLC. Although the mechanism of how antifolates work differently in *EGFR* mutant or wild-type tumors is largely unknown, previous reports provide some clues. Suehisa et al examined the effect of FU on lung adenocarcinoma cell lines with *EGFR* wild-type or mutation status to find that the sensitivity to FU was higher in *EGFR* wild-type versus mutant type tumors.[15] Mutant *EGFR*, such as exon 19 deletions and L858R, activate Akt and STAT signaling pathways, which protect cells against apoptosis and promote cell survival.[17] NSCLC cells expressing mutant EGFR were relatively resistant to apoptosis induced by conventional chemotherapeutic drugs, such as cisplatin and doxorubicin.[17] Of note, uracil-tegafur also acts as a cytotoxic drug on cancer cells through the induction of apoptosis.[18] Indeed, Tanaka et al reported that postoperative adjuvant therapy for NSCLC had a larger effect on the prolonged survival of patients with tumors that had a high apoptotic index compared with patients with tumors with a low apoptotic index.[19] Taken together, these results suggest that *EGFR* mutant tumors have a low sensitivity to uracil-tegafur and FU because of the highly activated status of their antiapoptotic pathway.

It remains unclear why the RFS benefit increased when wild-type *EGFR* is combined with high TMB for Pem/Cis. Damaged DNA is repaired by DNA damage repair (DDR) and homologous recombination repair (HRR) genes in cancer cells. Impaired DNA repair abilities of DDR and HRR genes are associated with TMB-high.[20] In contrast, altered excision repair abilities such as overexpression of ERCC1 or BRCA1/2 deficiency in tumor cells are known to be related to resistance and hypersensitivity of tumor cells to platinums, respectively.[21,22] Taken together, wild-type *EGFR* and TMB-high as the surrogate of impaired DNA repair ability are enriched the subpopulation sensitive to Pem/Cis treatment.

Recurrence-free survival benefit with adjuvant chemotherapy of Pem/Cis was more pronounced in Ns-NSCLC patients with wild *EGFR* genotype and high nonsynonymous TMB. This result might support that Pem/Cis should be used for NSCLC with high TMB as an adjuvant chemotherapy in clinical practice, although further discussion will be necessary in the future. This population could also achieve clinical benefit by immune checkpoint inhibitors. Therefore, it will be important to investigate how to combine both adjuvant treatments as the next step.

In conclusion, one could surmise that tumor mutation burden was predictive of recurrence-free survival benefit of Ns-NSCLC patients with adjuvant chemotherapy with pemetrexed plus cisplatin versus vinorelbine plus cisplatin. Further investigation will be required to determine whether TMB combined with *EGFR* mutation status could be a useful predictive biomarker.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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