



MET amplification results in heterogeneous responses to osimertinib in EGFR-mutant lung cancer treated with erlotinib

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Abstract

The third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) osimertinib is approved for untreated, or previously EGFR-TKI-treated T790M-positive EGFR-mutated non-small cell lung carcinoma (NSCLC). We investigated the heterogeneity of responses to osimertinib and its underlying mechanisms. A patient with EGFR-L858R-mutated NSCLC was treated with erlotinib. Following treatment, he developed brain and multiple bone metastases and was eventually diagnosed with NSCLC with EGFR-T790M mutation. The responses of various tumor specimens to osimertinib were heterogeneous. We investigated EGFR-T790M and MET amplification using PCR and FISH in autopsy specimens of the cervical spine, lumbar spine, and brain. We established the KNZ osimertinib-resistant (KNZ_OR) tumor cell line with MET amplification using a cervical spine lesion that was intrinsically resistant to osimertinib. We evaluated the effects of MET knockdown and MET inhibitor on KNZ_OR cell sensitivity to osimertinib in vitro and in vivo. Osimertinib-resistant lesions (cervical spine and brain) showed EGFR-L858R and MET amplification, but not EGFR-T790M, whereas osimertinib-sensitive lesions (lumbar spine) showed EGFR-L858R and -T790, but not MET amplification. Osimertinib decreased the association of amplified MET with L858R-mutated EGFR but increased that with human epidermal growth factor receptor 3 in KNZ_OR cells. MET knockdown or MET inhibitor sensitized KNZ_OR cells to osimertinib in vitro, indicating that MET amplification induced osimertinib resistance. Combination with osimertinib plus crizotinib induced tumor shrinkage in the KNZ_OR xenograft model. Hence, MET amplification might induce heterogeneous responses to osimertinib in EGFR-mutated NSCLC. Further investigations on mutated EGFR and amplified MET might lead to the development of effective therapies.

KEYWORDS

heterogeneity, L858R-mutated EGFR, MET amplification, osimertinib resistance, total EGFR

Abbreviations: CT, computed tomography; DAB, 3, 3'-diaminobenzidine; EGFR, epidermal growth factor receptor; HER3, human epidermal growth factor receptor 3; NSCLC, non-small cell lung carcinoma; NTRK1, neurotrophic receptor tyrosine kinase 1; PDC, patient-derived cell; TKI, tyrosine kinase inhibitor; TPM3, tropomyosin 3; TRK, tropomyosin receptor kinase.

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1 | INTRODUCTION

Osimertinib is an oral, third-generation, irreversible epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that selectively blocks both EGFR-TKI-sensitive mutations (such as *EGFR* exon 19 deletions or *EGFR* L858R mutation in exon 21) and *EGFR*-T790M resistance mutations but has little influence on wild-type EGFR.¹ The FDA-approved osimertinib is used for untreated or previously EGFR-TKI-treated *EGFR*-T790M mutation-positive, EGFR-mutated NSCLC.²⁻⁴ Osimertinib was shown to penetrate the mouse blood-brain barrier more effectively and provide better exposure in the cynomolgus monkey brain compared with other EGFR-TKIs.⁵ In fact, central nervous system (CNS) lesions responded better to osimertinib than to first- or second-generation EGFR-TKIs.⁶ However, patients frequently developed acquired resistance to osimertinib, and several EGFR-dependent and -independent mechanisms have been identified.⁷ The EGFR-dependent mechanisms include *EGFR* mutations, such as C797S, exon 20 insertions, S768I, and *EGFR* amplification.⁸⁻¹¹ The EGFR-independent resistance mechanisms include the activation of other receptor tyrosine kinases, the amplification of *MET* or human epidermal growth factor receptor 2 (*HER2*), the activation of downstream mutations (*PIK3CA*), and the mutation or amplification of other driver oncogenes and fusion genes, such as *KRAS*, *NRAS*, and *BRAF*.^{7,12,13}

In contrast, a population of *EGFR*-mutated NSCLC was reported to be intrinsically resistant to osimertinib.¹⁴ Heterogeneity is a known obstacle in cancer treatment.¹⁵ Previously *EGFR*-TKI-treated *EGFR*-mutated NSCLC is thought to be more heterogeneous than untreated NSCLC. Intertumor heterogeneity in the presence of *EGFR*-T790M is a mechanism of resistance.¹⁶ Moreover, intratumor heterogeneity consists of many concomitant resistance mechanisms.¹⁷ However, the heterogeneity of responses to osimertinib and the underlying molecular mechanisms in previously *EGFR*-TKI-treated T790M-positive NSCLC have not yet been investigated.

In the present study, we enrolled a male patient with *EGFR*-L858R-mutated NSCLC who was previously treated with erlotinib, following which he developed brain and multiple bone metastases. Eventually, the *EGFR*-T790M mutation was detected following pathological assessment by liquid biopsy. Osimertinib elicited heterogeneous responses in many lesions. We assessed *EGFR*-T790M and *MET* amplification in autopsy specimens of the cervical spine, lumbar spine, and brain using PCR and/or FISH. Moreover, we established an osimertinib-resistant (OR) tumor cell line (KNZ_OR) with *MET* amplification using a cervical spine lesion that was intrinsically resistant to osimertinib. We next examined the effects of *MET* knockdown and *MET* inhibitor treatment on the sensitivity of KNZ_OR cells to osimertinib in vitro and in vivo. Associations between L858R-mutated *EGFR* and amplified *MET* were assessed using an L858R-*EGFR*-specific antibody (Ab) in KNZ_OR cells.

2 | MATERIALS AND METHODS

2.1 | Molecular profiling

Molecules were screened at the Lung Cancer Genomic Screening Project for Individualized Medicine (LC-SCRUM) in Japan by performing PCR analysis of DNA extracted from frozen resistant tumors and OncoPrint™ Comprehensive Assay v3 (Thermo Fisher Scientific Inc).

2.2 | Cell lines, cell culture, and compounds

The KNZ_OR cell line was established from a bone metastatic tumor at C7 that was intrinsically resistant to osimertinib in the patient with *EGFR*-mutated lung cancer and plasma *EGFR*-T790M. The cells were maintained and cultured in ACL4 medium (Thermo Fisher Scientific Inc) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (50 µg/mL) in a humidified CO₂ incubator at 37°C. H3255 and PC-9 cells have an *EGFR*-L858R point mutation and exon 19 deletion, respectively. These two cell lines were cultured in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (50 µg/mL) in a humidified CO₂ incubator at 37°C. Mycoplasma infection of these cells was regularly monitored using the MycoAlert Mycoplasma Detection Kit (LONZA). Cell lines were authenticated by short tandem repeat analysis at the National Institute of Biomedical Innovation in May 2015. Osimertinib, gefitinib, crizotinib, golvatinib, JNJ38877605, and foretinib were purchased from Selleck Chemicals.

2.3 | Cell viability assay

Cells were seeded in RPMI-1640 at a density of 2×10^3 cells/100 µL containing 10% FBS per well in 96-well culture plates and incubated overnight. On the following day, *EGFR*-TKI and/or *MET*-TKI were added to each well at concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, and 3 µmol/L and incubated for 72 hours. Cell viability was assessed using 2 mg/mL of MTT (Sigma-Aldrich Corp.) as described.

2.4 | Abs and Western blotting

Cells were lysed with cell lysis buffer (Cell Signaling Technology) containing 1% (v/v) phosphatase inhibitor cocktail 3 (Sigma-Aldrich) in distilled water. Cell extracts (20 µg per lane) were resolved by SDS-PAGE using Mini-PROTEAN TGX Precast Gels, and the separated proteins were electrophoretically transferred onto Immun-Blot® PVDF membranes (Bio-Rad Laboratories). The primary antibodies used were as follows: phospho-*EGFR* (Y1068), protein kinase B

(AKT), phospho-AKT (Ser473), phospho-MET (Y1234/1235), MET (25H2), phospho-ErbB3 (Y1197), ErbB3 (D22C5), Gab1, β -actin (all from Cell Signaling Technology), EGFR, ERK1/ERK, and phospho-ERK1/ERK2 (Thr202/Thr204) (all from R&D Systems Inc). L858R-mutated-EGFR- and exon-19-deletion-positive-EGFR-specific Abs were obtained from Cell Signaling Technology. Membranes treated with primary Ab were incubated for 1 hour at room temperature with species-specific HRP-conjugated secondary Ab. Immunoreactive bands were visualized using the enhanced chemiluminescent SuperSignal West Dura Extend Duration Substrate (Pierce Biotechnology).

2.5 | RNAi assay

Silencer™ siRNA targeting MET (HSS106478) and Silencer™ Select Negative Control #1 siRNA (Ambion Inc) were used in RNAi experiments. Briefly, cells (1×10^5) in antibiotic-free medium (2 mL) were seeded into 6-well plates and incubated at 37°C for 24 hours. The cells were then transfected with siRNA (250 pmol) or scrambled RNA using Lipofectamine RNAiMAX (3 μ L; Invitrogen) as per the manufacturer's instructions. After 24 hours, the cells were washed twice with PBS and incubated for additional 48 hours in a medium containing antibiotics. The tumor cells were then used for cell proliferation assay. MET knockdown was confirmed by Western blotting.

2.6 | Immunoprecipitation

Proteins immunoprecipitated from cell extracts using Ab against MET, wild-type EGFR, and L858R-mutated EGFR were determined by immunoblotting for the indicated Abs.

Total cell lysates containing 500 mg of protein were incubated overnight at 4°C with Ab against MET (25H2), EGFR, or L858R-mutated EGFR. Immune complexes were precipitated by further incubation with a suspension of Protein G Sepharose (GE Healthcare Life Sciences) at 4°C overnight. Immunoprecipitates were isolated, washed, resolved by SDS-PAGE on a 7.5% gel, and immunoblotted as described above.

2.7 | DNA-FISH

We detected the c-MET gene in KNZ_OR cells and clinical samples, including primary lung tumor and metastatic lesions collected at autopsy using DNA-FISH. The MET 7q31.2 chromosomal locus was labeled with the C-MET Amplification Probe LPS004 (Cytocell Ltd.). Centromere 7 labeled with Spectrum Green Probe CEP7 (human locus D7Z1; Abbott Laboratories) was paired to control for copy numbers, and FISH was performed using the standard method.¹⁸ Only nuclei with unambiguous CEP7 signals were scored for MET

signal numbers. The C-MET gene and D7Z1 emitted red and green fluorescence, respectively.

2.8 | Droplet digital PCR (ddPCR)

We measured c-MET copy numbers in KNZ_OR cells using ddPCR. The DNA of negative and positive control cells for MET amplification and the DNA of KNZ_OR cells were extracted using the QIAamp® DNA Blood Mini kit (QIAGEN GmbH). The DNA was fractionalized by mixing it with LBx® Probe cMET CNV, LBx® Probe APOB CNV (reference), ddPCR™ Supermix for Probes, and Mse I, and ddPCR was performed using an automated droplet generator and a Veriti® Thermal Cycler (Applied Biosystems). All PCR steps were carried out at 50% ramp speed (2°C/s).

2.9 | Animal experiments

All animal experiments were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The Committee of Ethics of Experimental Animals and the Advanced Science Research Center of Kanazawa University (Kanazawa, Japan) approved the study protocol (approval number: AP-173867). KNZ_OR cells (5×10^6) and Matrigel® Matrix (354248) were subcutaneously implanted into the flanks of 5-week-old male SCID Hairless Outbred (SHO®) mice (Charles River Laboratories) to prepare subcutaneous tumor models. The size of the subcutaneous tumors and body weight of the mice were measured twice weekly, and the tumor volume was calculated as follows: tumor volume (mm^3) = ($\text{width}^2 \times \text{length}/2$). When the tumor volume reached 50-100 mm^3 , the mice were orally administered with vehicle, osimertinib (25 mg/kg), crizotinib (50 mg/kg), or a combinations of these agents daily for 18 consecutive days.

2.10 | Immunohistochemistry

The expression of phospho-MET was detected in deparaffinized formalin-fixed, paraffin-embedded tissue sections (4 μ m thick) using an MET Ab. Proliferating cells were detected by incubating tissue sections with a Ki-67 Ab (Clone MIB-1; DAKO Corp.). Apoptotic cells were detected by TUNEL staining of tissue sections using an in situ cell death detection kit (Roche Diagnostics GmbH). Antigens were retrieved by microwaving the tissue sections in 10 mmol/L citrate buffer (pH 6.0) and Tris-EDTA buffer (pH 9.0). After incubation with a secondary Ab, peroxidase activity was visualized via the DAB reaction using Histofine Simple Stain MAX-PO(R) kit (Nichirei). The sections were counterstained with hematoxylin. All sections were also stained with H&E.

2.11 | Quantification of immunohistochemical staining

Five areas containing the largest number of cells within each section were selected for histological quantification by light or fluorescence microscopy at 400 × magnification. Two authors (ST and AN) independently evaluated all findings.

2.12 | Clinical samples

Tissue samples of cervical spine, brain, and lumbar metastases were obtained at autopsy from the patient with plasma *EGFR*-T790M, who was treated with erlotinib followed by osimertinib. The primary lung tumor obtained at resection of the right lower lobe was also explored as a control surgical specimen.

2.13 | Statistical analysis

Data from the viability assay and tumor progression in the xenograft model are expressed as means ± SD. Differences among groups were analyzed by one-way ANOVA and Spearman's rank correlation using GraphPad Prism Ver. 6.0 (GraphPad Software Inc). Two-sided *P* values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Case report

A 55-year-old man who had been a light smoker (one pack a year) underwent right lower lobectomy for both diagnosis and treatment. He was diagnosed with T2aN2M0 (stage IIIA) lung adenocarcinoma and a primary tumor with an *EGFR* exon 21 L858R mutation. He underwent four courses of adjuvant chemotherapy with cisplatin plus pemetrexed. Metastases recurred in the mediastinal lymph nodes, lung, bone, and brain 6 months after completing adjuvant chemotherapy. He was then administered erlotinib (150 mg/d) and underwent whole brain irradiation for multiple brain metastases. However, 7 months after erlotinib administration, the brain and bone lesions worsened. Two brain metastatic lesions were treated using a γ knife. As the *EGFR*-T790M mutation was detected in his plasma using a COBAS *EGFR* Mutation Test[®] (Roche Molecular Systems Inc), the patient was started on osimertinib (80 mg/d). However, a neck pain developed 1 month after osimertinib administration, and CT findings revealed worsening metastases at the seventh cervical vertebra (C7) and exacerbation of brain metastases with brain edema (Figure 1A). Hence, we applied posterior decompression and C7 fusion to improve neck pain and quality of life. We obtained part of the resected tumor and established the KNZ_OR cell line as described below. However, subsequent treatments were ineffective and the patient died.

3.2 | Intertumor heterogeneity of *EGFR*-T790M detection and *MET* amplification

Molecular tests of the resistant tumor at C7, performed at LC-SCRUM, showed *MET* amplification and *EGFR*-L858R but not *EGFR*-T790M or *EGFR*-C797S detection (Figure 1B). We also assessed the status of *EGFR* mutation and *MET* amplification in the surgically resected primary lung tumor and brain, cervical, and lumbar spine specimens with metastatic lesions acquired from the patient at autopsy. The primary lung tumor had only an *EGFR*-L858R mutation. The brain and cervical spine tumors that were intrinsically resistant to osimertinib showed the *EGFR*-L858R mutation and *MET* amplification but not the *EGFR*-T790M mutation (Figure 1C, Figure S1). The lumbar spine, which was clinically stable during osimertinib therapy, displayed *EGFR*-L858R and *EGFR*-T790M mutations but not *MET* amplification. These results indicated the intertumor heterogeneity of the *EGFR*-T790M mutation and *MET* amplification and a correlation between *MET* amplification and intrinsic resistance to osimertinib.

3.3 | Establishment of *EGFR*-TKI-resistant KNZ_OR cells with *MET* amplification

We established a tumor cell line with stable growth by culturing part of the resected resistant C7 tumor in ACL4 medium with 1 μ mol/L Rho Kinase (ROCK) inhibitor (Figure 1D). Both FISH and PCR analyses of *MET* showed that the copy number of *MET* was 11.4, while that of the negative control gene (*APOB*) was 2 in KNZ_OR cells (Figure 1E, Table S1, Figure S2).

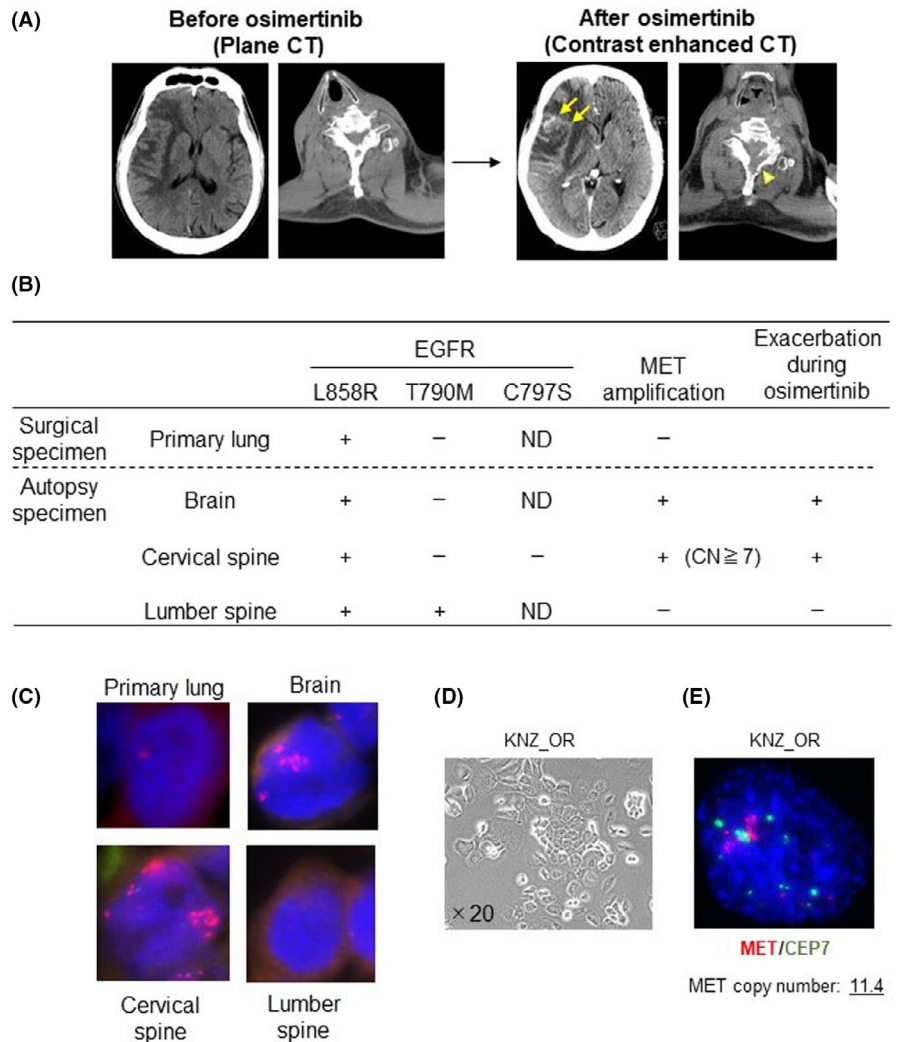
Next, we assessed the sensitivity of KNZ_OR cells to *EGFR*-TKIs in vitro. KNZ_OR cells were resistant to gefitinib or osimertinib compared with H3255 cells with *EGFR* exon 21 L858R mutation (Figure 2A,B). Western blot analysis showed that treatment with osimertinib inhibited the phosphorylation of *EGFR* in KNZ_OR cells but not that of other downstream molecules, such as AKT or ERK (Figure 2C).

We then determined the effects of *MET* knockdown in KNZ_OR cells using *MET*-specific siRNAs. Results showed that *MET* protein expression was effectively knocked down using siRNAs, and treatment with osimertinib remarkably inhibited AKT and ERK phosphorylation and reduced KNZ_OR cell viability (Figure 2D,E). These results indicated that KNZ_OR cells acquired osimertinib resistance through *MET* amplification.

3.4 | Dissociation of L858R-mutated *EGFR* from *MET* by osimertinib

Amplified *MET* associated with *HER3* restores survival signals via the adaptor protein Gab1 in *EGFR*-TKI-treated *EGFR*-mutated NSCLC cells with *MET* amplification.¹⁹ We therefore investigated interactions between *MET* and its related proteins using immunoprecipitation followed by immunoblotting. *EGFR* was detected

FIGURE 1 Analysis of clinical samples and KNZ_OR cells established from bone metastatic tumor at C7 that was intrinsically resistant to osimertinib. A, CT images 1 mo after the initiation of osimertinib treatment, showing enlarged brain metastatic lesions, brain edema (arrows), and cervical spine invasion by a bone metastatic lesion at C7 (arrowhead). B, Summary of mutation status of specimens and subjective and objective symptoms during osimertinib therapy. C, Detection of *MET* amplification (red dots) in specimens by FISH analysis. D, Establishment of KNZ_OR cell line. E, Amplification of *MET* in KNZ_OR cells as detected by FISH analysis. The copy number of *MET* was 11.4, while that of the reference gene was 2 in KNZ_OR cells. APOB, internal control; CN, copy number; CT, computed tomography; FISH, fluorescence in situ hybridization; ND, not determined



using anti-Leu25-Ser645-EGFR Ab that binds to the extracellular and transmembrane domains of EGFR irrespective of activating mutations (total EGFR), anti-E746-A750del-EGFR-specific Ab (Del19-EGFR), and anti-L858R-EGFR-specific Ab (L858R-EGFR). We found that PC-9 cells with an *EGFR* exon 19 deletion were positive for total EGFR and Del 19-EGFR Ab but not L858R-EGFR Ab. In contrast, H3255 and KNZ_OR cells with *EGFR*-L858R mutation were positive for total EGFR and L858R-EGFR Ab but not Del 19-EGFR Ab (Figure 3A), indicating the specificity of these Ab. Immunoprecipitation with anti-MET Ab followed by immunoblotting with anti-total EGFR Ab revealed that MET constitutively associated with EGFR (wild or L858R), and treatment with osimertinib did not affect this association in KNZ_OR cells (Figure 3B). However, HER3 hardly associated with MET in untreated KNZ_OR cells but strongly associated with MET in KNZ_OR cells treated with osimertinib. Moreover, the association between MET and Gab1 was not significantly affected by osimertinib. These associations were reproducible after immunoprecipitation with anti-total EGFR Ab followed by immunoblotting against MET, HER3, and Gab1 Ab. Immunoprecipitation with anti-L858R-EGFR Ab followed by immunoblotting with anti-MET Ab revealed that MET constitutively

associated with L858R-mutated EGFR. However, treatment with osimertinib notably reduced the association between L858R-mutated EGFR and MET, and this association was reproducible after immunoprecipitation with anti-MET Ab followed by immunoblotting with anti-L858R-EGFR Ab. Moreover, treatment with osimertinib inhibited the association between L858R-mutated EGFR and Gab1.

These results suggested that osimertinib inhibits the association of MET with L858R-mutated EGFR, but not total EGFR (presumably wild type), decreases the association between L858R-mutated EGFR and Gab1, and thus inhibits survival signals via L858R-mutated EGFR. Alternatively, osimertinib induces an association between MET and HER3 and maintains that between MET and Gab1, thus restoring the survival signals via Gab1 (Figure 3C).

Interestingly, in the immunoprecipitates with anti-L858R-EGFR Ab bound to anti-total EGFR and anti-L858R-EGFR Abs, treatment with osimertinib inhibited such binding. However, immunoprecipitates with anti-total EGFR Ab bound to anti-total EGFR and anti-L858R-EGFR Abs regardless of osimertinib treatment. These results suggested that osimertinib induces a conformational change in the L858R-mutated EGFR protein, and inhibits its binding to the anti-L858R-EGFR Ab, but not the anti-total-EGFR Ab, under nonreducing

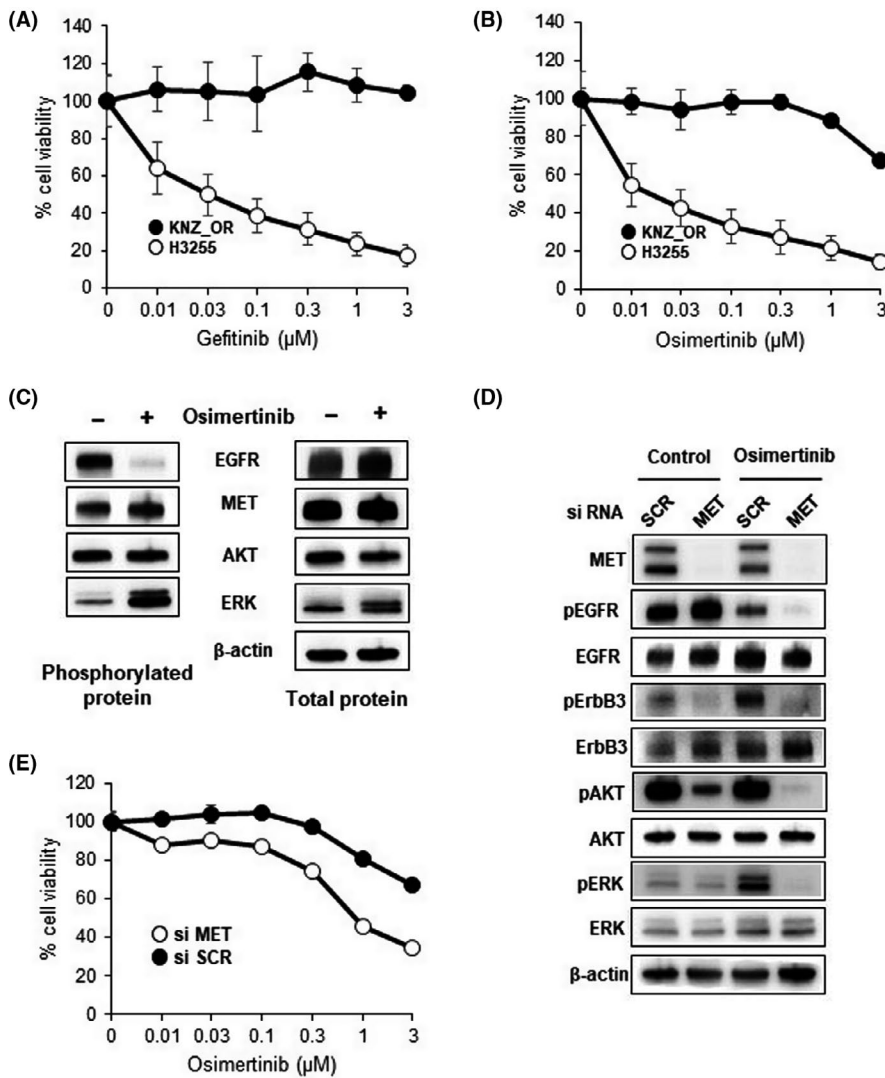


FIGURE 2 KNZ_OR was resistant to first- and third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) and MET knockdown sensitized KNZ_OR cells to osimertinib. A and B, Sensitivity of H3255 and KNZ_OR cells to gefitinib or osimertinib determined using MTT assay. Data are expressed as means \pm SD (of triplicate cultures) and representative of three independent experiments with similar results. C, KNZ_OR cells were incubated with osimertinib (1 μ mol/L) for 4 h, and harvested lysates were assessed by Western blotting. D, KNZ_OR cells were transfected with MET-specific siRNA or scrambled control, and cell lysates were harvested after 4 h and evaluated by Western blotting. E, KNZ_OR cells were treated with MET-specific siRNA or siSCR, and cell viability was determined by the MTT assay. Data are expressed as means \pm SD and representative of three independent experiments with similar results

conditions. Moreover, anti-L858R-EGFR and anti-total-EGFR Abs bind the immunoprecipitate under reduced conditions after SDS-PAGE.

3.5 | Sensitization of KNZ_OR cells to osimertinib by MET-TKIs in vitro

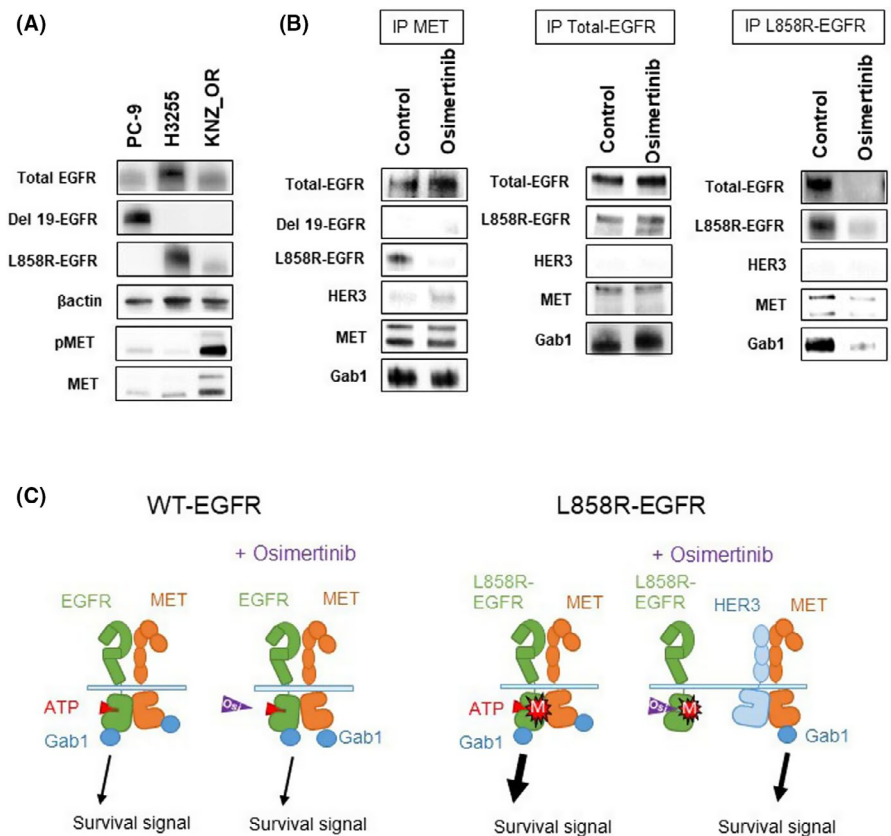
We next evaluated whether MET inhibitors could sensitize KNZ_OR cells to osimertinib. Results showed that treatment with crizotinib, golvatinib (E7050), JNJ38877605, and foretinib, all of which have inhibitory activity toward MET (26, 27) (Table S2), sensitized KNZ_OR cells to osimertinib (Figure 4A). Immunoblotting of KNZ_OR cell lysates showed that treatment with osimertinib alone remarkably inhibited the phosphorylation of EGFR but not that of AKT and ERK. Moreover, treatment with each of the MET-TKIs (crizotinib, golvatinib, JNJ38877605, and foretinib) alone inhibited the phosphorylation of MET remarkably and that of AKT and ERK discernibly. Furthermore, treatment with osimertinib plus MET-TKI (crizotinib, golvatinib, JNJ38877605, or foretinib) almost

completely inhibited the phosphorylation of EGFR, MET, AKT, and ERK (Figure 4B).

3.6 | Osimertinib combined with crizotinib diminished KNZ_OR cell tumor in vivo

Last, we examined the effects of combination treatment with osimertinib and the MET inhibitor crizotinib on KNZ_OR cells in vivo. KNZ_OR cells were subcutaneously implanted into SHO[®] mice, followed by treatment with vehicle (control), osimertinib alone, crizotinib alone, or osimertinib plus crizotinib combination. The results showed that the KNZ_OR tumors treated with osimertinib plus crizotinib obviously shrank (Figure 5A). Western blot analysis showed that osimertinib plus crizotinib treatment inhibited EGFR phosphorylation in KNZ_OR patient-derived cell (PDC) tumors (Figure 5B). Immunohistochemistry revealed that treatment with crizotinib alone as well as osimertinib plus crizotinib inhibited MET phosphorylation (Figure 5C). Moreover, osimertinib plus crizotinib combination treatment remarkably decreased the number of

FIGURE 3 Interaction between MET and L858R-mutated EGFR in KNZ_OR cells treated with osimertinib. A, Reactivity of Ab against total EGFR, L858R-EGFR, exon-19-deletion-EGFR, total MET, or phosphorylated-MET in the lysates of PC-9 cells with EGFR exon 19 deletion (E746-A750), H3255 cells with EGFR-L858R mutation, and KNZ_OR cells, as determined by Western blotting. B, KNZ_OR cells were incubated with osimertinib (1 μ mol/L) for 4 h, lysed, and immunoprecipitated with Ab against MET, total EGFR, or L858R-mutated EGFR. Immunoprecipitates were immunoblotted against indicated proteins. C, Schema of associations between MET and wild-type- or L858R-mutated EGFR



Ki-67-positive proliferating tumor cells (Figure 5D) and distinctively increased the number of TUNEL-positive tumor cells (Figure 5E) in KNZ_OR tumors. These results indicated that KNZ_OR tumors with MET amplification were sensitized in vivo by combination treatment with osimertinib and an MET inhibitor.

4 | DISCUSSION

Tumor heterogeneity presents a major obstacle in cancer treatment,¹⁵ and both inter- and intratumor heterogeneity are involved in targeted drug resistance.²⁰ A previous study on EGFR-T790M mutation and MET amplification in autopsy samples from six patients who achieved partial remission after treatment with EGFR-TKI showed that these two gene alterations were reciprocal and played complementary roles in acquired resistance to first-generation EGFR-TKIs.¹⁸ In the present study, intertumor heterogeneity was observed in response to osimertinib, and resistance mechanisms were detected in a patient with EGFR-mutated NSCLC, who was treated with erlotinib. For instance, while lumbar bone metastatic lesions with the EGFR-T790M mutation, but not MET amplification, were stable following osimertinib therapy, cervical bone metastatic lesions with MET amplification, but not the EGFR-T790M mutation, displayed progression. Moreover, MET knockdown sensitized KNZ_OR cells (derived from an OR cervical spine lesion in the patient) to osimertinib, suggesting that MET amplification induced osimertinib resistance in the patient. However, whether erlotinib induced MET

amplification before osimertinib was administered remains unclear. Heterogeneous resistance mechanisms have been previously detected in liquid biopsies. In a study involving 38 patients with EGFR sensitizing mutations, resistance gene alterations, including MET amplification, were detected in 50% of patients using next-generation sequencing of circulating tumor DNA.²¹ Hence, repeated liquid biopsies, which detect resistant gene alterations, including EGFR mutations and MET amplification, might be required for the early and optimal detection of resistance mechanisms.

The results of recent studies indicated that MET copy number 8 or higher, or fourfold or higher of the gene would result in EGFR-TKI resistance. We previously reported that an MET copy number of 8 or higher could induce gefitinib resistance in a PC-9 cell-based model.²² In addition, Suda K et al reported that a fourfold increase in MET copy number results in MET amplification and EGFR-TKI resistance in HCC827 cell-based models and clinical specimens.¹⁸ In line with these data, our results showed that KNZ_OR cells had an MET copy number of 11.4 and showed resistance to EGFR-TKIs. Many studies have reported a direct association between MET and EGFR in normal and cancer cells.²³ Heterodimerization between MET and EGFR has been identified in EGFR-L858R-transfected lung cancer cells without MET amplification.²⁴ While a direct association between amplified MET and HER3 has been detected in EGFR-TKI-resistant lung cancer,¹⁹ an association between amplified MET and mutated EGFR was not detected. In contrast, in the present study, a direct association between amplified MET and L858R-mutated EGFR was detected. Notably, treatment with

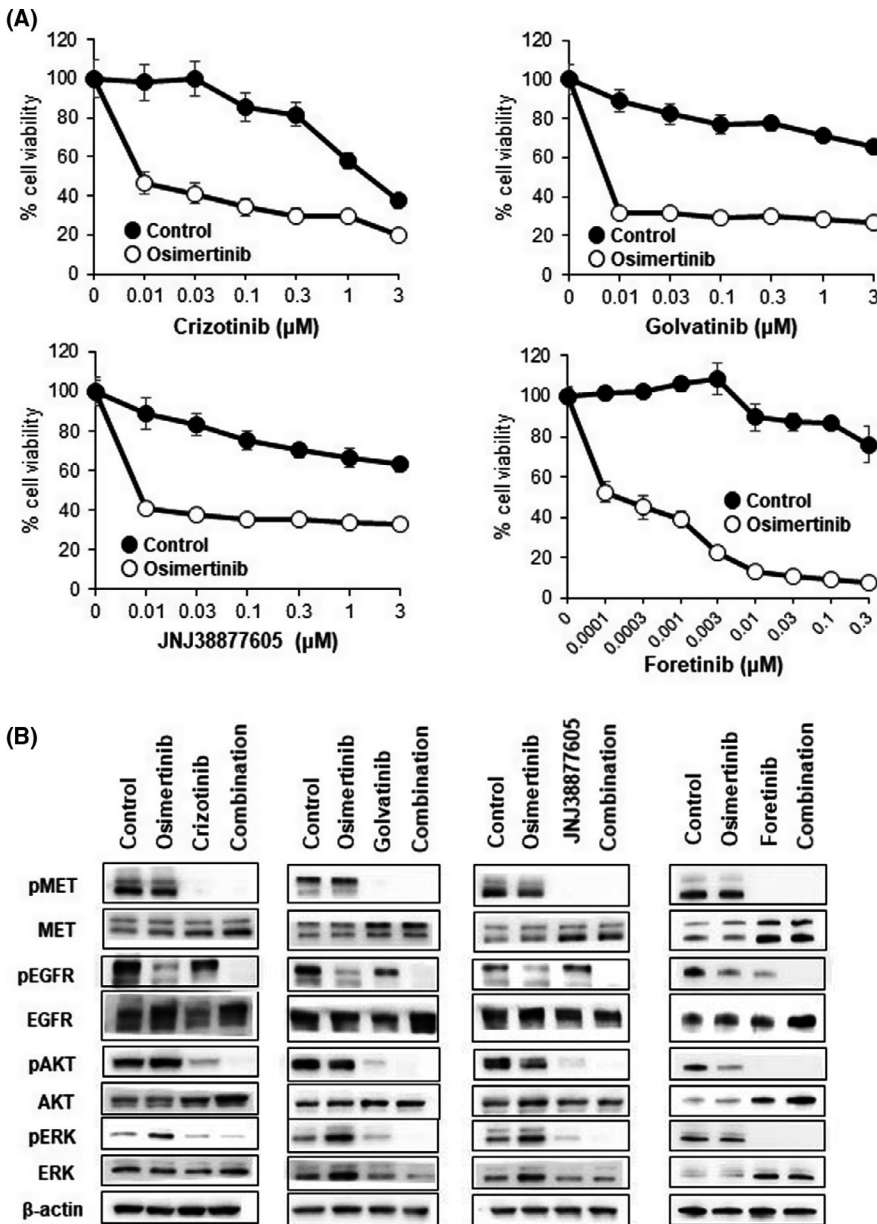


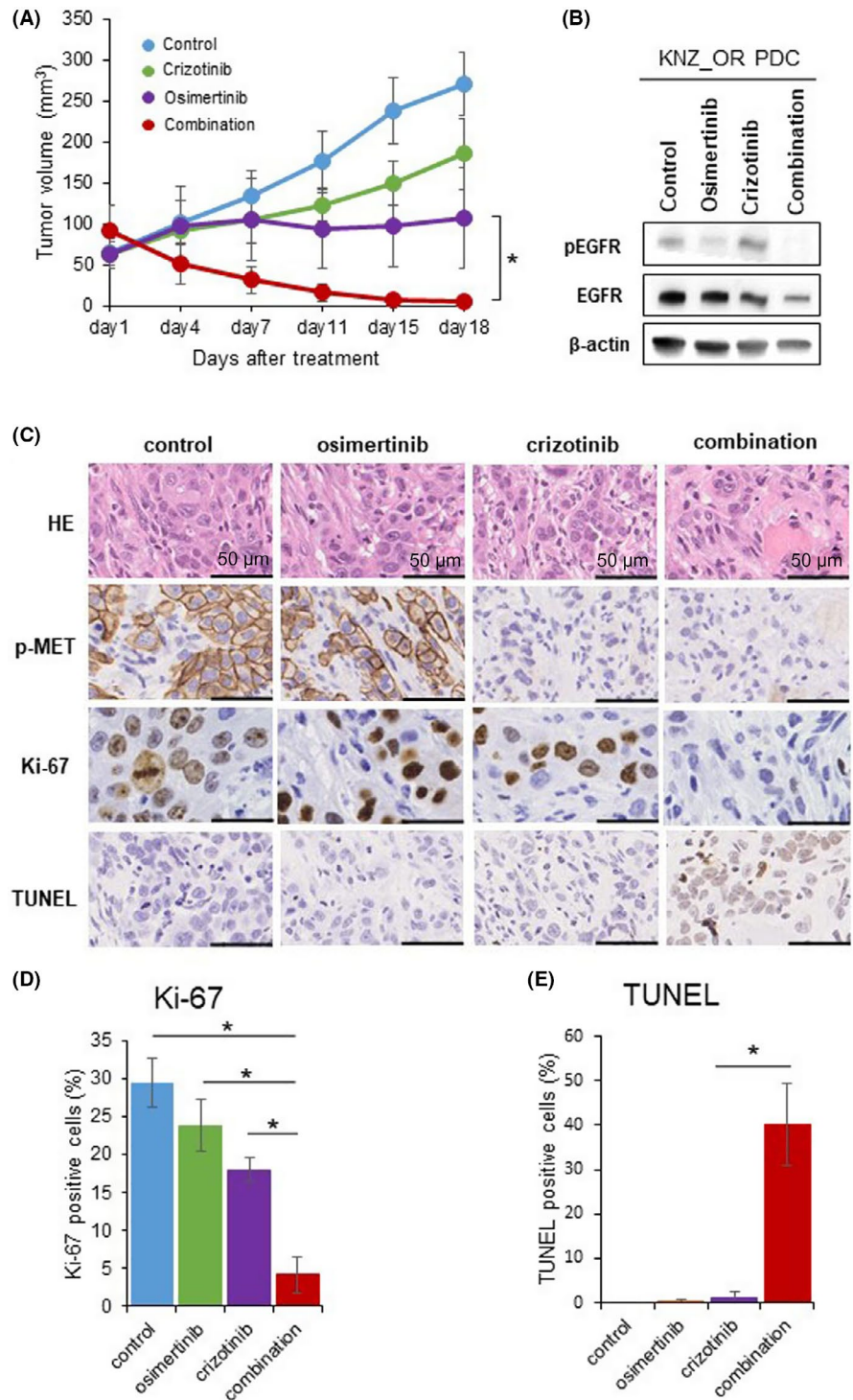
FIGURE 4 Effects of MET-TKI combined with osimertinib on KNZ_OR cells. A, KNZ_OR cells (2×10^3 cells/well) were incubated with various concentrations of MET-TKI (crizotinib, golvatinib, JNJ38877605, or foretinib) together with osimertinib ($1 \mu\text{mol/L}$) for 72 h, and cell viability was determined by the MTT assay. Error bars represent SD of triplicate cultures. B, KNZ_OR cells (2×10^5 cells/well) were incubated in six-well plates with osimertinib ($1 \mu\text{mol/L}$) with or without each MET-TKI (crizotinib, golvatinib, JNJ38877605, or foretinib; $1 \mu\text{mol/L}$). Cell lysates were immunoblotted with Ab against indicated molecules

osimertinib did not inhibit the association between MET and EGFR, as detected by an Ab that recognized both extracellular and transmembrane domains. However, osimertinib treatment remarkably inhibited the association between MET and L858R-mutated EGFR, suggesting a difference in behavior between wild-type and L858R-mutated EGFR. This might be due to the selective activity of osimertinib against mutated EGFR. These results indicate that functional analysis of mutated EGFR, such as association with other receptor tyrosine kinases or adaptor proteins in the presence of osimertinib, should apply methods that can distinguish wild-type from mutated EGFR. Such analyses might lead to the discovery of novel resistance mechanisms and development of novel drugs to circumvent resistance to osimertinib.

MET has attracted much attention as a target of cancer therapy because several MET alterations, including MET exon 14 skipping mutations and MET amplification, have been detected in populations

of solid tumors.²⁵ MET amplification is detectable in ~5% and ~15% of patients with EGFR-mutated lung cancer who acquired resistance to first- and third-generation EGFR-TKIs, respectively.²⁶ Various MET-TKIs, including crizotinib, cabozantinib, and capmatinib are under clinical development to treat lung cancers with MET alterations.^{27–29} In the present study, we found that several MET-TKIs have the potential to circumvent osimertinib resistance caused by MET amplification. Notably, crizotinib, a breakthrough therapy for MET exon 14 skipping positive lung cancer,³⁰ when combined with osimertinib induced shrinkage of subcutaneous tumors comprising KNZ_OR cells in a mouse model. One limitation of this study is that we did not evaluate the effects of MET-TKIs on tumors of various organs. Lung cancer patients with mutated EGFR frequently develop metastasis in several organs, including the brain and bone.³¹ The patient involved in the present study also had metastases in the brain and multiple bones. The activity of crizotinib against lesions in

FIGURE 5 Crizotinib combined with osimertinib exerted prominent antitumor activity on subcutaneous tumors derived from KNZ_OR cells. A, KNZ_OR cells were subcutaneously inoculated into SHO[®] mice ($n = 5$ per group). When the tumor volume reached 50–100 mm³, mice were orally administered with vehicle, osimertinib (25 mg/kg), crizotinib (50 mg/kg), or osimertinib combined with crizotinib daily for 18 consecutive days. Means \pm SE of tumor volumes are shown. * $P < .05$ vs crizotinib (Mann-Whitney U test). B, As an additional experiment, after 3 d of each therapy, mice were administered with drugs, then KNZ_OR tumors were resected 4 h later. Relative protein levels in tumor lysates were determined by Western blotting. C, Proliferating cells (Ki-67 staining) and apoptotic (TUNEL staining) cells in KNZ_OR PDC tumors of mice were identified by immunohistochemistry. Scale bar, 50 μ m. D and E, Quantification of the immunohistochemical staining data in (C). Data are expressed as means \pm SD of five areas. * $P < .05$ (Mann-Whitney U test)



the CNS is limited because of limited penetration.³² We previously reported that the TRK inhibitor foretinib was more effective than entrectinib, an FDA-approved TRK inhibitor, in a brain metastatic model comprising *TPM3-NTRK1* fusion-positive tumor cells.³³ Here, because foretinib was found to be active against MET and circumvented osimertinib resistance in KNZ_OR cells in vitro, it might be able to circumvent osimertinib resistance in CNS metastasis. In our future studies, we aim to determine the effects of foretinib on OR CNS metastases in vivo.

In summary, this study describes the intertumor heterogeneity of resistance mechanisms to osimertinib in a patient with *EGFR*-L858R-mutated lung cancer who had previously been treated with the first-generation *EGFR*-TKI, erlotinib. We established an *EGFR*-T790M-negative tumor cell line with acquired osimertinib resistance due to *MET* amplification. Our results showed that osimertinib combined with an *MET*-TKI circumvented resistance to osimertinib in vivo and in vitro. Using an L858R-mutated *EGFR*-specific Ab, we revealed a direct association between amplified *MET* and L858R-mutated

EGFR. While this association was inhibited by osimertinib treatment, the association between MET and HER3 might restore survival signals via Gab1, indicating that the behavior of L858R-mutated EGFR differs from that of wild-type EGFR. Our data suggest that further analyses of the mutated EGFR protein, including its association with amplified *MET*, using specific detection methods might lead to the discovery of a novel resistance mechanism and development of novel drugs to circumvent resistance in EGFR-mutated lung cancer.

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ETHICAL CONSIDERATION

The protocol for cell culture from patients samples was approved by the Institutional Review Board of Kanazawa University Hospital (2016-414).

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SUPPORTING INFORMATION

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

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