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RESEARCH

Immunological effects of amivantamab in EGFR or MET-expressing non-small cell lung cancer

Ryo Yoshichika[1,2] · Fumiaki Mukohara[1,2] · Kotaro Yamada[3] · Joji Nagasaki[1] · Hiroko Watanabe[1] · Youki Ueda[1] · Ken Suzawa[2] · Kazuhiko Shien[2] · Shinichi Toyooka[2] · Takamasa Ishino[1] · Yosuke Togashi[1,3,4]

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Abstract

Background *Epidermal growth factor receptor* (*EGFR*) mutations represent one of the most frequent oncogenic driver in non-small cell lung cancer (NSCLC). Amivantamab, a bispecific antibody targeting EGFR and MET proto-oncogene, receptor tyrosine kinase (MET), has demonstrated clinical benefit in *EGFR*-mutant NSCLC through dual blockade, but its immunological role in human clinical specimens, especially tumor-infiltrating lymphocytes (TILs), has not been directly evaluated.

Methods We analyzed surgically resected tumor samples from 40 patients with NSCLC to investigate immune responses and their associations with EGFR and MET expression. TILs were characterized by flow cytometry (FCM) and immunohistochemistry (IHC). To assess the immunomodulatory potential of amivantamab, fresh tumor digests containing live tumor cells and TILs were cultured ex vivo with CD3 and CD28 stimulation in the absence or presence of amivantamab, followed by FCM. EGFR and MET expression were also evaluated by IHC.

Results *EGFR* mutations and high EGFR protein expression were associated with a trend toward reduced $CD8^+$ T-cell and dendritic cell (DC) infiltration. In ex vivo TIL assays, exposure to amivantamab significantly activated $CD8^+$ T cells, such as programmed cell death-1 expression and cytokine production, and promoted DC maturation. These effects were most pronounced in tumors with high EGFR or MET protein expression rather than *EGFR* mutations.

Conclusions This study provides the first direct evidence from ex vivo fresh TIL assays using human NSCLC clinical specimens that amivantamab can activate immune responses. EGFR and MET expression may serve as potential biomarkers for amivantamab-induced immune responses.

Keywords Non-small cell lung cancer · Amivantamab · Antitumor immunity · EGFR · MET

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide [1]. Although smoking is a well-known major risk factor, environmental factors also contribute to disease pathogenesis. Indeed, a proportion of cases occur in never-smokers [2]. Along with changes in smoking prevalence, the epidemiology of lung cancer has changed, with increasing incidence observed particularly among young women and never-smokers. Therefore, lung cancer remains a life-threatening disease for a broad population [2]. Recent advances in the sequencing technologies have revealed the more presence of oncogenic driver mutations in such never-smoker populations [3]. Among them, the mutations in the *epidermal growth factor receptor* (*EGFR*), such as exon 19 deletions and L858R point mutation in exon 21, are the most commonly observed. In particular, approximately half

Ryo Yoshichika and Fumiaki Mukohara have contributed equally.

✉ Takamasa Ishino
t.ishino.0826@gmail.com

✉ Yosuke Togashi
ytogashi1584@gmail.com

1 Department of Tumor Microenvironment, Faculty of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama 700-8558, Japan

2 Department of General Thoracic Surgery and Breast and Endocrinological Surgery, Dentistry and Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Okayama University, Okayama 700-8558, Japan

3 Department of Respiratory Medicine, Okayama University Hospital, Okayama 700-8558, Japan

4 Faculty of Medicine, Kindai University, Osaka-Sayama, Osaka 589-0014, Japan

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of non-small cell lung cancer (NSCLC) cases in East Asian populations are known to have *EGFR* mutations [3].

EGFR is a receptor tyrosine kinase (RTK) which belongs to the erythroblastic leukemia viral oncogene homolog (ERBB) family. Activation of EGFR signaling leads to the induction of various downstream signaling pathways that promote cell proliferation and survival [4]. Because *EGFR* mutations result in ligand-independent activation of the downstream signaling, they are considered key oncogenic driver mutations. Therefore, various EGFR tyrosine kinase inhibitors (EGFR-TKIs) have been developed, leading to a paradigm shift in NSCLC treatment because of their high response rate and long progression-free survival (PFS) compared to conventional cytotoxic chemotherapies [3]. In addition, recent studies have revealed the involvement of the EGFR signaling pathway in the establishment of an immunosuppressive tumor microenvironment (TME) [5, 6]. Thus, EGFR-TKIs are considered to not only inhibit cancer cell proliferation but also enhance antitumor immune responses [5, 6]. However, even with osimertinib, a third-generation EGFR-TKI, PFS for *EGFR*-mutant NSCLC remains limited to approximately 12–16 months due to acquired resistance [7–9].

Activation of MET proto-oncogene, receptor tyrosine kinase (MET) signaling is known as one of the representative resistance mechanisms to EGFR-TKIs [6, 8]. MET is an RTK that uses hepatocyte growth factor (HGF) as a ligand and serves as a bypass signaling pathway for EGFR signaling, contributing to resistance against EGFR-TKIs. Therefore, MET has been considered a therapeutic target, leading to recent success of amivantamab, a bispecific antibody targeting EGFR and MET, in treating EGFR-mutant NSCLC [10–12]. In addition, high MET expression has been reported to contribute to the immunosuppressive TME [13]. Amivantamab inhibits EGFR and MET signaling pathways by internalizing and degrading EGFR and MET, as well as by inhibiting binding to epidermal growth factor (EGF) and HGF ligands [14]. In addition, amivantamab has been reported to induce antitumor immune responses through antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) activity, and trogocytosis by recruiting macrophages and natural killer cells via the Fc region [14, 15]. However, evidence about antitumor immunity is limited [14, 15].

In this study, we analyzed 40 NSCLC clinical samples to evaluate the infiltration and functions of $CD8^+$ T cells, regulatory T cells (Tregs), and dendritic cells (DCs) as well as the expression of EGFR and MET. We demonstrated that EGFR expression was associated with suppression of antitumor immune responses, consistent with its established immunosuppressive role. In addition, we established an original experimental system applying amivantamab to the digest of these fresh living tumor cells and tumor-infiltrating lymphocytes (TILs) with CD3 and CD28 stimulation, showing its potential to activate antitumor immune responses, particularly in cases with high EGFR or MET expression. These findings highlight the immunological role of amivantamab in human clinical samples, suggesting potential biomarkers of EGFR and MET expression for amivantamab-induced immune responses.

Materials and methods

Patient samples and ethical approval

Fresh tumor tissues were obtained from 40 patients with NSCLC who underwent surgical resection at Okayama University Hospital between December 2023 and December 2024 (Table S1). Fresh tumor specimens were divided into two halves at the maximal cross section immediately after surgical resection. One half was processed for ex vivo functional assays of TILs, while the other half was processed into formalin-fixed, paraffin-embedded (FFPE) blocks used for immunohistochemistry (IHC). Clinical data including *EGFR* mutation status and *MET* exon 14 skipping mutation, for which mutation tests were performed according to the physician's request as part of routine clinical care, were extracted from clinical records. All patients provided written informed consent, and the study protocol was approved by the Institutional Review Board of Okayama University.

TIL assay

Fresh tumor specimens were mechanically and enzymatically dissociated to generate single-cell suspensions with Tumor and Tissue Dissociation Reagent (BD Biosciences, Franklin Lakes, NJ, Cat# 661,563). Tumor digests containing tumor cells and TILs were cultured in RPMI-1640 medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan, Cat# 189–02025) supplemented with 10% AB serum (MP Biomedicals, Irvine, CA, Cat# 092930949) for 72 h. Proportion of each cell fraction and baseline status of them were evaluated based on unstimulated samples. For assessment of amivantamab-induced $CD8^+$ T-cell activation and cytokine production, cultures were stimulated with anti-CD3 (3 µg/mL) (OKT-3, BD Biosciences, RRID: AB_2869821) and anti-CD28 (5 µg/mL) (CD28.2, Thermo Fisher Scientific, RRID: AB_468926) antibodies in the presence or absence of amivantamab (Janssen Pharmaceutical, JNJ-61186372; 0.5 µg/mL), because CD3/CD28 stimulation was required to induce measurable cytokine. For assessment of amivantamab-induced maturation or activation of the other cell fractions, tumor digests were cultured without CD3/CD28 stimulation, and immune cell phenotypes were

compared in the presence or absence of amivantamab. Following culture, immune cell phenotypes were assessed by flow cytometry (FCM) (Fig. 1A).

FCM

FCM was performed as previously described [16]. Briefly, cells were washed with phosphate-buffered saline (PBS) (FUJIFILM Wako Pure Chemical Corporation, Cat# 048–29805) containing 2% fetal bovine serum (FBS) (Cytiva, Tokyo, Japan, Cat# SH30070.03) and subjected to staining with surface antibodies. Intracellular staining was performed with specific antibodies and the Fixation/Permeabilization Buffer Set (Thermo Fisher Scientific, Waltham, MA, Cat# 88–8824-00), according to the manufacturer's instructions. Antibodies were diluted according to the manufacturer's instructions before staining. Detailed information on the antibodies used is summarized in Table S2. Effector Tregs (eTregs) were identified as $CD45RA^{-}FoxP3^{high}CD4^{+}$ T cells. DCs were defined as Lin (CD3/14/19/56)$^{-}$HLA-$DR^{+}CD11c^{+}$ cells. For cytokine production, cells were suspended in 1 µL/mL of GolgiStop™ Protein Transport Inhibitor (BD Biosciences, RRID: 2,869,012), and cultured for 6 h at 37 °C. After incubation, cells were collected and stained. FCM was performed on a BD LSRFortessa X-20 (BD Biosciences, RRID: SCR_025285), and data were analyzed using FlowJo v10 software (BD Biosciences, RRID: SCR_008520).

IHC

Two samples were unavailable for IHC because of insufficient tissue volume, resulting in 38 samples, which were prepared from mirror samples corresponding to the fresh tumor tissues used for the ex vivo assays. FFPE tissue sections were cut into 4 µm slices, deparaffinized, and rehydrated. Antigen retrieval was carried out. After blocking endogenous peroxidase activity and nonspecific binding, sections were incubated overnight at 4 °C with an anti–human CD8 monoclonal antibody (mAb) (Cell Signaling Technology, Danvers, MA, RRID: AB_2800052), an anti–human CD11c mAb (Cell Signaling Technology, RRID: AB_2799286), an anti–human EGFR mAb (Cell Signaling Technology, RRID: AB_2246311), an anti–human cMET mAb (Cell Signaling Technology, RRID: AB_10858224), an anti-human phospho-EGFR (Tyr1068) mAb (Cell Signaling Technology, RRID:AB_2096270), or an anti-human phospho- Met (Tyr1234/1235) mAb (Cell Signaling Technology, RRID:AB_2143884) diluted in 5% BSA (Albumin, Bovine Serum, F-V, pH5.2; Nacalai tesque, Cat# 01863–48). The slides were then incubated with the SignalStain® Boost IHC Detection Reagent (Cell Signaling Technology, RRID: AB_10966207) and detected using the SignalStain® DAB Substrate Kit (Cell Signaling Technology, RRID: AB_10796606). All images were acquired using a BZ-X800 all-in-one microscope (Keyence, Osaka, Japan, RRID:SCR_023617) in bright-field mode. CD8 and CD11c positive cells were counted in five randomly selected high-power fields (× 200 magnification). For semi-quantitative evaluation of EGFR and MET expression, membranous staining intensity was classified into weak, moderate, and strong as previously reported [17, 18]. EGFR expression was scored by IHC as follows: 1 + for weak membrane staining in ≥ 10% of tumor cells, 2 + for moderate staining in ≥ 10%, and 3 + for strong staining in ≥ 10% as previously reported [17]. MET expression was scored by IHC as follows: 1 + for weak membrane staining in ≥ 50% of tumor cells, 2 + for moderate staining in ≥ 50%, and 3 + for strong staining in ≥ 50% as previously reported [18]. For evaluation of phospho-EGFR and MET expression, tumors containing ≥ 5% positive cells were defined as phosphorylation-positive as previously reported [19, 20]. Cell counts and expression scores were independently evaluated by two investigators blinded to mutation status.

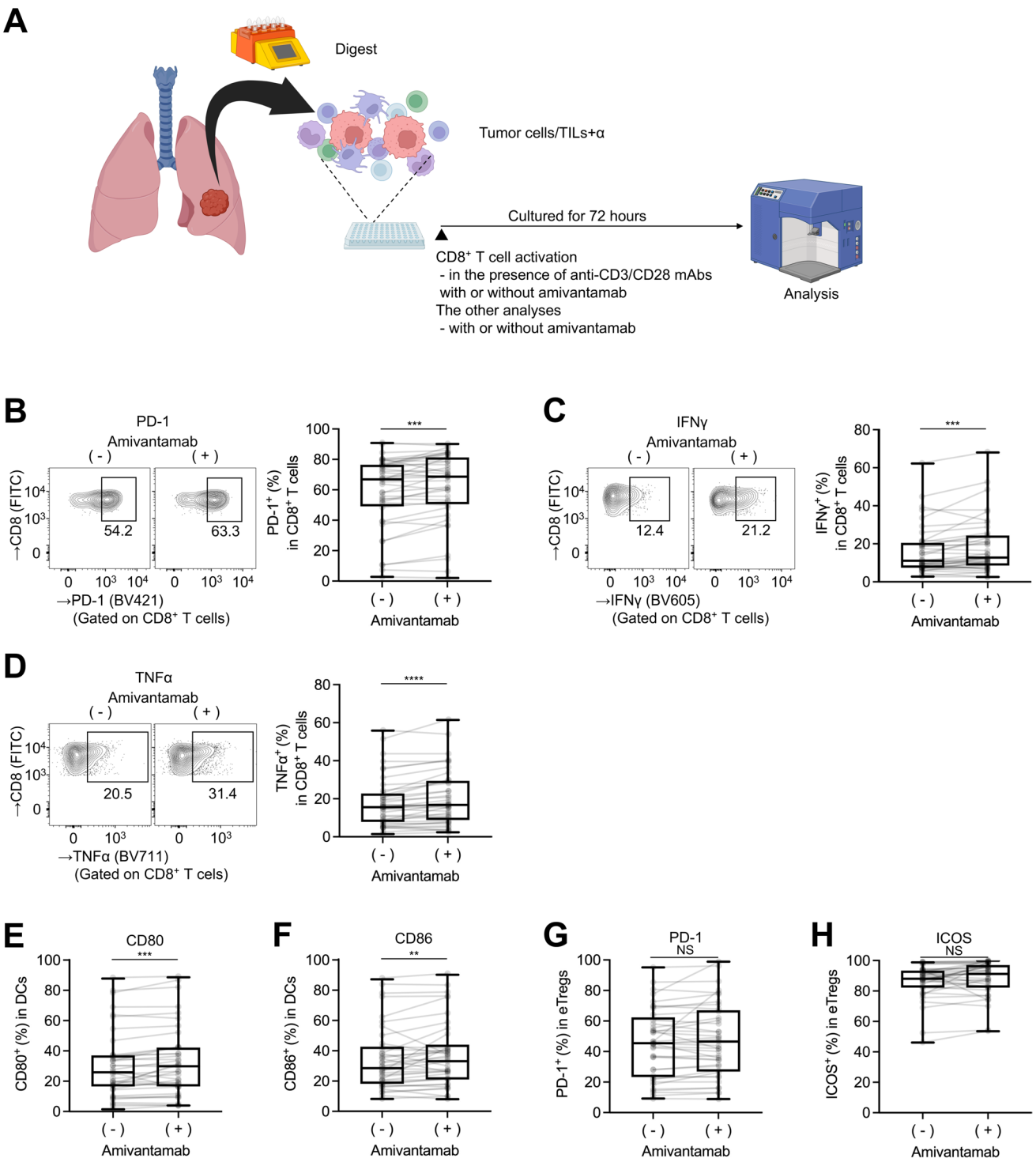
Statistical analysis

GraphPad Prism 9 (GraphPad Software, San Diego, CA, RRID: SCR_002798) was used for statistical analyses. Recurrence-free survival was defined as the time from the date of surgery to the date of the first documented recurrence or death from any cause, and was estimated using a Kaplan–Meier curve. Continuous variables were compared using the two-tailed Mann–Whitney U test and Wilcoxon signed-rank sum test. Categorical variables were analyzed with Fisher's exact test. P-values < 0.05 were considered statistically significant. All statistical details are provided in the figure legends.

Results

Antitumor immune responses appear to be impaired in *EGFR*-mutant NSCLC tumors

We analyzed tumor samples from 40 patients with NSCLC who underwent surgical resection and the patient characteristics are summarized in Table S1. All patients had good performance status (0 or 1) with median age of 75 years. Twenty-nine patients (73%) were male and 27 (68%) had a history of smoking. Adenocarcinoma was the predominant histological subtype (32 patients, 80%). Only two patients (5%) received neoadjuvant chemotherapy, both of which consisted of combination immunotherapy. Postoperative pathological staging revealed stage I in 23 patients (57.5%),



stage II in 12 (30%), stage III in four (10%), and stage IVA in one (2.5%). Genome analyses were performed at the request of the attending physician, which identified *EGFR* mutations in 35% (13/37), *MET* exon 14 skipping mutation in 13% (2/16), and *KRAS proto-oncogene, GTPase* (*KRAS*) exon 2 G12C mutation in 7% (1/16) (Table S1). Data on *EGFR* copy number alterations were not available in this study because they were not included in the clinical panel tests. During the observation period, postoperative recurrence was observed in only one patient, with no deaths observed (Table S1 and Fig. S1A).

◂**Fig. 1** Ex vivo activation of tumor-infiltrating lymphocytes (TILs) by amivantamab in fresh non-small cell lung cancer (NSCLC) clinical specimens (**A**) Experimental schema. Freshly resected NSCLC tumor digests containing living tumor cells and TILs were cultured for 72 h. Proportion of each cell fraction and baseline status of them were evaluated based on unstimulated samples. For assessment of amivantamab-induced $CD8^+$ T-cell activation and cytokine production, tumor digests were cultured without stimulation, or with anti-CD3/CD28 monoclonal antibodies (mAbs) in the absence or presence of amivantamab. For evaluation of dendritic cell (DC) maturation and effector regulatory T-cell (eTreg) activation, tumor digests were cultured without CD3/CD28 stimulation in the absence or presence of amivantamab. Tumor digests were subsequently analyzed by flow cytometry. (**B–D**) TILs activation by amivantamab. Representative flow cytometry staining (left) and summaries (right) of programmed cell death-1 (PD-1) expression (**B**), interferon-γ (IFNγ) production (**C**), and tumor necrosis factor-α (TNFα) production (**D**) in tumor-infiltrating $CD8^+$ T cells are shown. (**E–H**) TILs activation by amivantamab. Summaries of CD80 (**E**) and CD86 expression (**F**) in tumor-infiltrating DCs, and PD-1 (**G**) and inducible T-cell co-stimulator (ICOS) expression (**H**) in tumor-infiltrating eTregs are shown. Statistical analyses were performed by Wilcoxon signed-rank sum tests in (**B–H**). In box-and-whisker plots, the box spans from the first to the third quartile with a line at the median and the whiskers extend from the minimum to the maximum. Each dot represents an individual sample and connecting lines indicate paired before-after measurements for the same samples. NS: not significant; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

Because amivantamab is clinically indicated for *EGFR*-mutant NSCLC, we first compared the immune microenvironment between *EGFR*-mutant and wild-type (WT) tumors. $CD8^+$ T cells and DCs, which play central roles in antitumor immune responses, were evaluated by IHC. Two samples were unavailable for IHC because of insufficient tissue volume. As a result, compared with WT tumors, *EGFR*-mutant tumors exhibited a trend toward lower $CD8^+$ T-cell infiltration (Fig. S1B) and a significant reduction in DC infiltration (Fig. S1C). We also analyzed TILs by FCM, evaluating $CD8^+$ T cells, DCs, and eTregs (Fig. S1D). Consequently, the $CD8^+$ T cell to eTreg ratio, which has been reported to reflect antitumor immune responses [5], was lower in *EGFR*-mutant cases in our cohort, although the difference was not statistically significant (Fig. S1E). In addition, programmed cell death-1 (PD-1) expression in tumor-infiltrating $CD8^+$ T cells, a widely used activation marker that reflects antitumor immune responses [21–23], also tended to be lower in *EGFR*-mutant cases (Fig. S1F). Conversely, CD80 and CD86 expression, representative DC maturation markers [24], were not correlated with *EGFR* mutation status (Fig. S1G and H). We also evaluated expression of PD-1 and inducible T-cell co-stimulator (ICOS) in tumor-infiltrating eTregs and observed no significant differences regardless of *EGFR* mutation status (Fig. S1I and J).

Amivantamab can activate immune responses in NSCLC

We investigated whether antitumor immune responses can be affected by amivantamab using fresh TIL assay. For assessment of amivantamab-induced $CD8^+$ T-cell activation and cytokine production, the tumor digests were stimulated for 72 h in the presence of anti-CD3/CD28 antibodies with or without amivantamab (Fig. 1A). For assessment of amivantamab-induced maturation or activation of the other cell fractions, tumor digests were cultured without CD3/CD28 stimulation, and immune cell phenotypes were compared in the presence or absence of amivantamab (Fig. 1A). We could not include a condition with amivantamab stimulation alone without CD3/CD28 stimulation due to limited sample availability. As a result, amivantamab promoted $CD8^+$ TIL activation with increasing PD-1 expression and cytokine production such as interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) (Fig. 1B-D). Amivantamab also promoted tumor-infiltrating DC maturation (Fig. 1E and F), whereas little effect on eTregs was observed (Fig. 1G and H).

We also examined the relationship between immune cell infiltration and the effects of amivantamab on antitumor immune responses. Tumors were stratified into two groups based on the median values of $CD8^+$ T cell counts assessed by IHC. As a result, tumors with high $CD8^+$ T-cell infiltration tended to exhibit greater DC infiltration (Fig. S2A) and showed a significantly higher $CD8^+$ T cell to eTreg ratio (Fig. S2B). Regarding the effects of amivantamab, it significantly promoted $CD8^+$ T-cell activation (Fig. S2C-E) and exhibited a trend of increased DC maturation in tumors with high $CD8^+$ T-cell infiltration (Fig. S2F and G). Conversely, no significant changes in tumor-infiltrating eTregs were observed in both groups (Fig. S2H and I). In addition, we performed the same analysis based on DC infiltration counts. The tumors with high DC infiltration had significantly higher $CD8^+$ T-cell infiltration compared to low infiltration tumors (Fig. S3A), while the $CD8^+$ T cell to eTreg ratio was comparable between the groups (Fig. S3B). When evaluating the effects of amivantamab, tumor-infiltrating $CD8^+$ T-cell activation and DC maturation were induced regardless of DC infiltration level (Fig. S3C-G), whereas eTregs showed no significant changes (Fig. S3H and I). These results suggest that amivantamab can activate immune responses in NSCLC tumors, particularly in those with high $CD8^+$ T-cell infiltration.

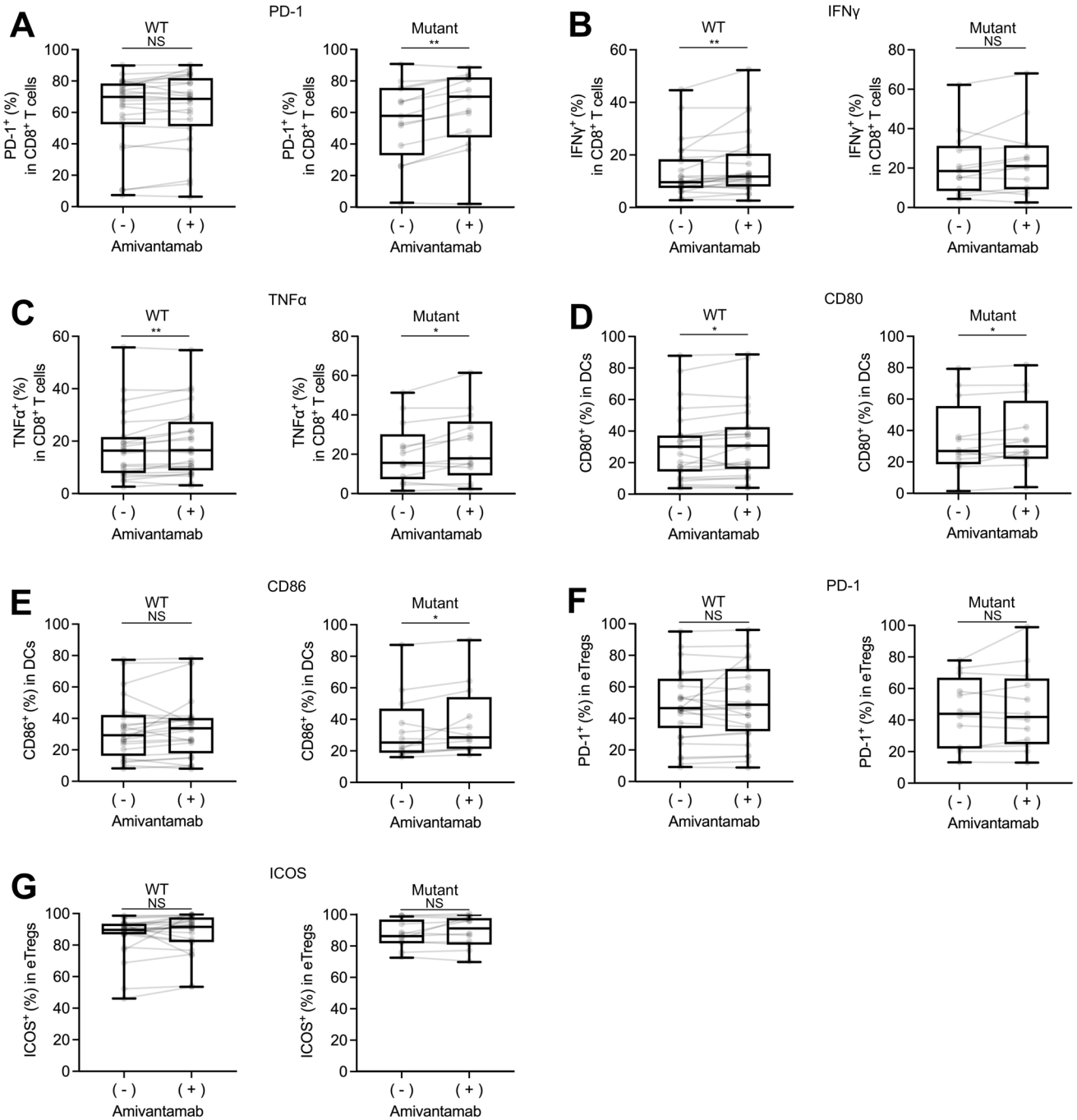


Fig. 2 Ex vivo activation of tumor-infiltrating lymphocytes (TILs) by amivantamab according to *epidermal growth factor receptor* (*EGFR*) mutation status Ex vivo TIL assays were performed as described in Fig. 1A. Summaries of programmed cell death-1 (PD-1) expression (**A**), interferon-γ (IFNγ) production (**B**), and tumor necrosis factor-α (TNFα) production (**C**) in tumor-infiltrating $CD8^+$ T cells, CD80 (**D**) and CD86 expression (**E**) in tumor-infiltrating dendritic cells (DCs), and PD-1 (**F**) and inducible T-cell co-stimulator (ICOS) expression (**G**) in tumor-infiltrating effector regulatory T cells (eTregs) according to *EGFR* mutation status (left, wild-type (WT); right, mutant) are shown. Statistical analyses were performed Wilcoxon signed-rank sum tests in (**A**–**G**). In box-and-whisker plots, the box spans from the first to the third quartile with a line at the median and the whiskers extend from the minimum to the maximum. Each dot represents an individual sample and connecting lines indicate paired before-after measurements for the same sample. NS: not significant; $^{*}P < 0.05$;.$^{**}P < 0.01$

Amivantamab potentially activates immune responses in NSCLC regardless of *EGFR* mutation

Because amivantamab is used clinically for *EGFR*-mutant NSCLC patients, we next evaluated the effect of amivantamab according to *EGFR* mutation status. In *EGFR*-mutant tumors, amivantamab promoted $CD8^+$ TIL activation, as indicated by PD-1 expression and cytokine production (Fig. 2A-C). However, amivantamab also promoted cytokine production even in *EGFR*-WT tumors (Fig. 2A-C). In addition, the maturation of tumor-infiltrating DCs was also promoted by amivantamab regardless of *EGFR* mutation status (Fig. 2D and E), whereas eTregs were unaffected in both groups (Fig. 2F and G). These findings suggest that amivantamab can activate immune responses not only in *EGFR*-mutant but also in *EGFR*-WT tumors.

Amivantamab activates immune responses in tumors with high EGFR or MET expression

Given the targets of amivantamab, we evaluated the relationship between EGFR expression and antitumor immunity. We evaluated EGFR expression with IHC (Fig. S4A), which was then classified into 4 categories (0–3 +) based on a previous report (Fig. 3A) [17]. In the entire cohort, 34% (13/38) of the tumors were classified as EGFR ≤ 1 + and 66% (25/38) were EGFR ≥ 2 + (Fig. 3A). As previously reported [25], the majority of the *EGFR*-mutant tumors highly expressed EGFR (85%, 11/13). However, more than half of *EGFR*-WT tumors also expressed high levels of EGFR (59%, 13/22) (Fig. 3B). There was a trend toward an association between *EGFR* mutation and EGFR expression, although not statistically significant (Fisher's exact test: P = 0.150).

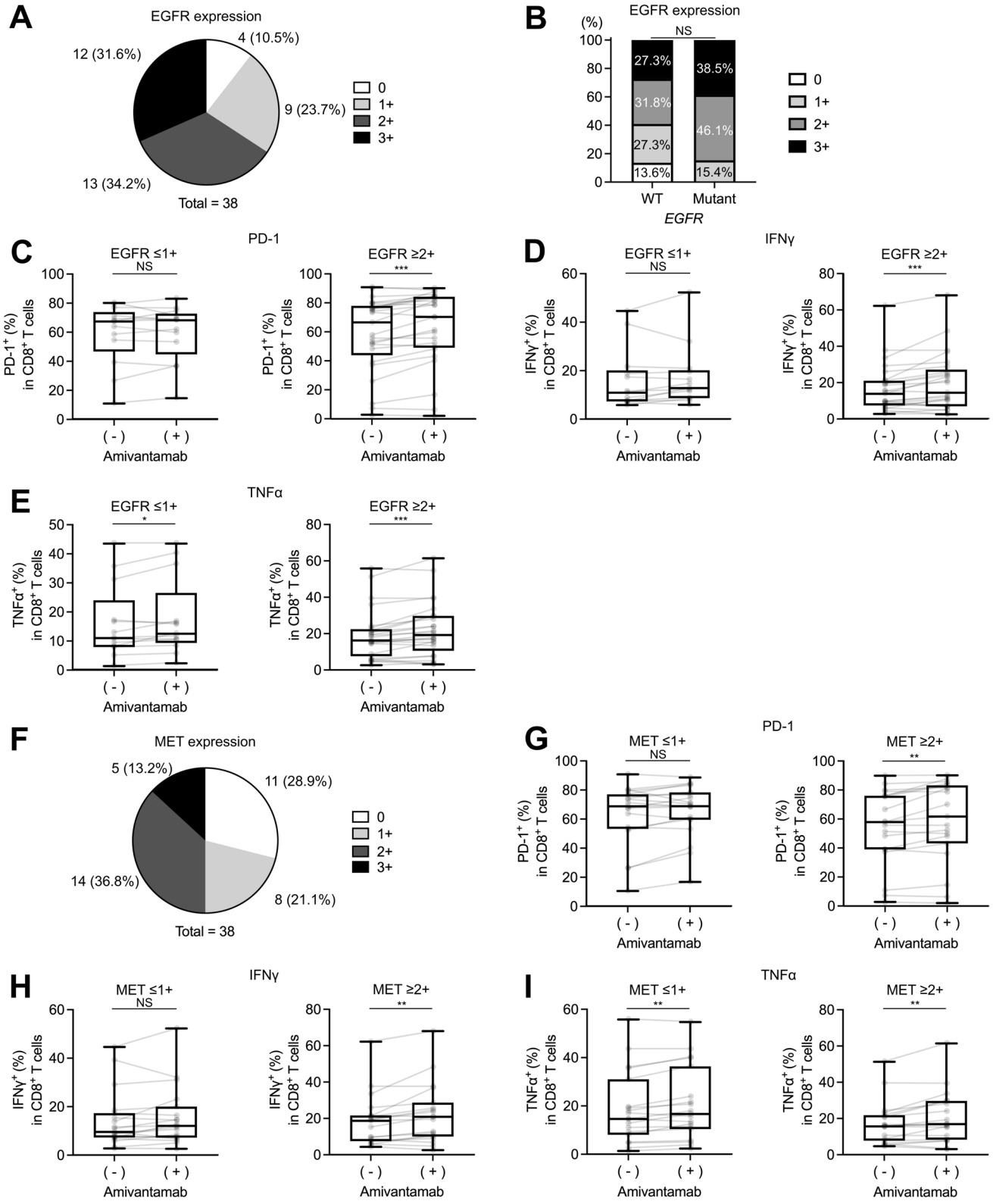
We next compared the immune microenvironment and immune cell phenotypes according to EGFR expression. As a result, infiltration of $CD8^+$ T cells and DCs was significantly reduced in EGFR ≥ 2 + tumors (Fig. S4B and C). In contrast, the ratio of $CD8^+$ T cells to eTregs, as well as the activation status of $CD8^+$ T cells, DCs, and eTregs, was comparable irrespective of EGFR expression levels (Fig. S4D-I). We further analyzed the relationship between EGFR expression and amivantamab-mediated immune activation. Amivantamab increased PD-1 expression and cytokine production in $CD8^+$ TILs from EGFR ≥ 2 + tumors, whereas these effects were limited in EGFR ≤ 1 + tumors (Fig. 3C-E). Similarly, tumor-infiltrating DC maturation was significantly promoted by amivantamab in EGFR ≥ 2 + tumors but remained limited in EGFR ≤ 1 + tumors (Fig. S4J and K). In contrast, tumor-infiltrating eTregs were unaffected by amivantamab in EGFR ≥ 2 + tumors (Fig. S4L and M). These findings suggest that high EGFR expression is associated with an immunosuppressive TME, but amivantamab can promote immune activation in such a TME.

Similar to EGFR, we also examined MET, another target of amivantamab, by classifying its expression in tumors into four categories (0–3 +) based on the previous report [18]. In the entire cohort, MET was highly expressed (2 + or 3 +) in half of the cases (19/38) (Fig. 3F and Fig. S5A). The comparison of immune features between MET ≥ 2 + and MET ≤ 1 + tumors revealed no significant differences in $CD8^+$ T-cell or DC infiltration (Fig. S5B and C) and in the $CD8^+$ T cell to eTreg ratio (Fig. S5D). In addition, the activation status of $CD8^+$ T cells, DCs, and eTregs were also comparable between the two groups (Fig. S5E-I). However, in MET ≥ 2 + tumors, amivantamab increased PD-1 expression and cytokine production in $CD8^+$ TILs, whereas such effects were limited in MET ≤ 1 + tumors (Fig. 3G-I). Tumor-infiltrating DC maturation tended to be promoted by amivantamab regardless of MET expression (Fig. S5J and K), while tumor-infiltrating eTregs remained unchanged MET ≥ 2 + tumors (Fig. S5L and M). Taken together, these findings suggest that, similar to EGFR, MET expression can be associated with immune-activating effects of amivantamab.

We also stratified tumors using a more stringent 3 + cut-off for EGFR and MET expression (0–2 + vs. 3 +). As a result, amivantamab treatment significantly increased PD-1 expression and cytokine production in $CD8^+$ TILs from EGFR 3 + tumors, whereas cytokine production was also induced in EGFR 0–2 + tumors (Fig. S6A-C), suggesting that a certain level of EGFR expression may be sufficient to support amivantamab-mediated immune activation. Conversely, amivantamab did not induce a significant increase in PD-1 expression or cytokine production in MET 3 + tumors (n = 5) (Fig. S6D-F), which may be due to the limited number of samples.

Amivantamab promotes immune responses in EGFR or MET expressing tumors, even with WT *EGFR*

Given the individual importance of EGFR and MET expression in amivantamab-mediated immune activation, we next analyzed their combined association. The cohort was divided into four groups based on EGFR and MET expression as described in Fig. 4A, which exhibited a significant association between EGFR and MET expression levels (Fisher's exact test: P = 0.038). Although EGFR and MET expression were positively correlated, 32% (12/38) of the tumors exhibited EGFR or MET expression scored as ≥ 2 + (Fig. 4A). We subsequently divided the tumors into two groups according to EGFR and MET expression



levels (Group I: both $\leq 1+$; Group II: either $\geq 2+$) (Fig. 4A), and compared their immune microenvironment and immune cell phenotypes. The results revealed that tumors in group II exhibited a trend toward lower $CD8^+$ T-cell infiltration (Fig. S7A) and significantly reduced DC infiltration (Fig. S7B). In contrast, the $CD8^+$ T cell to eTreg ratio and the activation status of immune cells were comparable between the two groups (Fig. S7C–H). We

◂**Fig. 3** Ex vivo activation of tumor-infiltrating lymphocytes (TILs) by amivantamab according to epidermal growth factor receptor (EGFR) or MET proto-oncogene, receptor tyrosine kinase (MET) expression level (**A**) Distribution of EGFR protein expression levels in tumor samples. EGFR expression was scored by immunohistochemistry (IHC) as follows: 1 + for weak membrane staining in ≥ 10% of tumor cells, 2 + for moderate staining in ≥ 10%, and 3 + for strong staining in ≥ 10%. A pie chart is shown. (**B**) Association between *EGFR* mutation status and EGFR protein expression. A bar graph is shown. (**C**–**E**) Tumor-infiltrating $CD8^+$ T-cell activation stratified by EGFR expression level. Ex vivo TIL assays were performed as described in Fig. 1A. Summaries of programmed cell death-1 (PD-1) expression (**C**), interferon-γ (IFNγ) production (**D**), and tumor necrosis factor-α (TNFα) production (**E**) in tumor-infiltrating $CD8^+$ T cells according to EGFR expression level (left, ≤ 1 +; right, ≥ 2 +) are shown. (**F**) Distribution of MET protein expression levels in tumor samples. MET expression was scored by IHC as follows: 1 + for weak membrane staining in ≥ 50% of tumor cells, 2 + for moderate staining in ≥ 50%, and 3 + for strong staining in ≥ 50%. A pie chart is shown. (**G**–**I**) Tumor-infiltrating $CD8^+$ T-cell activation stratified by MET expression level. Ex vivo TIL assays were performed as described in Fig. 1A. Summaries of PD-1 expression (**G**), IFNγ production (**H**), and TNFα production (**I**) in tumor-infiltrating $CD8^+$ T cells according to MET expression level (left, ≤ 1 +; right, ≥ 2 +) are shown. The correlation between EGFR expression levels (≤ 1 + and ≥ 2 +) and *EGFR* mutation status was analyzed using Fisher's exact test in (**B**). Wilcoxon signed-rank sum tests were performed in (**C**–**E**) and (**G**–**I**). In box-and-whisker plots, the box spans from the first to the third quartile with a line at the median and the whiskers extend from the minimum to the maximum. Each dot represents an individual sample and connecting lines indicate paired before-after measurements for the same sample. NS: not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

also examined the association of EGFR and MET expression with the immune-activating effects of amivantamab. In group II tumors, amivantamab increased PD-1 expression and cytokine production in $CD8^+$ TILs. In contrast, in group I tumors, amivantamab-induced $CD8^+$ TIL activation was limited (Fig. 4B-D). Similarly, tumor-infiltrating DC maturation was promoted by amivantamab in group II tumors, but not in group I tumors (Fig. S7I and J). Tumor-infiltrating eTregs were unaffected by amivantamab in group II tumors (Fig. S7K and L). Collectively, these findings indicate that high expression of EGFR or MET is important for amivantamab-induced immune responses.

We further hypothesized that EGFR and MET expression might influence the efficacy of amivantamab even in *EGFR*-WT tumors. *EGFR*-mutant and *EGFR*-WT tumors were each stratified into four groups based on EGFR and MET expression as described in Fig. 5A, and 72.7% (16/22) tumors exhibited ≥ 2 + expression of either EGFR or MET even among *EGFR*-WT tumors (Fig. 5A). No significant correlation was observed between EGFR mutation status and EGFR or MET expression (Fisher's exact test: P = 0.680). Then, using the same EGFR and MET expression criteria, EGFR–WT tumors were further divided into group I (both ≤ 1 +) and group II (either ≥ 2 +) (Fig. 5A). We then examined the association of EGFR and MET expression with the immune-activating effects of amivantamab in *EGFR*-WT tumors. Even in group II tumors with EGFR-WT, amivantamab significantly increased PD-1 expression and cytokine production in $CD8^+$ TILs (Fig. 5B–D). A trend toward amivantamab-induced DC maturation was observed only in group II, but not in group I (Fig. S7A and B). In contrast, amivantamab did not induce significant changes in tumor-infiltrating eTregs in either group (Fig. S8C and D). These findings suggest that even *EGFR*-WT tumors, the presence of EGFR or MET expression can allow amivantamab to promote immune responses.

Amivantamab activates immune responses in tumors with EGFR phosphorylation

Considering the importance of EGFR and MET signaling in antitumor immunity [5, 6, 13], We evaluated the relationship between EGFR phosphorylation (pEGFR) (Fig S9A) and the immunomodulatory effects of amivantamab. In our cohort, 45% (17/38) of the tumors exhibited pEGFR expression. We stratified the cohort according to pEGFR into positive and negative groups, which was evaluated using a previous method [19]. As previously reported [26], the majority of the *EGFR*-mutant tumors exhibited pEGFR, with a significant trend toward an association between EGFR mutation and pEGFR (Fisher's exact test: P = 0.0425) (Fig. S9B). Although a previous report suggested that EGFR expression and phosphorylation do not necessarily correlate [27], a significant correlation was observed in our cohort (Fisher's exact test: P = 0.0151) (Fig. S9B). We further analyzed the relationship between pEGFR and amivantamab-mediated immune activation. As a result, amivantamab increased PD-1 expression and cytokine production in $CD8^+$ TILs from pEGFR-positive tumors, whereas it increased only TNFα production in $CD8^+$ TILs from pEGFR-negative tumors (Fig. S9C-E). Tumor-infiltrating DC maturation also tended to be promoted by amivantamab in pEGFR-positive tumors (Fig. S9F and G). In contrast, tumor-infiltrating eTregs were unaffected by amivantamab regardless of pEGFR status (Fig. S9H and I). These results support the importance of EGFR signaling in regulating immune responses. Similar to pEGFR, we evaluated the relationship between MET phosphorylation (pMET) (Fig. S10A) and the immunomodulatory effects of amivantamab [20]. Only 13% (5/38) of the cases were positive for pMET, which was not significantly correlated with total MET expression (Fig. S10B). Immune activation induced by amivantamab was not observed in pMET-positive tumors (n = 5) (Fig. S10C-I), which may be attributable to the limited sample size.

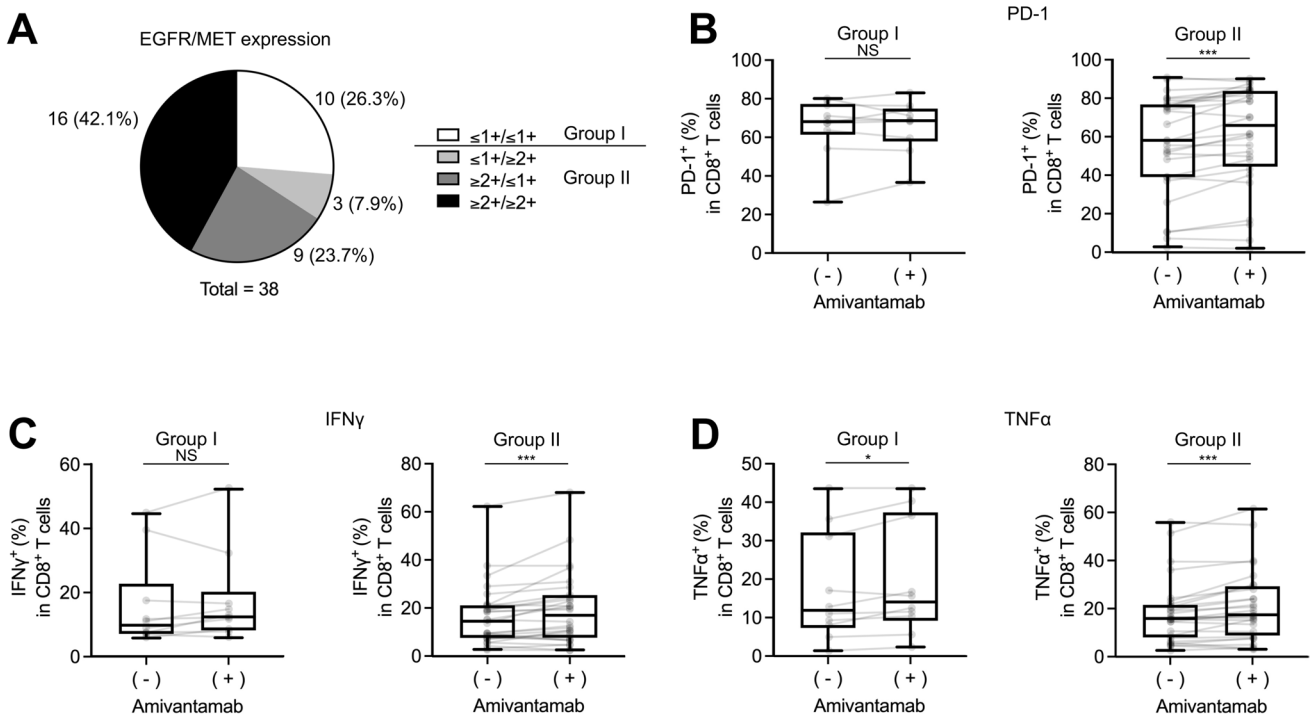


Fig. 4 Ex vivo activation of tumor-infiltrating lymphocytes (TILs) by amivantamab according to epidermal growth factor receptor (EGFR)/MET proto-oncogene, receptor tyrosine kinase (MET) expression level (**A**) Distribution of EGFR/MET protein expression levels in tumor samples. The EGFR/MET expression was stratified as described in Fig. 3. A pie chart is shown. (**B**–**D**) Tumor-infiltrating $CD8^+$ T-cell activation stratified by EGFR/MET expression level categorized as group I (both ≤ 1 +) or group II (either ≥ 2 +). Ex vivo TIL assays were performed as described in Fig. 1A. Summaries of programmed cell death-1 (PD-1) expression (**B**), interferon-γ (IFNγ) production (**C**), and tumor necrosis factor-α (TNFα) production (**D**) in tumor-infiltrating $CD8^+$ T cells according to EGFR/MET expression level (left, group I; right, group II) are shown. Statistical analyses were performed by Wilcoxon signed-rank sum tests in (**B**–**D**). In box-and-whisker plots, the box spans from the first to the third quartile with a line at the median and the whiskers extend from the minimum to the maximum. Each dot represents an individual sample and connecting lines indicate paired before-after measurements for the same sample. NS: not significant; $^{*}P < 0.05$; $^{***}P < 0.001$

Discussion

EGFR-mutant NSCLCs are characterized by the immunosuppressive TME and poor responses to immune checkpoint inhibitors (ICIs) [28, 29]. *EGFR* mutations induce ligand-independent phosphorylation of RTK and activate EGFR signaling pathways, which contribute not only to cancer cell proliferation but also to the establishment of immunosuppressive TME. Specifically, EGFR activation regulates chemokine production, leading to reduced infiltration of cytotoxic $CD8^+$ T cells while promoting the recruitment of Tregs [5, 6]. This pathway also increases tumor-promoting cytokines and EGFR ligands, suppressing $CD8^+$ T-cell function and DC maturation while enhancing the immunosuppressive activity of Tregs and tumor-associated macrophages [5, 6]. Consistently, in our cohort, *EGFR*-mutant tumors exhibited reduced infiltration of $CD8^+$ T cells and DCs, reinforcing the concept of the immunosuppressive TME in these tumors. As with EGFR, MET has also been reported to suppress antitumor immune responses [13]. Therefore, blockade of the EGFR and MET pathways could potentially counteract this immunosuppressive TME. Consistently, amivantamab, a bispecific antibody targeting both EGFR and MET, has demonstrated a tail plateau pattern in clinical responses [30, 31], which suggests that amivantamab can activate antitumor immunity. However, amivantamab-induced immune activation has been demonstrated only in vitro and in mouse models [14, 15], with no direct evidence from human clinical specimens. In this study, we provide the first direct evidence from ex vivo fresh TIL assays using human NSCLC clinical specimens that amivantamab can activate antitumor immune responses, particularly through T-cell-mediated responses.

Although the mechanisms underlying amivantamab-induced immune activation remain incompletely understood, studies of other receptor-targeting antibodies may offer relevant insights. Human epidermal growth factor receptor 2 (HER2)-targeted antibodies, which are reported to show clinical responses when combined with ICIs for HER2-positive gastric cancer [32], have been shown to alleviate immune suppression. The immunomodulatory

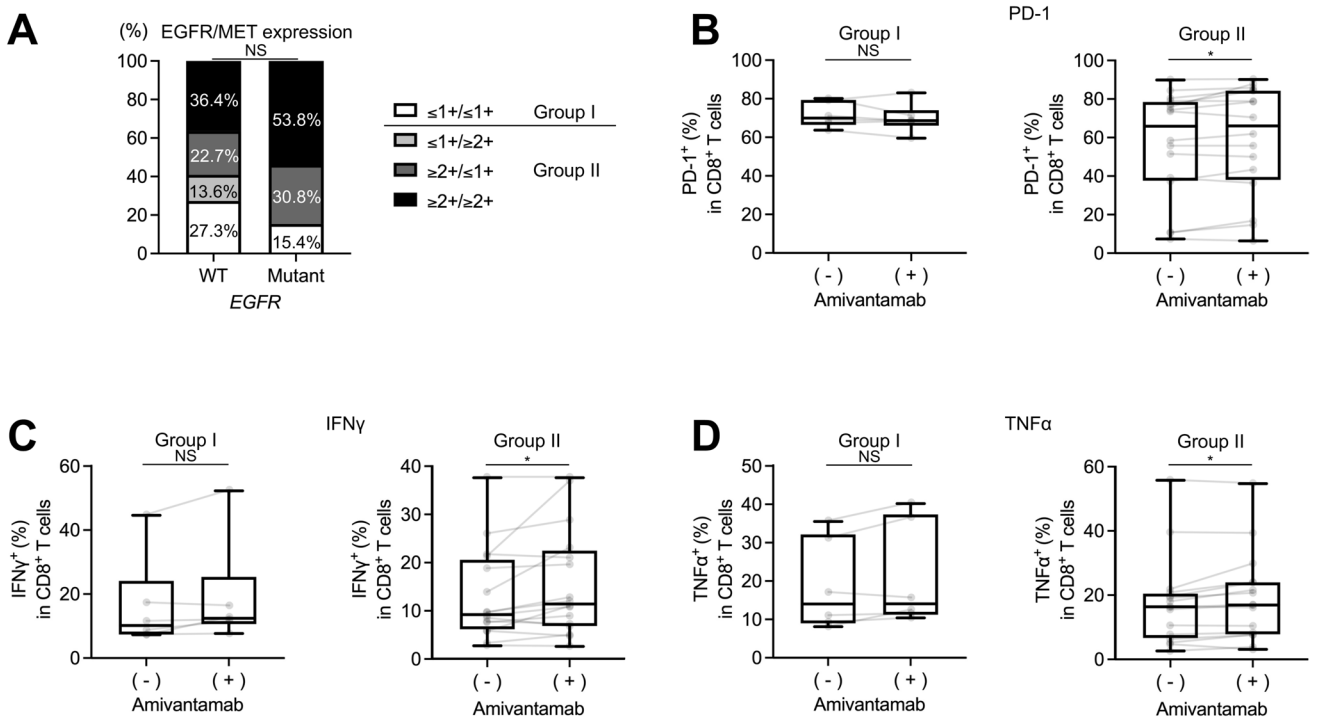


Fig. 5 Ex vivo activation of tumor-infiltrating lymphocytes (TILs) by amivantamab based on epidermal growth factor receptor (EGFR)/MET proto-oncogene, receptor tyrosine kinase (MET) expression level in *EGFR-wild type (WT) tumors* (**A**) Association between *EGFR* mutation status and EGFR/MET protein expression. The EGFR/MET expression was stratified as described in Fig. 3. A bar graph is shown. (**B**–**D**) Tumor-infiltrating $CD8^+$ T-cell activation stratified by EGFR/MET expression level categorized as described in Fig. 4 in *EGFR*-WT tumors. Ex vivo TIL assays were performed as described in Fig. 1A. Summaries of programmed cell death-1 (PD-1) expression (**B**), interferon-γ (IFNγ) production (**C**), and tumor necrosis factor-α (TNFα) production (**D**) in tumor-infiltrating $CD8^+$ T cells according to EGFR/MET expression level (left, group I; right, group II) are shown. The correlation between *EGFR* mutation status and EGFR/MET expression classified into group I and group II was analyzed using Fisher's exact test in (**A**). Wilcoxon signed-rank sum tests were performed in (**B**–**D**). In box-and-whisker plots, the box spans from the first to the third quartile with a line at the median and the whiskers extend from the minimum to the maximum. Each dot represents an individual sample and connecting lines indicate paired before-after measurements for the same sample. NS: not significant; $^*P < 0.05$

effects are reportedly mediated by inhibition of HER2 signaling and Fc-dependent mechanisms, including ADCC and ADCP activity [6, 33]. Similarly, because both EGFR and MET signaling pathways are involved in immune suppression [13, 34, 35], their blockade by amivantamab may relieve immune suppression and promote immune activation. Consistently, in our cohort, amivantamab-induced immune activation correlated with EGFR phosphorylation status. In addition, amivantamab is engineered to enhance ADCC and ADCP activity through reduced fucosylation [15, 36, 37], and we also observed DC maturation following amivantamab treatment although its correlation with EGFR or MET expression was less clear than that observed for T cells. Because tumor-infiltrating natural killer (NK) cells are generally small, their potential contribution was not evaluated in our cohort, highlighting the need for further investigation.

Although amivantamab is currently approved only for *EGFR*-mutant NSCLC, previous reports have suggested efficacy in *EGFR*-WT tumors [38]. Consistently, we observed that amivantamab promoted TIL activation in tumors with high EGFR or MET expression, regardless of mutation status. This finding is aligned with its pharmacological mechanism and underscores the potential of EGFR/MET expression as biomarkers for amivantamab-induced antitumor immunity. Indeed, several ongoing clinical trials are already including *EGFR*-WT cohorts [39–41], and our results support such trial designs. Importantly, these findings suggest that tumors with high EGFR/MET expression may benefit from amivantamab even without *EGFR* mutations.

From a methodological perspective, mouse models frequently fail to predict human clinical outcomes, and peripheral blood–based assays cannot recapitulate the chronic antigen exposure and suppressive cues that shape TIL function in the TME. In contrast, our fresh TIL assay preserves the viability of tumor and immune cells, enabling real-time assessment of therapeutic antibody activity within a physiologically relevant context. Unlike assays relying on TIL expansion, organoid generation, or cryopreservation, our approach is very simple, minimizing artifacts and reflecting

in vivo conditions more faithfully [42]. Voabil et al. also developed a similar tumor fragment platform, showing the advantages [43]. This approach not only provides a more faithful reflection of the in vivo TME but also holds broader implications for drug development. Moreover, with further development, this approach could evolve into a patient-specific tool, enabling clinicians to project therapeutic responses to treatment.

In summary, this study provides the first ex vivo human evidence that amivantamab activates TILs together with the unique strengths of our fresh TIL assay. These effects extended beyond *EGFR*-mutant tumors to *EGFR*-WT tumors with high EGFR or MET expression, underscoring the potential of EGFR/MET expression as biomarkers for amivantamab-induced antitumor immunity. Further basic and translational research is warranted to validate these findings and guide future clinical applications.

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Author contributions RY: Data curation, formal analysis, investigation, methodology, writing—original draft. FM: Data curation, formal analysis, investigation, methodology. KY: Formal analysis and investigation. JN: Conceptualization, data curation, formal analysis, methodology. HW: Data curation, formal analysis, investigation. YU: Methodology. K. Suzawa: Investigation. K. Shien: Investigation. ST: Supervision. TI: Data curation, formal analysis, investigation, visualization, methodology, writing—original draft, writing—review and editing. YT: Conceptualization, data curation, funding acquisition, methodology, writing—original draft, project administration, writing-review and editing.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information. Additional data are accessible from the corresponding author upon reasonable request.

Declarations

Conflict of interest S. Toyooka received research grants from TAIHO PHARMA, Eli Lilly, Chugai Pharmaceutical and Astellas Pharma, and honoraria from TAIHO PHARMA, Eli Lilly, Chugai Pharmaceutical, GUARDANT, AstraZeneca, Illumina, MERCK, CSL Behring, Nippon Kayaku, Daiichi-Sankyo, Ono Pharmaceutical, Medtronic, Ziosoft, NOVARTIS, Sysmex and Riken Genesis outside of this study. Y. Togashi received a research grant from Janssen Pharmaceutical K.K. for this study. Y. Togashi received research grants from AstraZeneca, TAIHO PHARMA, Takeda, Chugai Pharmaceutical, Daiichi-Sankyo, and KORTUC, and honoraria from Ono Pharmaceutical, Bristol-Myers Squibb, AstraZeneca, Chugai Pharmaceutical, Eisai and MSD outside of this study. The other authors declare that they have no research support relevant to financial competing interests.

Consent to publish All patients provided written informed consent.

Consent to participate All patients provided written informed consent.

Ethical Approval.
The study protocol was approved by the Institutional Review Board of Okayama University.

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