Integrative Genomic Analysis of Drug Resistance in *MET* Exon 14 Skipping Lung Cancer Using Patient-Derived Xenograft Models

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Abstract

Background

Non-small cell lung cancer (NSCLC) driven by *MET* exon 14 skipping (*MET*ex14) occurs in 3-4% of NSCLC cases and defines a subset of patients with distinct characteristics. *MET* targeted therapy has led to strong clinical responses, however little is known about acquired resistance to drugs in these patients. Patient derived xenograft (PDX) models are recognized as excellent preclinical models to facilitate the understanding of the mechanisms underlying drug resistance.

Methods

We describe a patient case harboring *MET*ex14 who exhibited drug resistance after treatment with crizotinib. Subcutaneous xenografts were generated from pretreatment and post-resistance patient specimens. PDX mice were then treated with *MET* inhibitors (crizotinib and tepotinib) to evaluate their drug response. DNA and RNA sequencing analysis was performed on patient tumor specimens and matching xenografts.

Results

PDXs preserved most of the histological and molecular profiles of the parental tumors. Drug resistance to *MET* targeted therapy was confirmed in PDX models through *in vivo* drug analysis. Newly acquired *MET* D1228N mutations and *EGFR* amplified were detected in patient-resistant tumor specimens. Although the mutations were not detected in the PDX, *EGFR* overexpression was observed in RNA sequencing analysis indicating possible off target resistance through the *EGFR* bypass signaling pathway.

Conclusions

We established and characterized a pair of *MET*ex14 NSCLC patient-derived xenografts (PDXs), including the first crizotinib resistant *MET*ex14 PDX. This model will be a powerful tool for testing hypotheses of drug resistance mechanisms and investigations into novel therapeutic strategies.

Introduction

Non-small cell lung cancer (NSCLC) is a heterogeneous disease that accounts for 85% of lung cancer diagnoses, accompanied by various alterations in known oncogenes including *EGFR*, *ALK*, *ROS* and *MET*. *MET* exon 14 skipping (*MET*ex14), often reported without co-occurring oncogenic driver mutations, has been identified in 3-4% of NSCLC cases and is associated with poor prognosis [1, 2].

MET is a proto-oncogene that encodes for a tyrosine kinase receptor and plays key functions in embryonic development, organogenesis and wound healing. Somatic mutation in introns 13 or 14 of *MET* could lead to an alternative splicing result in the skipping of exon 14 [3]. Exon 14 encodes for the cytoplasmic juxtamembrane domain responsible for ubiquitination of MET protein, which maintains the dynamic balance between MET activation and its removal from the cell surface. A lack of the juxtamembrane domain disrupts the regulation of MET signaling causing persistent activation of the downstream signaling pathway [4, 5].

Considering that *MET*ex14 tends to occur in the absence of other oncogenic mutations, MET activity is likely the key event in tumorigenesis. MET selective inhibitors have subsequently become an attractive treatment choice for patients with *MET*ex14 mutations. Clinical evidence suggests that MET specific tyrosine kinase inhibitors (TKIs) are highly effective against tumors bearing *MET*ex14. Capmatinib and tepotinib have recently been approved for the treatment of NSCLC patients harboring *MET*ex14, and NCCN guidelines also note that crizotinib may be useful in certain circumstances and is recommended as a category 2A treatment option [6–8]. In addition, several other MET inhibitors have already shown clinical benefits for *MET*ex14 NSCLCs, including savolitinib, merestinib and bozitinib [9].

Despite the dramatic initial response to MET inhibitor therapy, patients eventually develop drug resistance through activation of the bypass signaling pathway (off target) or MET resistant mutations (on target) [10]. This has greatly limited the potential impact of this form of therapy. Thus, identification of the precise drug resistance mechanism(s) affecting MET inhibitors will be essential to enable clinicians to accommodate therapeutic strategies in pursuance of improved clinical outcomes. Preclinical models such as a patient-derived xenograft (PDX) model have become increasingly important in translational research. The reason is that PDX models are believed truly to resemble the original disease, therefore providing researchers with invaluable insights.

Previously, our group has successfully generated an ALK+ and EGFR+ targeted TKI resistance PDX model using novel microfluidic technology to investigate underlying resistance mechanisms [11]. In the present study, we established two PDX models from a *MET*ex14 NSCLC patient's tumor specimen that were taken before treatment and after resistance to crizotinib developed. To date and as far as we are aware, there have been no published articles on PDX models representing TKI-resistant *MET*ex14 patients. By investigating these models, we aim to improve the understanding of the underlying mechanisms involved in TKI drug resistance.

Patient And Methods

Patient specimens

Primary tumor specimens at diagnosis and resistance specimens after crizotinib treatment were obtained using CT-guided biopsy. Tumor specimens and their paired peripheral blood samples were collected to establish PDX and to conduct DNA whole exome-sequencing (WES) and RNA sequencing.

Animals

Female NOD SCID mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd and were between 6-8 weeks of age at the time of implantation. Mice were hosted in a specific pathogen-free (SPF) environment of a vivarium facility and acclimatized to their new environment for at least 3 days prior to initiation of any experiments following IACUC protocols.

Establishment of xenograft models

Tumor biopsy specimens of the patient were implanted subcutaneously in the right flanks of female NOD SCID mice using an 18-gauge trocar needle. The first implanted passage was defined as P0 mice (tumor), the next passage implanted from P0 tumor was defined as P1 mice (tumor), and further passages were generated by serial implantation. Tumor sizes were measured routinely in two dimensions using a caliper, and the volume was expressed in mm³ using the formula: $V = 0.5a \times b^2$, where a and b are the long and short diameters of the tumor, respectively.

In vivo drug treatments

Tumor fragments (30 mm³) harvested from the PDX model were implanted subcutaneously in the right flanks of mice. When average tumor sizes reached 100-150 mm³, tumor-bearing mice were randomly divided into study groups with 5 or 6 mice in each group. Treatment was started on the day of randomization. Mice were dosed orally with vehicle control, crizotinib at 50 mg/kg and tepotinib at 10 mg/kg. All drugs were administered following a strict daily schedule.

The tumor size was used for the calculations of both T/C and TGI values. The T/C value (percentage) is an indication of antitumor effectiveness, and T and C are the mean volume of the treated and control groups, respectively. On a given day, TGI was calculated for each group using the formula:

$$\text{TGI (\%)} = [1 - (T_i - T_0) / (V_i - V_0)] \times 100$$

where T_i is the average tumor volume of a treatment group on a given day, T_0 is the average tumor volume of the treatment group on the first day of treatment, V_i is the average tumor volume of the vehicle control group on the same day with T_i , and V_0 is the average tumor volume of the vehicle group on the first day of treatment.

Specimen extraction and sequencing

WES and various analyses were performed at the Genomics Laboratory of GenomiCare Biotechnology (Shanghai, China). Specimen extraction and sequencing was performed using a previously published protocol [12–14]. DNA in frozen tissue specimens and blood was extracted from thawed materials using the Maxwell RSC Blood DNA Kit (cat# AS1400, Promega, Madison, WI, USA) on a Maxwell RSC system (cat# AS4500, Promega). DNA of formalin-fixed, paraffin-embedded (FFPE) tissue was extracted using the MagMAX FFPE DNA/RNA Ultra Kit (cat# A31881, ThermoFisher, Waltham, MA, USA) on a KingFisher

Flex system (ThermoFisher). The extracted DNA was sheared using a Covaris L220 sonicator and the exome DNA was captured using the SureSelect Human All Exon V7 kit (cat# 5991-9039EN, Agilent). The SureSelectXT Low Input Target Enrichment and Library Preparation system (cat# G9703-90000, Agilent, Santa Clara, CA, USA) was used for target enrichment and library preparation. DNA aliquots were then sequenced on an Illumina NovaSeq-6000 sequencer (Illumina, San Diego, CA, USA) to generate 2 x 150 bp paired-end reads. Image analysis and base calling was conducted using onboard RTA3 software (Illumina) [12, 13].

RNA from FFPE specimens was purified using the MagMAX FFPE DNA/RNA Ultra Kit (cat# A31881, ThermoFisher) on a KingFisher Flex system (ThermoFisher) and used as the template to synthesize cDNA using the NEBNext RNA First Strand Synthesis Module (cat# E7525S, NEB, Waltham, MA, USA) and the NEBNext mRNA Second Strand Synthesis Module (cat# E6111S, NEB) sequentially. The library preparation, sequencing and base calling were conducted similarly (*vide supra* the WES section) [14].

Somatic variant identification

After removing adapters and low-quality reads, the commercial Sentieon (version 201911) [15] running environment with default parameters was implemented to process the following steps sequentially: reads alignment to NCBI human genome reference assembly hg19 using the Burrows-Wheeler Aligner (BWA) algorithm, duplication sorting, realignment and recalibration, and somatic mutation calling including single nucleotide variations (SNVs) and short insertion/deletions (INDELs). During the mutation calling stage, the reads from the tumor specimens were compared with paired blood from the same patient to generate the somatic mutation list. The called somatic mutations were then filtered to retain only the mutations with the variant allele frequency (VAF) ≥ 0.05 and supported by at least 3 reads, and annotated using the Variant Effect Predictor (VEP) package [12, 13].

Copy number variation (CNV)

By following the ExomeCNV package [16], a normalized depth-of-coverage ratio approach was used to identify CNV from the WES results of paired specimens. A standard normal distribution was used to account for 5 sources of bias that would affect raw read counts, which included the size of exonic regions, batch effect, the quantity and quality of the sequencing data, local GC content and genomic mappability. Genes with a haploid copy number ≥ 3 or ≤ 1.2 were defined as amplified or deleted, respectively and a minimum tumor content (purity) of 20% was required [12, 13].

RNA differential gene expression analysis

RNA-seq reads were mapped to the NCBI human genome reference assembly hg19 using STAR (version 020201) [17] and a count matrix was generated. The raw read counts were further normalized by log2-counts per million normalization (TPM) [14].

Results

1. Patient Case

In December, 2019, a 68-year-old female Chinese patient who never smoked but with a history of cavernous sinus hemangioma surgery presented at the Shanghai Chest Hospital. A CT scan showed a 5.2 x 4.5 x 6.2 cm mass in the left upper lobe (**Figure 1A**), combined with mediastinal lymph nodes, bone and brain metastasis. The upper left lung nodule was biopsied and subjected to pathological tests, next-generation sequencing (NGS) and the establishment of a xenograft at the initial diagnosis. The patient was diagnosed as NSCLC-NOS (not otherwise specified) (**Figure 1B**). As the patient harbored *MET*ex14 mutations, she received crizotinib treatment from January 2020. The best outcome was a partial response, the tumor size was reduced by 35.5% , which resulted in a durable response for 7 months until evidence of disease progression occurred, with a larger primary tumor and hepatic metastasis (**Figure 1A**). At time of disease progression, the patient again underwent a CT-guided biopsy of the left upper lung lesion for pathology tests, NGS and the establishment of a xenograft. Histopathological examination of the biopsy specimens revealed squamous cell carcinoma characteristics, which were negative for TTF-1, NapsinA and CD56, and positive for CK and P40 (**Figure 1C**). Due to her poor physical condition and ECOG score, the patient could not tolerate the toxicity associated with chemotherapy and received the best supportive care for 4 more months until she died in December 2020.

2. Establishment of patient-derived xenograft models for human *MET*ex14 NSCLC with and without crizotinib resistance

The subcutaneous patient-derived xenograft models were established from biopsy specimens obtained before (PDX pretreatment) or after (PDX resistant) crizotinib resistance had developed. PDXs were serially passaged in animals 3-5 times for tissue expansion. Representative tumor-bearing mice are shown in **Figure 2A**. No significant body mass loss was observed in mice bearing both PDX tumors.

The histology and degree of differentiation associated with PDX pretreatment was slightly different from the parent tumor histology of which led to a diagnosis of NSCLC-NOS, whereas PDX was diagnosed as squamous cell carcinoma (**Figure 2B**). Histology of PDX-resistant tissue matched well with its parent tumor (**Figure 2C**). In addition, both PDX tumors maintained intra-tumor heterogeneity, resembling the original tumors.

To evaluate the responses of PDX models to the standard-of-care agent, we conducted *in vivo* efficacy studies of crizotinib and tepotinib in 2 PDX models. Crizotinib and tepotinib administration all proved to be highly effective in suppressing PDX pretreatment tumor growth. The quantified TGI (PG-D21) were 115.25% and 112.31%, respectively (**Figure 3A**). While drug resistant PDXs showed decreased drug sensitivity to crizotinib (TGI = 65.32%, PG-D35) and tepotinib (TGI = 50.40%, PG-D35), they resembled the patient's drug resistance to crizotinib (**Figure 3B**).

3. Sequencing data validated the PDX model and reported on acquired target *MET* mutation and *EGFR* gene amplification in a patient crizotinib-resistant specimen

WES and RNA-sequencing (RNA-seq) were performed on patient biopsy specimens before treatment and after resistance to crizotinib developed and on their corresponding PDX tissue. RNA-seq results confirmed

that both PDX models retained *MET*ex14 (**Figure 4B**). DNA results revealed that both PDX tumors retained the overall pattern of the somatic mutations and copy number variation of their parent tumor tissue. **Figure 4A** gives a list of a total of 27 mutations, either oncogenic or with higher relevance to the disease. Most of the oncogene alterations in the parent primary tumor were preserved in the PDX specimens including *MET*ex14, *MTOR*^{L2500F}, *RAD54L*^{G235R}, *BRCA1*^{Q1240*} and *TP53*^{H193R} (**Figure 4A**). We noticed the frequency of several mutations, including *MET*ex14 (splice site p.E1009fs), *TP53*^{H193R}, *ARID3A*^{P373L} and *PRSS1*^{T137M}, in PDX tissue increased to 100% which may be a result of a homozygous mutation in the parent tumor.

We then compared resistant specimens to non-resistant specimens in patient and PDX models. *MTOR*^{L2500F}, *RAD54L*^{G235R}, *BRCA1*^{Q1240*}, *TP53*^{H193R} and *MET*ex14 (splice site p.E1009fs) were fairly consistent between the 2 patient specimens, while mutation of *KMT2C*^{G4660E}, *FGFR1*^{N546K}, *PBX1*^{E60K} and *CSF1R*^{L368V} were lost in patient-resistant specimens. *MET*^{D1228N} and *APC*^{D396N} newly occurred in patient-resistant specimens at mutational frequencies of 6.44% and 5.08%, respectively. However, these 2 mutations were lost in PDX-resistant tissue at both the DNA and RNA level, possibly implying clonal selection during establishment. *EGFR* amplification, a classic drug resistance gene alteration, was acquired in patient-resistant specimens. The copy number of *EGFR* significantly increased from 2.24 to 7.44 (**Figure 4C**). Although gene amplification was lost in PDX-resistant tissue, we observed a significant increase in the RNA expression level of *EGFR* in PDX specimens compared to non-resistant tissue. TPM increased from 37.6 to 102.6, which was consistent with patient specimens. Since *EGFR*, *FGFR3*, *IGF1R* and *MET* all belong to the RTK family, we further investigated the RNA expression levels in these RTK-related molecules. RNA-seq data showed increased expression levels of *EGFR*, *FGFR3* and *IGF1R* in resistant specimens compared to pretreatment specimens indicating activation of the bypass signaling pathway (**Figure 4C**).

Discussion

Before treatment, the patient's tumor tissue contains mixed adenocarcinoma and squamous cell carcinoma components with partly TTF1 and NapsinA positive and partly P40 positive. However, the specimen after crizotinib treatment was characterized as squamous cell carcinoma. The specific mechanism for this transition, termed adenocarcinoma and squamous cell carcinoma transdifferentiation (AST), is still under investigation [18–20].

MET exon 14 skipping is known to be an important recurrent alteration that is responsible for driving tumorigenesis in NSCLC. Much research has proven that this oncogenic driver is mutually exclusive with other NSCLC driver mutations such as *EGFR*, *ERBB2*, *BRAF*, *MET* or *KRAS* [21]. Cohort studies also reported distinct mutation patterns coincident with driver mutations suggesting distinct molecular subtypes in NSCLC [22]. Common co-occurring mutations in *MET*ex14 patients included *TP53*, *NF1*, *BRAF* and *CDKN2A*; however, these co-alterations did not show a correlation to the development of *MET* inhibitor resistance [23]. Resistance to TKI treatment can be mainly categorized into two types of

mechanism, namely on target and off target resistance. On target resistance usually occurs with a second-site mutation of the targeted oncogene or acquired oncogene gain or loss. Off target resistance occurs through mutational activation of the downstream signaling pathway or the parallel bypass signaling pathway including the RAS-MAPK, PI3K-AKT and JAK-STAT pathways [24].

The *de novo* on target second site mutation MET^{D1228N} in patient-resistant specimens has been reported in several cases in response to crizotinib treatment. MET^{D1228N} was first reported by the Heist group as an underlying crizotinib resistance mechanism in MET_{ex14} patients. Protein crystallization of crizotinib with the kinase domain of MET suggested point mutation at this location could affect the binding affinity of crizotinib, due to the electrostatic change that resulted from the substitution [25]. *In vitro* experiments also confirmed the loss of sensitivity towards crizotinib in MET^{D1228N} compared to MET^{WT} in a HEK293 cell line from measurements of MET phosphorylation [26]. Although this mutation has been reported to be sufficient to cause resistance to crizotinib, the PDX model of the resistant tumor did not retain this mutation. The low allelic fraction of this *de novo* mutation in NGS results, as well as differences in histology, indicated that clonal selection, sites of biopsy or clonal evolution during tumor establishment and passaging were likely the cause for the discrepancy [27, 28]. This result led us to propose the involvement of other contributing factors in the process of gaining drug resistance.

Acquired *EGFR* gene amplification in patient-resistant tissue suggested that activation of the EGFR related pathway could be another contributor to crizotinib resistance in this case. *EGFR* amplification has been found in MET_{ex14} patients as a resistance mechanism during MET targeted therapies [29]. As the primary resistance mechanism to the EGFR inhibitors, acquired MET amplification has been extensively described in the literature, stressing the biological link between EGFR and MET [30, 31]. *In vivo* experiments on a MET-resistant gastric cancer cell line revealed that EGFR signaling became the primary and independent driver for the downstream signaling pathway mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) [20]. MET activation also signals through the MAPK/ERK pathway regulating cell proliferation, cell motility and cell cycle progression [33]. Combined inhibition of EGFR and MET that successfully overcame the resistance towards MET targeted TKI resistance has been described *in vitro*, *in vivo* and in clinical reports [29, 32]. These results all suggested that MET and EGFR are functionally redundant in maintaining the activation of the downstream signaling pathway in targeted TKI inhibitor resistance [34].

Our PDX model derived from patient-resistant tissue retained *EGFR* overexpression at the transcriptome level only. Inconsistency between *EGFR* amplification and overexpression has been observed in many cases, but *EGFR* amplification is sufficient but not necessary for transcriptome level overexpression. Other factors such as transcriptional or posttranscriptional activation have been proposed to explain this upregulation. Theories on advantages of clonal selection and increased EGFR ligand expression have been thoroughly researched but the underlying mechanisms still remain unclear [35]. In addition, *in vivo* efficacy studies using our PDX model exposed to crizotinib and tepotinib confirmed MET resistance, thus supporting our theory on the role of EGFR activation in MET targeted TKI resistance. Recent studies on bispecific antibody (BsAb) targeting both MET and EGFR have shown promising results, blocking

downstream pathways. Researchers have speculated that BsAb targeting both MET and EGFR will benefit patients who have acquired drug resistance [36, 37]. Our models, including the first crizotinib resistant *MET*ex14 NSCLC PDX, would be ideal candidates to test this hypothesis.

List Of Abbreviations

NSCLC: non-small-cell lung carcinoma

EGFR: epidermal growth factor receptor

ALK: anaplastic lymphoma kinase

ROS1: c-ros proto-oncogene 1

METex14: MET exon 14 skipping

MTOR: mechanistic target of rapamycin kinase

RAD54L: RAD54 like

BRCA1: Breast cancer type 1

TP53: Tumor protein p53

KMT2C: Lysine Methyltransferase 2C

FGFR: fibroblast growth factor receptor

IGF1R: Insulin-like growth factor 1 receptor

PBX1: Pre-B-cell leukemia transcription factor 1

APC: Adenomatous polyposis coli

CSF1R: Colony Stimulating Factor 1 Receptor

ERBB2: Erb-B2 Receptor Tyrosine Kinase 2

BRAF: v-Raf murine sarcoma viral oncogene homolog B

KRAS: Kirsten rat sarcoma

NF1: Neurofibromatosis type 1

CDKN2A: Cyclin Dependent Kinase Inhibitor 2A

Ras-MAPK: Rat sarcoma virus GTPase/mitogen-activated protein kinase

PI3K-AKT: phosphatidylinositol 3-kinase/protein kinase B

JAK-STAT: Janus kinase/signal transducer and activator of transcription

ERK: extracellular signal-regulated kinases

TPM: Transcripts Per Million

RTK: receptor tyrosine kinases

TKI: tyrosine kinase inhibitor

PDX: pNatient derived xenograft

NCCN: National Comprehensive Cancer Network

NGS: Next generation sequencing

ECOG: Eastern Cooperative Oncology Group

NSCLC-NOS: non-small-cell lung carcinoma-not otherwise specific

BsAb: bispecific antibody

Declarations

Ethics approval and consent to participate

The current research work was reviewed and approved by the Shanghai Chest Hospital Research Ethics Committee (KS1950). Written informed consent was obtained from patient prior to sample collection. During the study, the care and use of animals were conducted in accordance with the regulations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Availability of data and materials

Data are available under BioProject accession code PRJNA765468

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Authors' contributions

YHX, LPG, HJ, and SL conceived this study. YHX, LPG, YQL, RYZ, HJ, WHX, LL, HWW and ZHC collected data. YHX, LPG, YQL, RYZ, HJ, YCH, WHX, LL and SL performed data analysis and interpretation. YHX, LPG, YQL, RYZ and YCH drafted the manuscript. YHX, LPG, YQL, RYZ, YCH and SL revised and commented on the draft. All authors read and approved the final manuscript.

Disclosure

The authors declare that they have no competing interests.

Consent for publication

All authors approved the final version of this manuscript.

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Figures

Figure 1

Chest computed tomography (CT) scans and histopathology of patient tumor before and after crizotinib treatment

(A) CT scans of the primary tumor of patient at baseline (left), best response (middle) and progression disease (right) after crizotinib treatment. Red arrows point to malignant loci. (B) Hematoxylin and eosin (H&E) staining and immunohistochemistry staining of the tumor biopsies from patient before crizotinib treatment, which was CD56 negative, CK positive and partially positive for TTF1, P40 and NapsinA; the scale bar represents 40 μ m. (C) H&E staining and immunohistochemistry staining of tumor biopsy specimens from a patient in disease progression, which was negative for CD56, NapsinA and TTF-1, positive for CK and P40; the scale bar represents 40 μ m.

Figure 2

Tumor-bearing mice and histopathology staining

(A) Representative images of tumor-bearing PDX mice established from patient pretreatment tumor (left) and drug-resistant tumor (right), respectively. (B) Hematoxylin and eosin (H&E) staining and immunohistochemistry staining of tumor derived from pretreatment PDX (passage 3), which was negative for CD56, NapsinA and TTF-1, and positive for CK and P40; the scale bar represents 40 μ m. (C) H&E and immunohistochemistry staining of tumor derived from drug-resistant PDX (passage 2), which is negative for CD56, NapsinA and TTF-1, and positive for CK and P40; the scale bar is 40 μ m.

Figure 3

The effect of TKIs on two PDX models

(A) Tumor change in pretreatment PDX mice treated with vehicle, 50 mg/kg crizotinib and 10 mg/kg tepotinib once a day for 3 weeks. (B) Tumor change in drug resistant PDX mice treated with vehicle, 50 mg/kg crizotinib and 10 mg/kg tepotinib once a day for 5 weeks. Tumor volume was measured and expressed as the mean $\pm$ standard error.

Figure 4

Genetic and transcriptomic characterization of tumors from the patient and the corresponding PDX models

(A) Co-mutation plot of genomic alterations across tumor specimens from patient and PDX models. The known oncogenes and other most frequently altered genes are shown. (B) Sashimi plot of *MET* exon14 skipping event in patient and PDX tumor specimens. The Y axis indicates read density, the number on arc represents junction reads, and portion of schematic *MET* transcripts with exon number is the plot at the bottom. The figure was adapted from IGV [38]. (C) Expression level and copy number of RTK related genes in patient and PDX model tumor specimens. Numbers are presented in TPM.