Received: 16 June 2022 | Revised: 20 September 2022 | Accepted: 22 September 2022

DOI: 10.1111/cas.15608

Cancer Science WILEY

ORIGINAL ARTICLE

# High levels of AXL expression in untreated *EGFR*-mutated non-small cell lung cancer negatively impacts the use of osimertinib

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## Abstract

For non-small cell lung cancer (NSCLC) patients with epidermal growth factor receptor (*EGFR*) mutations, the initial therapeutic interventions will have crucial impacts

**Abbreviations:** 19del, exon 19 deletion; CD274, cluster of differentiation 274; CI, confidence interval; CR, complete response; EGFR, epidermal growth factor receptor; ICI, immune checkpoint inhibitor; L858R, exon 21 L858R point mutation; NSCLC, non-small-cell lung cancer; ORR, objective response rate; OS, overall survival; PD, progressive disease; PD-L1, programmed death-ligand 1; PFS, progression-free survival; PR, partial response; PS, performance status; RB1, RB transcriptional corepressor 1; SD, stable disease; TCGA, The Cancer Genome Atlas; TKI, tyrosine kinase inhibitor; TP53, tumor protein p53.

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**Funding information**
Japan Society for the Promotion of Science, Grant/Award Number: 19K08608 and 22K15588; Joint Research with the Cancer Research Institute of Kanazawa University; Ono Pharmaceutical Company Limited; Takeda Science Foundation

on their clinical outcomes. Drug tolerant factors reportedly have an impact on EGFR-tyrosine kinase inhibitor sensitivity. This prospective study investigated the impacts of drug tolerant-related protein expression in tumors based on the efficacy of osimertinib in the first-setting of *EGFR*-mutated advanced NSCLC patients. A total of 92 patients with *EGFR*-mutated advanced or postoperative recurrent NSCLC were analyzed and treated with osimertinib at 14 institutions in Japan. AXL, p53, and programmed death-ligand 1 (PD-L1) expression in patient tumors was determined using immunohistochemistry. The AXL signaling pathway was investigated using a cell line-based assay and AXL-related gene expression in The Cancer Genome Atlas (TCGA) database. High levels of AXL and positive-p53 expression were detected in 26.1% and 53.3% of the pretreatment *EGFR*-mutated NSCLC tumors, respectively. High AXL expression levels were significantly associated with a shorter progression-free survival compared with low AXL expression levels, irrespective of the *EGFR* activating mutation status ($p = 0.026$). Cell line-based assays indicated that the overexpression of AXL protein accelerated PD-L1 expression, which induced insensitivity to osimertinib. In the TCGA database, AXL RNA levels were positively correlated with PD-L1 expression in the lung adenocarcinoma cohort. The results show that high AXL expression levels in tumors impact clinical predictions when using osimertinib to treat *EGFR*-mutated NSCLC patients. Trial Registration: UMIN000043942.

**KEYWORDS**
AXL, *EGFR* mutation, lung cancer, osimertinib, PD-L1

## 1 | INTRODUCTION

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) can provide effective treatment for non-small cell lung cancer (NSCLC) patients harboring *EGFR* mutations, such as the exon 19 deletion (19del) and exon 21 L858R point mutation (L858R).[1–3] Based on the results of several clinical trials, osimertinib was approved for use with untreated *EGFR*-mutated advanced NSCLC patients and *EGFR*-T790M mutated NSCLC patients after the resistance of the initial EGFR-TKIs in several countries.[4–7] However, almost all patients ultimately develop acquired resistance to osimertinib and approximately 30% of *EGFR*-mutated NSCLC patients experienced early relapse within 12 months.[4] This indicates that the initial therapeutic intervention plays a crucial role in the survival of NSCLC patients with *EGFR* mutations.

Recently, several concomitant gene alterations, such as tumor protein p53 (TP53) mutations, RB transcriptional corepressor 1 (RB1) mutations, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha mutations, phosphatase and tensin homolog alterations, and other driver oncogenes, were reported as predictive negative factors for initial EGFR-TKI treatment in *EGFR*-mutated NSCLC patients.[8,9] Of these, p53 mutations are the most common gene alterations in *EGFR*-mutated NSCLC patients, occurring in 55%–65% of cases. In a meta-analysis of *EGFR*-mutated NSCLC patients receiving first-line EGFR-TKIs, p53 comutations with activating *EGFR* mutations were identified as factors indicating a poorer prognosis.[10] Furthermore, *EGFR*-mutant NSCLC patients with co-occurring TP53 mutations had shorter progression-free survival (PFS) under initial EGFR-TKI treatment than those without TP53 mutations.[11] Thus, loss of function due to TP53 alterations was related to poor clinical outcomes for *EGFR*-mutant NSCLC patients treated with first- and second-generation EGFR-TKIs. However, it remains unclear whether p53 protein expression is associated with the efficacy of osimertinib in *EGFR*-mutated NSCLC patients.

The coexistence of drug tolerant factors, which are reversible nongenetic mechanisms, is reportedly related to poor clinical outcomes in EGFR-TKI monotherapies for *EGFR*-mutated NSCLC tumors.[12,13] Of them, overexpression of AXL, a tyrosine kinase receptor belonging to the TAM family, is correlated with poor prognosis in several cancers.[14–16] The activation of AXL signaling in cancers induced acquired resistance to chemotherapeutic agents and targeted molecular therapy drugs, including EGFR-TKIs for *EGFR*-mutated NSCLC.[14,17–19] Our preclinical research identified the pivotal role of AXL activation in the intrinsic resistance to osimertinib and the emergence of osimertinib-tolerant cells in *EGFR*-mutated NSCLC cells.[12,20] However, clinical evidence of the impacts of AXL expression on osimertinib treatments in *EGFR*-mutated NSCLC patients is still lacking.

Tumor programmed death-ligand 1 (PD-L1), also known as cluster of differentiation 274 (CD274), is utilized as a biomarker for immunotherapy-containing treatments in NSCLC patients.[21] In contrast, immune-checkpoint inhibitors (ICIs) are generally less effective in NSCLC patients harboring driver mutations, such as *EGFR* and *ALK*.[22,23] Elevated PD-L1 expression in tumors is reportedly a

negative predictive factor for EGFR-TKI outcomes in NSCLC patients with *EGFR* mutations.[24–26]

In this prospective study, we investigated the effect of drug tolerant-related protein expression in tumors on osimertinib efficacy in the first-setting for *EGFR*-mutated advanced NSCLC patients.

## 2 | MATERIALS AND METHODS

Details of the materials and methods are available in Appendix S1.

## 3 | RESULTS

### 3.1 | Patient characteristics

Ninety-two untreated *EGFR*-mutated NSCLC patients, from whom tissue blocks were obtained prior to the osimertinib treatment, were enrolled. Patient characteristics are summarized in Table 1. The median age was 71.0 years (range, 35.0–87.0); 65 patients (70.7%) were female, 77 patients (83.7%) indicated performance status (PS) of 0 and 1, and 56 patients (60.9%) were nonsmokers. The most prevalent history of disease included incidence of adenocarcinoma (95.7%); 17 patients (18.5%) had relapse after surgery. The *EGFR* mutation status of the patients indicated that 47 (51.1%) had 19del and 43 (46.7%) had L858R. The median follow-up time was 26.4 months (range, 1.3–35.0 months).

### 3.2 | Evaluation of AXL and p53 expression in *EGFR*-mutated NSCLC tumors

We evaluated AXL and p53 expression levels in the 92 *EGFR*-mutant NSCLC specimens obtained from the 92 patients. Strong (3+), intermediate (2+), weak (1+), and negative (0) tumor AXL expression was observed in 24 (26.1%), 29 (31.5%), 29 (31.5%), and 10 (10.9%) patients, respectively. We defined tumor AXL expression 3+ as the AXL-high group (26.1%), and tumor AXL expression 2+, 1+, and 0 as the AXL-low group (73.9%), and these were utilized in the subsequent study. Positive and negative p53 expression was observed in 49 (53.3%) and 43 (46.7%) patients, respectively (Figures 1A and S1). The site for biopsy and the method of analysis at diagnosis did not cause any significant differences in AXL or p53 expression (Table S1). Moreover, there were no significant differences between the AXL-high and -low groups in clinicopathologic features, except clinical staging (Table S2).

### 3.3 | Impacts of AXL and p53 expression on clinical outcomes of osimertinib treatment

We examined the impacts of AXL and p53 expression levels in tumors based on the clinical outcomes of initial osimertinib

**TABLE 1** Characteristics of patients with non-small cell lung cancer ($n = 92$)

| | $n = 92$ |
|---|---|
| Median age, years (range) | 71.0 (35.0–87.0) |
| Age categorization, years; *n* (%) | |
| <75 | 60 (65.2) |
| ≥75 | 32 (34.8) |
| Sex, *n* (%) | |
| Male | 27 (29.3) |
| Female | 65 (70.7) |
| PS, *n* (%) | |
| 0, 1 | 77 (83.7) |
| 2, 3 | 15 (16.3) |
| Stage, *n* (%) | |
| III, IV | 75 (81.5) |
| Postoperative relapse | 17 (18.5) |
| Brain metastasis, *n* (%) | |
| Positive | 27 (29.3) |
| Negative | 65 (70.7) |
| Histology, *n* (%) | |
| Adenocarcinoma | 88 (95.7) |
| Others | 4 (4.3) |
| *EGFR* mutation, *n* (%) | |
| 19del | 47 (51.1) |
| L858R | 43 (46.7) |
| Others | 2 (2.2) |
| Smoking status, *n* (%) | |
| Current or former | 36 (39.1) |
| Never | 56 (60.9) |
| Response, *n* (%) | |
| CR | 3 (3.3) |
| PR | 65 (70.7) |
| SD | 15 (16.3) |
| PD | 4 (4.3) |
| NE | 5 (5.4) |
| ORR (95% CI) | 78.2% (68.0%–86.3%) |
| DCR (95% CI) | 95.4% (88.6%–98.7%) |
| IHC for AXL, *n* (%) | |
| 3+ | 24 (26.1) |
| 2+ | 29 (31.5) |
| 1+ | 29 (31.5) |
| 0 | 10 (10.9) |
| IHC for p53, *n* (%) | |
| Positive | 49 (53.3) |
| Negative | 43 (46.7) |
| PD-L1 TPS, *n* (%) | |
| ≥50% | 13 (14.1) |

TABLE 1 (Continued)

| | n = 92 |
|---|---|
| 1%–49% | 24 (26.1) |
| <1% | 27 (29.3) |
| Unknown | 28 (30.4) |

Abbreviations: 19del, exon 19 deletion; CI, confidence interval; CR, complete response; DCR, disease control rate; IHC, immunohistochemistry; L858R, exon 21 L858R point mutation; NE, not evaluable; ORR, objective response rate; PD, progressive disease; PD-L1, programmed death-ligand 1; PR, partial response; PS, performance status; SD, stable disease; TPS, tumor proportion score.

treatments for NSCLC patients with an *EGFR* mutation. In all *EGFR*-mutated NSCLC patients, the objective response rate (ORR) of the osimertinib treatment was 78.4% (Figure S2). The ORR value of the osimertinib treatment in patients with low AXL expression levels was 82.8%, whereas for those with high AXL expression it was 66.7% ($p = 0.145$). There was no remarkable difference in the ORR with osimertinib treatment, regardless of the p53 expression level (Figure S2). With the highest percentage change for the osimertinib treatment, there was no significant difference in the tumors of patients with high and low levels of AXL or positive and negative p53 (Figure S3).

Of the 90 patients evaluable for primary resistance, 12 *EGFR*-mutant NSCLC patients were classified as having a primary resistance to the osimertinib treatment. The frequency of the primary resistance to the osimertinib treatment tended to be higher in patients with high AXL levels than in those with low AXL levels (25.0% vs. 8.8%, respectively, $p = 0.073$). Patients who were p53-positive also tended to have an increased frequency of primary resistance to osimertinib, when compared to those who were p53-negative (18.4% vs. 7.3%, respectively, $p = 0.212$) (Figure S2).

The median PFS with osimertinib was 17.4 months in all *EGFR*-mutated NSCLC patients (Figure S2). Furthermore, the median PFS with osimertinib was significantly shorter in patients with high levels of AXL expression than in those with low levels (8.9 months [95% CI, 6.1–17.4 months]; and 21.5 months [95% CI, 15.4–24.0 months], respectively, $p = 0.026$). Patients who were p53-positive tended to have shorter PFS with osimertinib than those who were p53-negative (14.7 months vs. 21.5 months, respectively, $p = 0.144$) (Figure 1B). Subgroup analyses showed that the median PFS of the osimertinib treatment was significantly shorter in patients with a poor PS as well as high AXL and high PD-L1 expression (Table 2, Figure S4).

The median overall survival (OS) with osimertinib was not reached by any *EGFR*-mutated NSCLC patient (Figure S2). For those who did, the median OS with osimertinib was not significantly affected by AXL expression levels in tumors (HR 1.60; 95% CI, 0.67–3.84; $p = 0.290$). Meanwhile, patients who were p53-positive had significantly shorter PFS with osimertinib than those who were p53-negative (HR 2.22; 95% CI, 1.00–4.91; $p = 0.044$) (Figure 1C).

In contrast, for p53-positive patients in the AXL-high group, there was no significant difference in clinical outcomes with osimertinib treatment (Figure S5).

These observations indicate that AXL and p53 protein expression in pretreatment tumors could potentially be useful for the prediction of *EGFR*-mutated NSCLC patients with poor outcomes under osimertinib treatment.

### 3.4 | AXL expression impacts the clinical outcomes of osimertinib treatment according to *EGFR* mutation status

We investigated the correlations between AXL expression and the clinicopathologic features of *EGFR*-mutant NSCLC patients with 19del and L858R mutations. There was no significant difference in the incidence of high AXL expression levels or clinicopathologic features between those with 19del and L858R mutations (Figure S6, Table S3). The ORR value for the osimertinib treatment in patients with the 19del mutation was higher than in those with the L858R mutation (89.1% vs. 70.0%, respectively, $p = 0.032$). In addition, the ORR value for osimertinib treatment in patients with the 19del mutation with low AXL expression levels was relatively higher than in those with high AXL expression levels (94.3% vs. 72.7%, respectively, $p = 0.080$). For patients with the L858R mutation, there was no remarkable difference in the ORR value with osimertinib treatment, regardless of AXL expression level (71.4% vs. 66.7%, $p = 1.000$) (Figure S6). The median PFS with osimertinib tended to be prolonged in patients with the 19del mutation compared with those with the L858R mutation (21.8 months vs. 14.7 months, $p = 0.112$) (Figure 2A). In contrast, there was no significant difference in OS between the two groups ($p = 0.992$) (Figure 2B).

We further investigated the impact of AXL expression on the outcome of osimertinib treatment according to the *EGFR* mutation status. The median PFS with osimertinib when ranked from highest to lowest in patients was as follows: 19del mutation plus low AXL expression; L858R mutation plus low AXL expression; and high AXL expression. Although the groups with high AXL expression levels tended to have the worst PFS among those examined, there was no remarkable difference in the PFS between those with the 19del or L858R mutation or high AXL expression levels (10.1 months [95% CI, 4.4 months–not reached] and 8.9 months [95% CI, 3.0–23.2 months], respectively) (Figure 2C). In contrast, there was no significant difference in OS among these groups. Of them, patients with the 19del mutation plus high AXL expression levels tended to have relatively a shorter OS (Figure 2D).

### 3.5 | AXL expression level is positively related to PD-L1 protein in NSCLC tumors

Our previous data indicated that high PD-L1 expression levels are associated with shorter PFS with osimertinib in untreated advanced NSCLC patients harboring an *EGFR* mutation. [26] We further investigated the high levels of PD-L1 in relation to AXL and p53 for the *EGFR*-mutated NSCLC patients. Interestingly, the

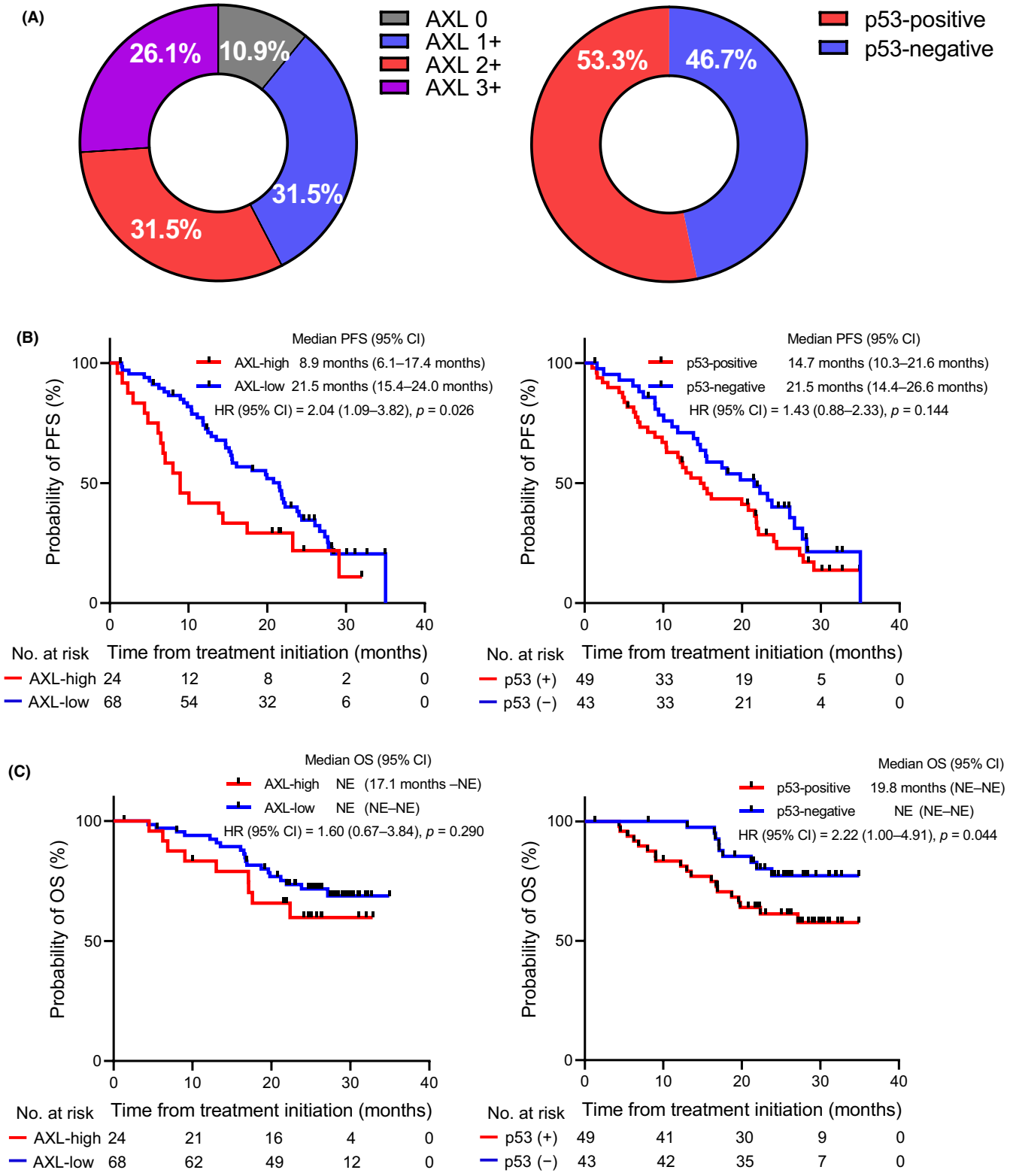



**FIGURE 1** Evaluation of AXL and p53 expression in relation to the clinical outcomes of osimertinib treatment in epidermal growth factor receptor (*EGFR*)-mutated non-small cell lung cancer (NSCLC) tumors. (A) AXL and p53 expression levels in tumors from patients with *EGFR*-mutated NSCLC. (B,C) Kaplan–Meier survival curves for (B) progression-free survival (PFS) and (C) overall survival (OS) of *EGFR*-mutated NSCLC patients receiving osimertinib treatment between the low and high AXL expression groups, and negative and positive p53 expression groups. CI, confidence interval; HR, hazard ratio; NE, not evaluable

**TABLE 2** Univariate analysis for progression-free survival (PFS) among patients with non-small cell lung cancer ($n = 92$)

| | No. of patients | Median PFS, months (95% CI) | *p* value |
|---|---|---|---|
| Age categorization, years | | | |
| <75 | 60 | 17.4 (12.5–22.8) | 0.656 |
| ≥75 | 32 | 15.6 (11.8–23.2) | |
| Sex | | | |
| Male | 27 | 16.5 (13.5–23.2) | 0.857 |
| Female | 65 | 18.1 (11.9–23.8) | |
| PS | | | |
| 0, 1 | 77 | 21.6 (14.7–24.0) | <0.001 |
| 2, 3 | 15 | 8.9 (2.4–15.4) | |
| Disease stage | | | |
| III, IV | 75 | 15.6 (11.9–21.8) | 0.610 |
| Postoperative relapse | 17 | 23.8 (13.5–26.6) | |
| Brain metastasis | | | |
| Positive | 27 | 12.9 (8.0–18.1) | 0.058 |
| Negative | 65 | 21.5 (14.4–24.4) | |
| Histology | | | |
| Adenocarcinoma | 88 | 18.1 (14.4–22.1) | 0.444 |
| Others | 4 | 4.0 (1.5–NE) | |
| *EGFR* mutation | | | |
| 19del | 47 | 21.8 (15.4–26.6) | <0.001 |
| L858R | 43 | 14.7 (9.9–21.6) | |
| Others | 2 | 3.0 (1.0–NE) | |
| Smoking status | | | |
| Current or former smoker | 36 | 13.8 (9.0–21.6) | 0.169 |
| Never smoker | 56 | 21.5 (14.7–24.4) | |
| IHC for AXL | | | |
| High | 24 | 8.9 (6.1–17.4) | 0.026 |
| Low | 68 | 21.5 (15.4–24.0) | |
| IHC for p53 | | | |
| Positive | 49 | 14.7 (10.3–21.6) | 0.144 |
| Negative | 43 | 21.5 (14.4–26.6) | |
| PD-L1 TPS | | | |
| ≥50% | 13 | 6.5 (2.4–14.4) | 0.005 |
| <50% | 51 | 17.4 (12.9–21.8) | |
| Unknown | 28 | 26.1 (14.7–29.1) | |

Abbreviations: 19del, exon 19 deletion; CI, confidence interval; IHC, immunohistochemistry; L858R, exon21 L858R point mutation; NE, not evaluable; PD-L1, programmed death-ligand 1; PFS, progression-free survival; PS, performance status; TPS, tumor proportion score.

frequency of patients with high PD-L1 levels was significantly higher in patients with high AXL levels than in those with low AXL levels (45.0% vs. 9.1%, $p = 0.002$), but not p53 (18.9% vs. 22.2%, $p = 0.763$) (Figure 3A). Moreover, tumor AXL expression was significantly associated with PD-L1 expression levels in patients with *EGFR* mutations ($r = 0.4126$, $p < 0.001$). However, tumor p53 expression did not correlate with PD-L1 expression ($r = 0.0048$, $p = 0.970$) (Figure 3B). The Cancer Genome Atlas (TCGA) database revealed that AXL mRNA expression positively correlated with CD274 (PD-L1) in patients with lung adenocarcinoma ($r = 0.4701$, $p < 0.0001$) (Figure 3C). Additional analysis showed that the combination of AXL and PD-L1 expression in tumors was substantially correlated with PFS, which indicated the positive effect of their combined use. For the prognostic factors in *EGFR*-mutated NSCLC patients receiving osimertinib monotherapy (AXL-low vs. AXL-high with PD-L1-low, $p = 0.376$, or AXL-high with PD-L1-high, $p = 0.028$), there was no significant difference in the OS among the three groups (Figure 3D,E).

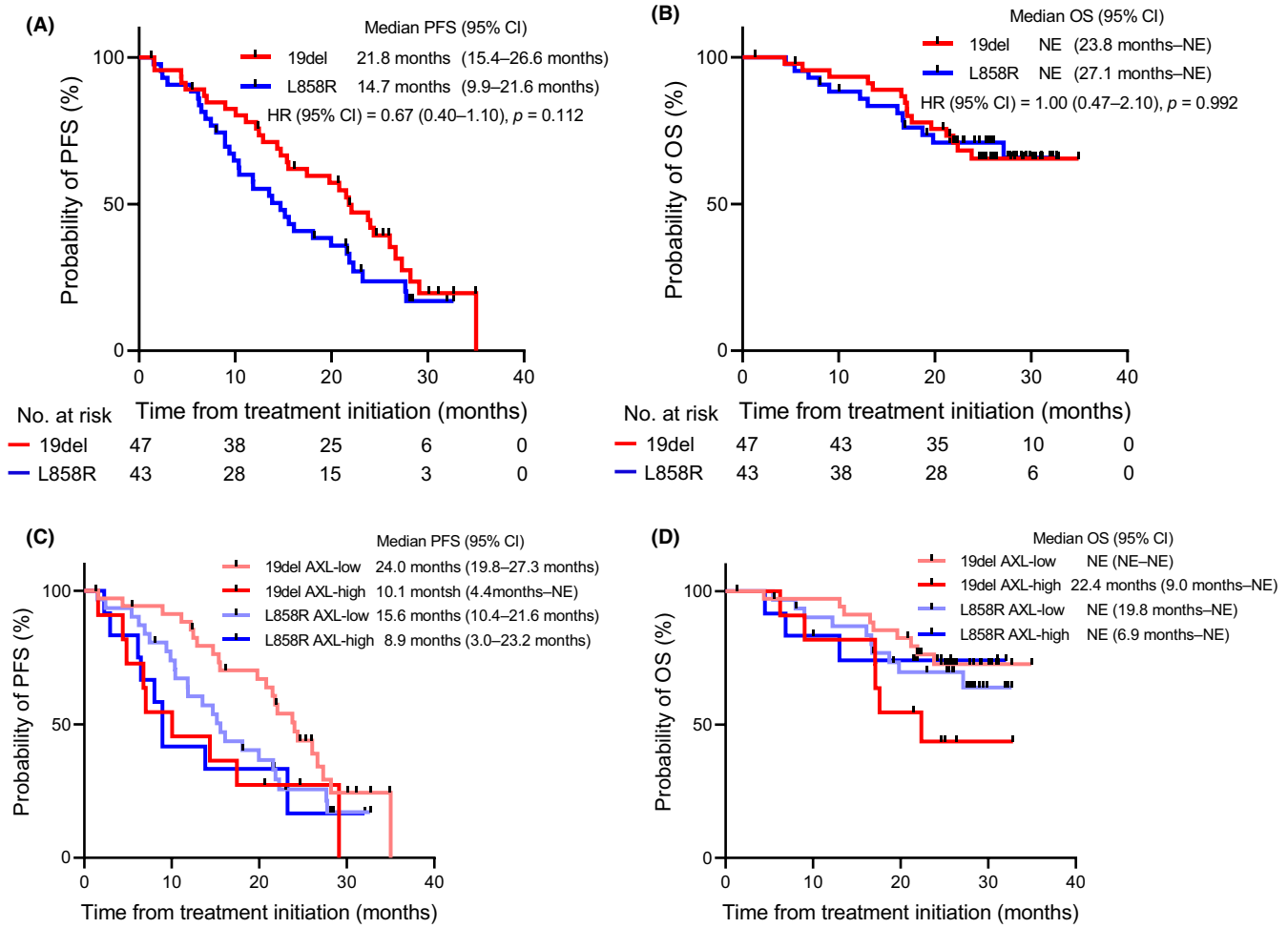



**FIGURE 2** Evaluation of AXL expression in relation to the clinical outcomes of osimertinib treatment according to epidermal growth factor receptor (*EGFR*) mutation status. (A,B) Kaplan–Meier survival curves for (A) progression-free survival (PFS) and (B) overall survival OS of *EGFR*-mutated non-small cell lung cancer (NSCLC) patients with exon 19 deletion (19del) and exon 21 L858R point mutation (L858R) mutations receiving osimertinib treatment. (C,D) Kaplan–Meier survival curves for (C) PFS and (D) OS of *EGFR*-mutated NSCLC patients receiving osimertinib treatment according to *EGFR* mutation status and AXL expression levels. CI, confidence interval; HR, hazard ratio; NE, not evaluable

These observations indicated a positive correlation between the tumor expression of AXL and PD-L1, which are related to the poor outcomes of osimertinib monotherapy, in *EGFR*-mutated NSCLC patients treated with osimertinib.

### 3.6 | AXL activation promoted osimertinib resistance and was involved in cMyc and PD-L1 expression in EGFR-mutated in vitro models

To further elucidate the underlying mechanisms of AXL-induced osimertinib resistance, we undertook a pathway analysis using AXL activation in Ba/F3 cells. Cell line-based analysis showed that AXL overexpression promoted resistance to osimertinib in Ba/F3 cells harboring both 19del and L858R mutations in *EGFR* (Figure 4A). AXL overexpression increased the expression levels of the transcription factors cMyc and PD-L1 in Ba/F3 cells harboring both 19del and L858R mutations (Figure 4B). Treatment with osimertinib had little impact on cMyc and PD-L1 expression in Ba/F3 cells harboring 19del and L858R mutations, regardless of AXL overexpression (Figure 4C). Specific siRNA-mediated AXL knockdown restored cMyc and PD-L1 expression in *EGFR*-mutated cells, whereas cMyc knockdown restored the expression of PD-L1, but not AXL, in *EGFR*-mutated cells when AXL was overexpressed. This indicated that AXL activates the cMyc-PD-L1 axis (Figure 4D). Moreover, a cell growth assay showed that cMyc or PD-L1 knockdown marginally affected osimertinib sensitivity in AXL-overexpressing *EGFR*-mutated cells (Figure 4E). These findings indicated that AXL activation promotes insensitivity to osimertinib and is involved in the expression of cMyc and PD-L1, which are downstream molecules in cells harboring *EGFR* activating mutations. The TCGA data for patients with lung adenocarcinoma revealed that tumors with high AXL mRNA expression showed remarkably increased CD274 mRNA levels compared with those with intermediate and low AXL mRNA expression ($p < 0.0001$), but there was no significant correlation in mRNA levels between MYC and AXL (Figures 4F,G and S7).

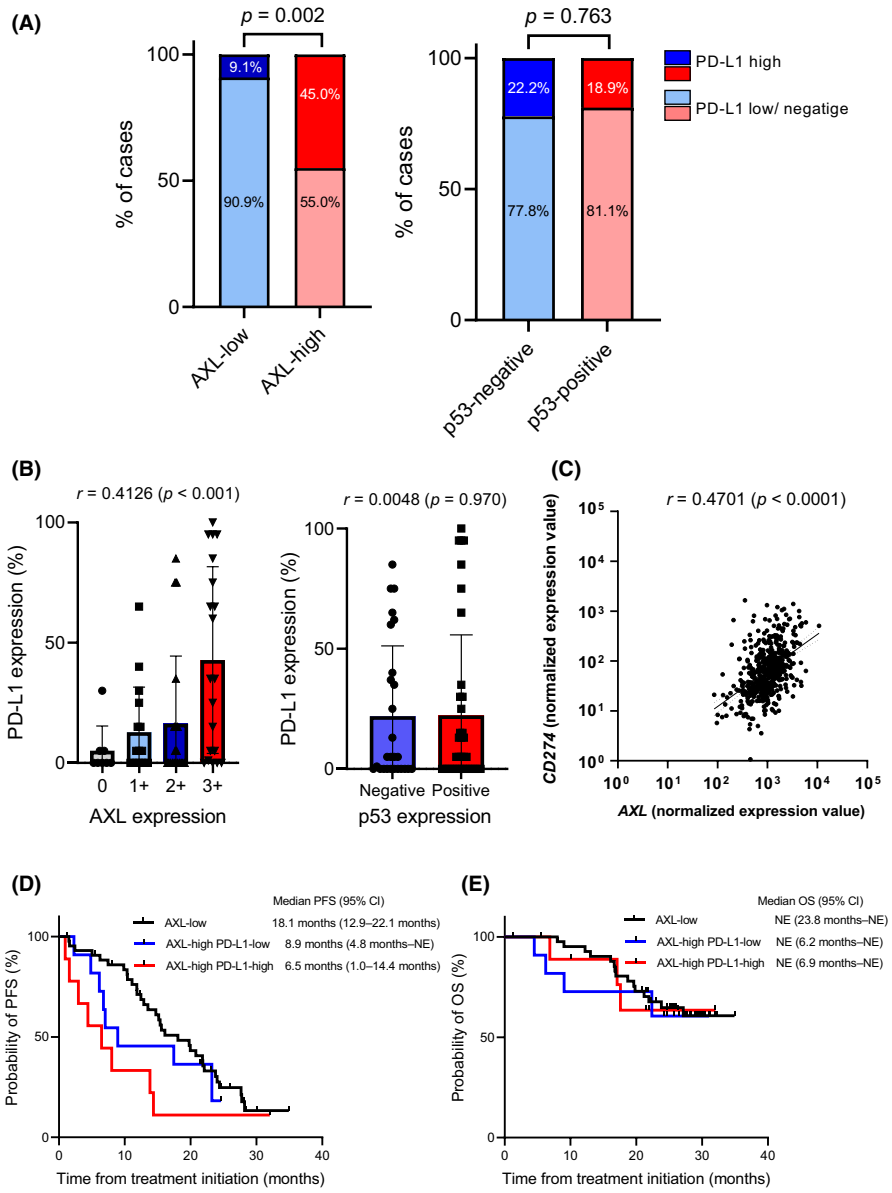
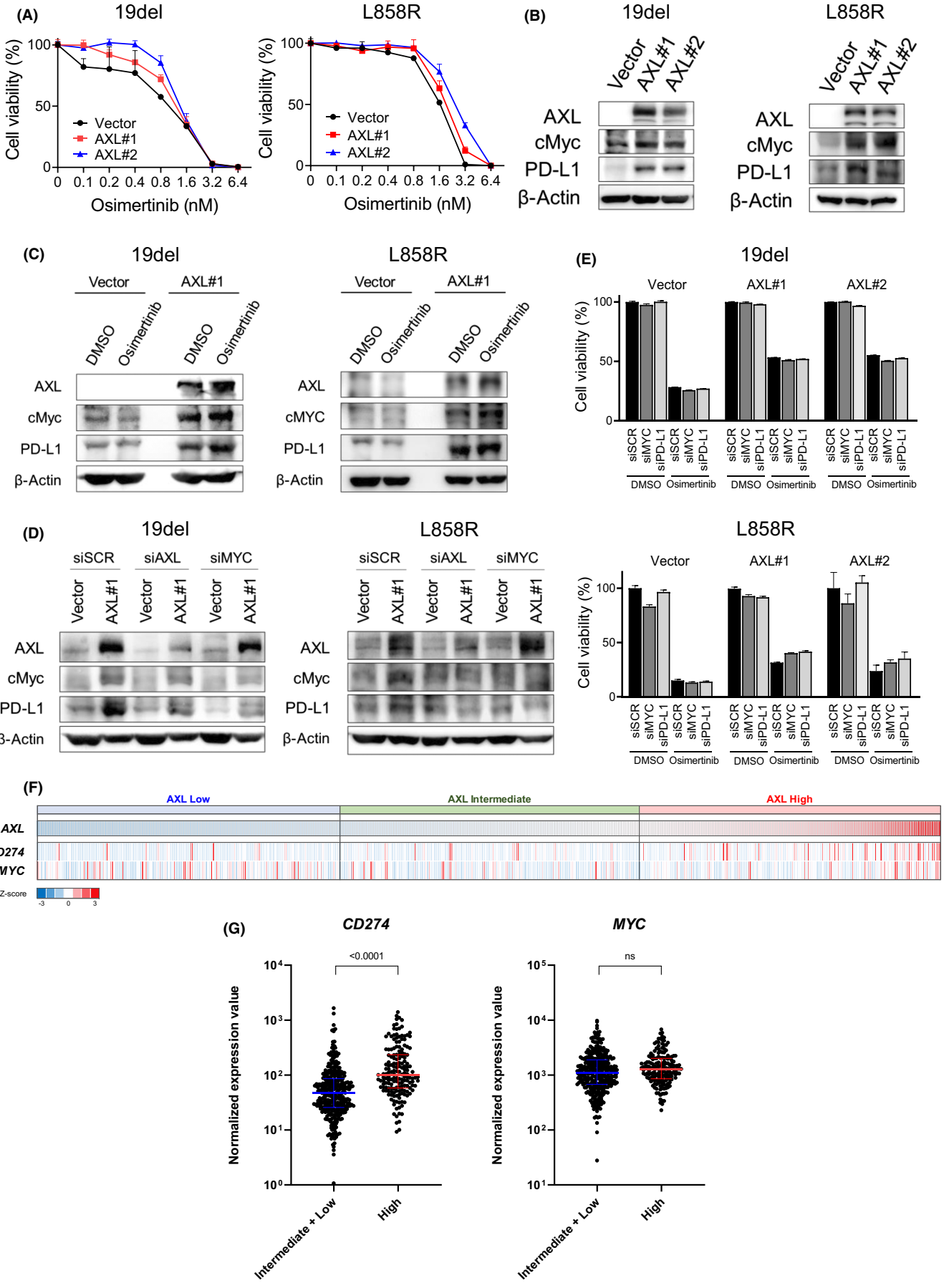



**FIGURE 3** AXL expression is positively related to programmed death-ligand 1 (PD-L1) protein expression in non-small cell lung cancer (NSCLC) tumors. (A) Distribution of tumor PD-L1 expression (% of cases) in epidermal growth factor receptor (*EGFR*)-mutated NSCLC patients according to their AXL and p53 expression levels. (B) Distribution of tumor PD-L1 expression (% of positive cells) in *EGFR*-mutated NSCLC patients according to the AXL and p53 expression levels. (C) Correlation between the log10-transformed AXL and CD274 expression data, using the RNA sequencing data of The Cancer Genome Atlas datasets. Positive correlation was determined using the Pearson correlation ($r = 0.4701$, $p < 0.0001$). (D,E) Kaplan–Meier survival curves for (D) progression-free survival (PFS) and (E) overall survival (OS) of *EGFR*-mutated NSCLC patients receiving osimertinib treatment among the AXL-low, AXL-high–PD-L1-low, and AXL-high-PD-L1-high expression groups. CI, confidence interval, NE, not evaluable

**FIGURE 4** AXL activation promoted osimertinib resistance and was involved in cMyc and programmed death-ligand 1 (PD-L1) expression in epidermal growth factor receptor (*EGFR*)-mutated non-small cell lung cancer (NSCLC). (A) After the exon 19 deletion (19del) and exon 21 L858R point mutation (L858R) mutations were introduced into *EGFR*, Ba/F3 cells transfected with the pWPXL plasmid expressing either empty vector (Vec) or human AXL (AXL-wt) were incubated with osimertinib for 72 h and the cell viability was determined using WST-8 assays. (B) The cells were lysed, and the indicated proteins detected by western blot analysis. (C) Ba/F3 cells harboring the 19del and L858R mutations were further transfected with either Vec or AXL-wt and incubated with or without osimertinib (1.0 nmol/L) for 48 h, lysed, and the indicated proteins were detected by western blotting. (D) Nonspecific siRNA was used as a control, specific siRNAs for AXL and MYC siRNAs were introduced into Ba/F3 cells harboring the 19del and L858R mutations, and the indicated proteins were detected by western blotting. (E) The indicated siRNAs were introduced into Ba/F3 cells transfected harboring the 19del and L858R mutations. After 24 h, the cells were incubated with or without osimertinib (1.0 nmol/L) for 72 h and cell viability was determined using WST-8 assays. (F) Heatmap indicating the expression levels of AXL, CD274, and MYC across 510 patients according to their AXL expression level classification. Patients were sorted in descending order of their AXL expression data, which were converted to a *z*-score. (G) Expression data from The Cancer Genome Atlas were used to compare the expression levels of CD274 and MYC between the groups with high AXL expression levels and the groups with intermediate and low AXL expression levels. Two-sided Welch's *t*-test was used for the statistical evaluation. Tumors expressing high levels of AXL mRNA had significantly increased levels of CD274 mRNA when compared with the others ($p < 0.0001$), but there was no significant correlation for the MYC or AXL mRNA levels ($p < 0.4562$)


(A)
19del
L858R
Cell viability (%)
Vector
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Osimertinib (nM)
(B)
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AXL#2
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cMyc
PD-L1
β-Actin
(C)
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Osimertinib
AXL
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PD-L1
β-Actin
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Cell viability (%)
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siPD-L1
DMSO
Osimertinib
(F)
AXL Low
AXL Intermediate
AXL High
AXL
CD274
MYC
Z-score
(G)
CD274
MYC
Normalized expression value
<0.0001
ns
Intermediate + Low
High

## 4 | DISCUSSION

In *EGFR*-mutated NSCLC patients, intervention with osimertinib promotes tumor evolution and induces acquired resistance, following which there is no optimal therapeutic strategy. Several therapeutic strategies that are potentially followed by therapy with osimertinib have been approved in several countries as first-line treatments for *EGFR*-mutated NSCLC patients, including combination with an antiangiogenesis agent or chemotherapy.[27,28] Therefore, predictive factors to detect nonresponders to osimertinib monotherapy are needed to ensure that the most promising initial therapeutic strategy for *EGFR*-mutated NSCLC patients can be selected.

Our preclinical study revealed the AXL activation in response to osimertinib treatment elicited an intrinsic resistance to osimertinib and the emergence of osimertinib-tolerant cells in *EGFR*-mutated NSCLC cells.[12] Moreover, a cell-line-derived tumor xenograft model showed that the combination of a novel AXL inhibitor ONO-7475 with osimertinib was effective for the initial treatment phase in AXL-overexpressing *EGFR*-mutated NSCLC cells.[29] These preclinical observations led to the initiation of a phase I clinical trial of osimertinib and ONO-7475 combination therapy for advanced *EGFR*-mutated NSCLC patients in Japan (jRCT2051210045).

In this prospective study, we validated the impact of tumor AXL and p53 protein expression levels, which are potentially related to the primary resistance to osimertinib and clinical outcomes of *EGFR*-mutated NSCLC tumors. We revealed that high AXL expression levels in pretreatment tumors were associated with a shorter PFS with osimertinib monotherapy in *EGFR*-mutated NSCLC patients. To the best of our knowledge, this is the first prospective study reporting the importance of high AXL expression levels in tumors as a clinically relevant predictive factor for osimertinib monotherapy in untreated *EGFR*-mutated NSCLC patients.

Expression of PD-L1 in tumors is used as a positive predictive biomarker for advanced NSCLC patients treated with ICIs.[21] In contrast, subpopulations with *EGFR* mutations tend to show a reduced response to PD-1/PD-L1 inhibitors, and tumor PD-L1 expression does not predict sensitivity to ICIs.[30] Several predictive biomarkers for clinical outcomes with immunotherapy in *EGFR*-mutated NSCLC patients have been reported, including the duration of response and a shorter response to a prior EGFR-TKI treatment.[22,31,32] Kunimasa et al. reported that changes in intratumoral heterogeneity influenced the therapeutic response of *EGFR*-mutated NSCLC tumors showing high PD-L1 expression to ICIs and first-generation EGFR-TKI erlotinib.[33] These observations suggest that the effectiveness of these therapies might be influenced by the resident tumor burdens with neither *EGFR* mutations nor PD-L1 expression for NSCLC tumors harboring *EGFR* mutations. Our observations showed that, when combined with the PD-L1 status, high AXL expression levels were associated with the worst PFS in the *EGFR*-mutated NSCLC tumors treated with osimertinib monotherapy. In addition, cell line-based analysis revealed that the underlying AXL signaling pathway, through the cMyc-PD-L1 axis, induces osimertinib resistance in Ba/F3 cells harboring *EGFR* activating mutations. Notably, PD-L1 knockdown did not reverse AXL-induced resistance to osimertinib, which suggested that PD-L1 might not work as an effector for AXL activation but for resistance to osimertinib in *EGFR*-mutated cells. However, how AXL and PD-L1 affect the association of clinical outcomes during osimertinib treatment remains unknown. Further evidence is required to understand the impact of tumor PD-L1 expression and to determine whether AXL signal transduction in tumors is related to the outcomes of osimertinib for *EGFR*-mutated NSCLC patients.

Recently, much attention has been paid to the efficacy of osimertinib in the first-line setting and the clinical differences between 19del and L858R mutations in *EGFR* due to differences in their molecular structures and tumor heterogeneity.[34–36] Our current observations showed a superior PFS of osimertinib in NSCLC patients with the 19del mutation compared with those with the L858R mutation, consistent with previous reports. In addition, tumors with high AXL expression had a poor prognosis regardless of the type of *EGFR* mutation, and the difference in clinical outcomes between the AXL-low and -high groups was more remarkable for the 19del mutation than for the L858R mutation.

Comutations with TP53 tumor suppresser genes were reported as predictive biomarkers for shorter PFS in first-generation EGFR-TKIs for NSCLC patients with *EGFR* mutations.[11] Although we did not evaluate the correlation between the protein and gene expression of TP53, the expression of tumor p53 proteins was a prognostic factor for OS in patients treated with osimertinib. Thus, it is suggested that tumor p53 expression might have an influence on the clinical outcomes of *EGFR*-mutated NSCLC patients receiving osimertinib treatment. Among the mechanisms of acquired resistance to osimertinib, the frequency of histologic transformation to small-cell lung cancer has been reported to be approximately 5%, which is related to the gain of TP53 mutations.[11,37] A clinical trial of osimertinib plus carboplatin and etoposide is being carried out for *EGFR*-mutated NSCLC patients with TP53 and RB1 mutations (NCT03567642). Further investigations are thus required to validate whether enriched p53 proteins in tumors leads to small cell transformation after acquired resistance to osimertinib.

There were several limitations to this study. First, the enrolled cohort was limited to 92 cases. Second, the *EGFR* mutation status was detected using PCR analysis, which is limited in its ability to identify compound mutations. Finally, follow-up times were insufficient to evaluate the OS of untreated *EGFR*-mutated NSCLC patients. However, several novel findings were notable, and further large-cohort investigations are warranted to confirm the roles of pretreatment tumor AXL and PD-L1 expression levels in the clinical outcomes of osimertinib treatment.

In summary, we have uncovered the clinical impacts of tumor AXL and p53 expression levels in patients receiving osimertinib treatment for *EGFR*-mutated lung cancer. High levels of AXL and positive p53 expression were detected in 26.1% and 53.3% of the pretreatment *EGFR*-mutated NSCLC tumors, respectively. The high levels of AXL expression were associated with significantly shorter PFS with osimertinib than low levels of AXL expression, irrespective of *EGFR* activating mutation status. Our observations

revealed that high AXL and PD-L1 expression levels in pretreatment tumors were predictors of poor PFS with osimertinib. A cell line-based assay indicated that AXL protein overexpression accelerated PD-L1 expression and induced insensitivity to osimertinib. In the TCGA database, AXL RNA levels were positively correlated with PD-L1 expression in a lung adenocarcinoma cohort. Based on our observations, further clinical verifications are expected to confirm the relationship between high pretreatment AXL expression levels and reduced sensitivity to osimertinib monotherapy in *EGFR*-mutated NSCLC patients.

## AUTHOR CONTRIBUTIONS

Conceptualization: T. Yamada. Data curation: A. Yoshimura. Formal analysis: A. Yoshimura, T. Yamada, M. Serizawa, H. Uehara. Funding acquisition: A. Yoshimura, T. Yamada. Investigation: all authors. Methodology: A. Yoshimura, T. Yamada. Project administration: A. Yoshimura, T. Yamada. Resources: A. Yoshimura, Y. Okuma, A. Fukuda, S. Watanabe, N. Nishioka, T. Takeda, Y. Chihara, S. Takemoto, T. Harada, O. Hiranuma, Y. Shirai, T. Shukuya, A. Nishiyama, Y. Goto, S. Shiotsu, K. Kunimasa, K. Suda, T. Mitsudomi, S. Yano, H. Kenmotsu, T. Takahashi. Software: N/A. Supervision: T. Yamada, K. Takayama. Validation: T. Yamada. Visualization: N/A; Roles/Writing - original draft: A. Yoshimura, T. Yamada. Writing - review and editing: T. Yamada, K Takayama.

## ACKNOWLEDGMENTS

We greatly thank the patients, their families, and all investigators involved in this study. Additionally, we thank Editage for help with English language editing.

## FUNDING INFORMATION

This study was supported by research grants from Ono Pharmaceutical Company Limited (to T. Yamada), Takeda Science Foundation (to T. Yamada), Joint Research with the Cancer Research Institute of Kanazawa University (to A. Yoshimura), and grants from JSPS KAKENHI (Grant Number 19 K08608 [to T. Yamada] and 22K15588 [to A. Yoshimura]).

## DISCLOSURE

S. Yano and T. Mitsudomi are editorial board members of *Cancer Science*. T. Yamada received commercial research grants from Pfizer, Ono Pharmaceutical, Chugai Pharmaceutical, Janssen, and Takeda Pharmaceutical, and has received speaking honoraria from Eli Lilly. Y. Okuma received commercial research grants from AbbVie, G.K., and Chugai Pharmaceutical, and has received speaking honoraria from Astra Zenca, Chugai Phamaceutical, Eli Lilly, Boehringer Ingelheim, Ono Pharmaceutical, Pfizer, Taiho, and Takeda Pharmacuetical. T. Shukuya received commercial research grants from AstraZeneca, Chugai Pharmaceutical, Boehringer Ingelheim, Novartis, and MSD, and has received speaking honoraria from AstraZeneca, Chugai Pharmaceutical, Boehringer Ingelheim, Novartis, MSD, Taiho, Daiichi-Sankyo, Ono Pharmaceutical, Bristol-Myers Squibb, Nippon Kayaku, and Pfizer. S. Watanabe has received research grants from Boehringer Ingelheim and Chugai Pharma and personal fees from Eli Lilly, Novartis Pharma, Chugai Pharma, Boehringer Ingelheim, Ono Pharmaceutical, Taiho, Pfizer, AstraZeneca, Bristol-Myers, MSD, and Daiichi Sankyo. K. Suda received personal fees from AstraZeneca, Chugai Pharmaceutical, and Taiho. T. Mitsudomi received research grants from Boehringer-Ingelheim, Chugai Pharmaceutical, Ono Pharmaceutical/BMS, Taiho Pharmaceutical, MSD Bridge Biotherapeutics, and AstraZeneca, and personal fees from AstraZeneca, Chugai, MSD, Eli Lilly, Boehringer-Ingelheim, Daiichi-Sankyo, Merck, Novartis, Amgen, Taiho, Ono Pharmaceutical/BMA, Daiichi-Sankyo, Janssen, Boehringer-Ingelheim, Takeda, Pfizer, Bayer, Invitae, and Thermo Fisher. S. Yano obtained commercial research grants from Chugai Pharmaceutical and Boehringer-Ingelheim, and has received speaking honoraria from Chugai Pharmaceutical, Boehringer-Ingelheim, Novartis, and Pfizer. H. Kenmotsu obtained commercial research grants from AstraZeneca, Chugai Pharmaceutical, Eli Lilly, LOXO Oncology, Novartis Pharma, and Ono Pharmaceutical, and has received speaking honoraria from Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharmaceutical, Daiichi-Sankyo, Eli Lilly, Kyowa Hakko Kirin, Merk, MSD, Novartis Pharma, Ono Pharmaceutical, Pfizer, Taiho, and Takeda Pharmaceutical. T. Takahashi obtained commercial research grants from AstraZeneca, Chugai Pharmaceutical, Eli Lilly, Amgen, Merck Biopharma, MSD, Ono Pharmaceutical, and Pfizer, and has received speaking honoraria from AstraZeneca, Boehringer Ingelheim, Chugai Pharmaceutical, MSD, Ono Pharmaceutical, Pfizer, Takeda Pharmaceutical, and Yakult Honsha. K. Takayama received research grants from Chugai Pharmaceutical and Ono Pharmaceutical and personal fees from AstraZeneca, Chugai Pharmaceutical, MSD-Merck, Eli Lilly, Boehringer-Ingelheim, and Daiichi-Sankyo. The other authors declare no potential conflicts of interest.

## ETHICS STATEMENT

Approval of the research protocol by an institutional review board: The study was approved by the institutional review board of Kyoto Prefectural University of Medicine and each respective hospital.

Informed consent: Written informed consent was obtained from all participants.

Registry and the registration no. of the study/trial: University hospital Medical Information Network, UMIN000043942.

Animal studies: N/A.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Yoshimura A, Yamada T, Serizawa M, et al. High levels of AXL expression in untreated *EGFR*-mutated non-small cell lung cancer negatively impacts the use of osimertinib. *Cancer Sci*. 2023;114:606-618. doi: 10.1111/cas.15608