Identification of a novel MET mutation in high-grade glioma resulting in an auto-active intracellular protein

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Abstract MET has gained interest as a therapeutic target for a number of malignancies because of its involvement in tumorigenesis, invasion and metastasis. At present, a number of inhibitors, both antibodies against MET or its ligand hepatocyte growth factor, and small molecule MET tyrosine kinase inhibitors are in clinical trials. We here describe a novel variant of MET that is expressed in 6% of high-grade gliomas. Characterization of this mutation in a glioma cell line revealed that it consists of an intronic deletion, resulting in a splice event connecting an intact splice donor site in exon 6 with the next splice acceptor site being that of exon 9. The encoded protein lacks parts of the extracellular IPT domains 1 and 2, encoded by exons 7 and 8, resulting in a novel pseudo-IPT and is named METΔ7–8. METΔ7–8 is located predominantly in the cytosol and is constitutively active. The auto-activating nature of METΔ7–8, in combination with a lack of transmembrane localization, renders METΔ7–8 not targetable using antibodies, although the protein is efficiently deactivated by MET-specific tyrosine kinase inhibitors. Testing of MET-expressing tumors for the presence of this variant may be important for treatment decision making.

Keywords MET · Glioma · Mutation · Protein localization · Genetic deletion · Auto-active · Intracellular location · Biomarker

Introduction

The MET proto-oncogene (chromosome 7q31.2) encodes the tyrosine kinase membrane receptor MET (also called Scatter Factor Receptor), which is essential during development. Signaling from the receptor controls epithelial-to-mesenchymal transition (EMT) of myogenic precursor cells during differentiation into skeletal muscle cells [5], a process that involves migration over long distances in the embryo. In adults, MET is involved in tissue regeneration upon injury [6].

MET is produced as a glycosylated single-chain precursor protein of ~190 kDa which, during transport to the membrane, undergoes furin-mediated cleavage in the trans-Golgi network [8, 29]. The resulting mature receptor consists of an extracellular 50 kDa α-chain, covalently attached via a disulfide bond to a membrane-spanning 140 kDa β-chain [20, 56]. The extracellular segment consists of an N-terminal Sema domain that is involved in ligand binding, a small cysteine-rich domain, and four IPT (Immunoglobulin-like fold shared by plexins and transcription...
factors) domains, which connect the Sema and cysteine-rich domains with the C-terminal β-subunit [18].

Upon binding of the ligand hepatocyte growth factor (HGF, scatter factor), receptor dimerization occurs followed by trans-phosphorylation in the intracellular tyrosine kinase (TK) domain at tyrosine (Y) residues 1230, 1234 and 1235 [14, 34]. The TK domain subsequently induces auto-phosphorylation of Y1349 and Y1356, which act as docking sites for signal transduction molecules including GAB1, GRB2, phospholipase-C and SRC [50]. Phosphorylated GAB1 interacts with molecules like PI3-K and SHP2, which together induce several downstream signaling pathways. MET signaling is mediated by, among others, the PI3-K/AKT and RAS/MAPK pathways, which induce cell cycle progression, survival, cytoskeletal changes and invasion (reviewed in [17]). In addition to its role downstream of HGF, MET can also be involved in signaling of other transmembrane receptors, including VEGFR2, CD44v6, and 1235 [14, 34]. The TK domain subsequently induces auto-phosphorylation of Y1349 and Y1356, which act as docking sites for signal transduction molecules including GAB1, GRB2, phospholipase-C and SRC [50]. Phosphorylated GAB1 interacts with molecules like PI3-K and SHP2, which together induce several downstream signaling pathways. MET signaling is mediated by, among others, the PI3-K/AKT and RAS/MAPK pathways, which induce cell cycle progression, survival, cytoskeletal changes and invasion (reviewed in [17]). In addition to its role downstream of HGF, MET can also be involved in signaling of other transmembrane receptors, including VEGFR2, CD44v6, EGFR and Plexin B1 [19, 26, 35, 44]. Upon ligand-induced receptor dimerization, MET is internalized via endocytosis and may be recycled [27]. Phosphorylation of Y1003 in the juxtamembrane (JM) domain of the receptor leads to ubiquitination and subsequent proteasomal degradation [25]. Thus, levels of MET in the cell are tightly regulated.

Aberrant activation of MET signaling is a tumor-promoting event in a variety of malignancies and can be induced by several mechanisms, including alternative mRNA splicing, exon skipping and crosstalk with other receptors [13]. In high-grade gliomas, the frequently occurring oncogenic EGFR mutant EGFRvIII can induce overexpression of both MET and HGF, a process that is balanced by wild-type EGFR activation [33]. MET amplifications have been found in a number of tumor types including glioblastoma (GBM) [9, 10] and missense mutations in the Sema, the TK and juxtamembrane (JM) domain have been reported to affect HGF binding, kinase activation and receptor degradation, respectively [1, 30, 32, 36, 38, 43, 48, 49]. Recently, gene fusions between the protein tyrosine phosphatase PTPRZ1 and MET, resulting in constitutive activation of MET, were described in 16 % of secondary GBMs [1]. Activation of MET signaling has been proposed as a mechanism of resistance to EGFR inhibitors, likely a result of the similarities in downstream signaling events from both receptors [3].

The significant role that MET plays in tumor progression and metastasis has made it a prime therapeutic target in oncology. MET tyrosine kinase inhibitors and therapeutic antibodies against the extracellular domain of MET and against HGF, all preventing HGF-mediated MET activation, are currently in clinical trial (www.clinicaltrials.gov). In a previous study, we have shown that the combined VEGFR2/MET tyrosine kinase inhibitor cabozantinib (XL-184, CoMETRIQ) potently inhibits MET phosphorylation, cell proliferation and migration and consequently prolongs survival of mice carrying orthotopic E98 glioma xenografts [42]. Here, we identify a novel intragenic MET deletion in E98 cells, which results in a truncated protein that is constitutively active and lacks membranous expression, thereby having important implications for therapeutic strategies targeting MET. We show that this mutation occurs in 6 % of glioblastomas and, like the EGFR mutation EGFRvIII [4], is relatively specific for this tumor type.

Materials and methods

Immunohistochemistry

Immunohistochemistry on formalin-fixed, paraffin-embedded (FFPE) tissue sections was performed as previously described using antibodies against MET and P-MET (clone D1C2 and D26, respectively, both CST) [42]. Antibodies were visualized via sequential incubations with biotinylated secondary antibodies, avidin–biotin complexes (Vector laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine solution (Power-DAB, ImmunoLogic, Duiven, The Netherlands).

Cell lines

The E98 cell line and xenograft model and genetic analysis thereof have been described before [12, 42]. E98, U87, A549, HEK-293T and TOV-112D or TOV-112D-MET cells [22] were cultured in DMEM + 4.5 g/l glucose medium (PAA Laboratories, Pasching, Austria) supplemented with 10 % fetal calf serum (FCS) (PAA) and gentamycin (40 μg/ml). All cell lines were maintained at 37 °C in the presence of 5 % CO2. To examine HGF-induced MET activation, E98 and A549 cells were seeded in 6 wells plates. The next day, cells were serum-starved overnight, followed by a 10 min treatment with 50 ng/ml HGF (Miltenyi Biotech, Bergisch Gladbach, Germany). In some experiments, prior to HGF incubation cells were incubated with the anti-MET llama VHH G2 [22] or cabozantinib (XL-184, Exelixis, San Francisco, CA, USA) for 60 min.

Genetic analysis of E98

Genomic DNA from E98 cells was analyzed by semi-conductor sequencing (IonPGM, Life Technologies) using the comprehensive cancer panel (Life Technologies) that targets 409 cancer-related genes. The IonPGM E98 library generation was performed according to the manufacturer’s protocol. In short, 10 ng of DNA per pool was amplified in 21 cycles by PCR using the Ion AmpliSeqTM mastermix, followed by barcode and adapter ligation. Amplified products were purified with Agencourt AMPure XP beads.
(Beckman Coulter Genomics, High Wycombe, UK). The library was diluted to 20 pM. Emulsion PCR was performed using the Ion OneTouchTM 200 Template kit following the protocol of the Ion OneTouchTM System. Next, Ion Sphere Particles (ISPs) were recovered and enriched for template-positive ISPs using Dynabeads MyOne Streptavidin C1 beads (Life Technologies) in the Ion OneTouchTM ES instrument (Life Technologies). ISP enrichment was quantified using the Qubit 2.0 fluorometer (Life Technologies). Sequencing primer and polymerase were added to the final enriched spheres before loading onto an Ion 318 chip according to the Ion PGTM 200 sequencing kit protocol. The gene copy number analysis was performed as follows. The relative number of sequence reads aligned to a specific gene was determined by dividing by the total number of aligned reads of E98, and was divided over the relative number of sequence reads of the same gene in non-neoplastic blood cells. The relative ratios are plotted in a graph based on the genomic position of the gene.

To perform confirmative mapping of the intronic deletion in MET on the nucleotide level, genomic DNA was isolated from E98 and U87 cells using the DNeasy Blood & Tissue Kit (QIAGEN), according to the manufacturer’s protocol. 10 ng was subjected to PCR, using primer MET1997Fw (5'-CTCCTTGGAAATGAGAGCTG-3'), forward, exon 6, genome location chr7:q:g.116395517-36) and reverse primer MET2414Rev (5'-GGGATCTTCACGGTAACTATTAC-3') under a cover slip. Sections were washed in ddH2O, dried and hybridized with 10 ml hybridization mix (1 ml cep7 Spectrum Green (06J37007), 7 ml hybridization buffer in MQ, all Vysis) under a cover slip. Sections were rinsed in 10 mM HCl and PBS and postfixed for 5 min in 10 % paraformaldehyde (PFA, Merck)/PBS. Sections were rinsed in 10 mM HCl. Proteins were digested with pepsin (200 U/ml, Sigma) for 15 min at 37 °C. Subsequently, slides were rinsed in 10 mM HCl and PBS for 30 min in 1 % paraformaldehyde (PFA, Merck)/PBS. Sections were washed in ddH2O, dried and hybridized with 10 μl probe mix (1 μl cep7 Spectrum Green (06J37007) + 1 μl LSI MET Spectrum Red (06N05-001) + 7 μl hybridization buffer in MQ, all Vysis) under a cover slip. Sections were denatured at 80 °C for 10 min, followed by hybridization o/n at 37 °C in a Hybridizer (Dako). After removing the coverslip by soaking for 5 min in 2x SSC buffer (Maxim Biotech) at 42 °C, slides were washed 3 times in 2x SSC buffer at 73 °C, once in 2xSSC (5 min) and once in ddH2O. After dehydration in EtOH, slides were air-dried in the dark and mounted in Vectashield/DAPI (3 parts Vectashield/DAPI + 1 part Vectashield, all vector). Slides were analyzed on a Leica Fluorescence microscope.

**RT-PCR and cloning**

RNA was isolated from cell lines E98 and TOV112D-MET [22] using TRIzol Reagent (Life Technologies) and reverse transcribed with MMLV-RT (New England Bio-labs) using oligo-dT primers, according to the manufacturer’s instruction. HGF was PCR amplified from cDNA using Phusion High-Fidelity DNA polymerase (Finnzymes, ThermoFisher Scientific, Waltham, USA) and primers HGF-Fw204 (5'-CTGCAGCATGTCCTCC TGCA-3') and HGF-Rv504 (5'-GAGGTCAATTCATGGCCAA-3') (30 cycles, annealing at 55 °C, 20 s; extension at 72 °C, 20 s). Control PCR reactions were performed for housekeeping gene HMBS (Hydroxymethylbilane Synthase) using primers p361-Fw (5'-TGCCAGAGAAGTGGTGTTGTG-3') and p425-Rv (5'-GTTAAGCTGGCTGCAACATC-3'). MET open reading frames were PCR amplified from cDNA using Phusion DNA polymerase and primers flanking start and stop codons (MET173EcoR1-Fw: 5'-CGAATTCGATAAACC TCTCATAATGAAGG-3' and MET4421NotI-Rv 5'-AGCGG CCGCCTATGATGTCTCCCAGAAGG-3'). PCR products were purified on agarose gel, digested with NotI and cloned as blunt-NotI fragment in pIREs-neo-EcoRV-NotI (Clontech Laboratories, Inc, CA, USA) to yield pIREs-neo-MET and pIREs-neo-MET^57-8^ Full sequences were obtained by Sanger sequencing via the sequencing facility of Radboud UMC.

The extracellular parts of MET were PCR amplified from E98 and TOV112D-MET cDNA using primers MET173EcoR1-Fw and MET3028NheI-Rv (5'-CGCTAGCCCTAGCT GTTG ACTATTACG-3') and cloned in vector pHlscc-BAPHIS, a derivative from pHLSec-HIS (Addgene, Cambridge, MA, USA). The resulting vector adds a biotin acceptor peptide and His-tag to the carboxyterminus of the MET extracellular domains, resulting in pHLSec-MET^BAPHIS^ and the E98-derived variant pHLSec-MET^BAPHIS^ (Addgene) in HEK293T cells and cells...
were cultured in the presence of 10 μM biotin (Sigma-Aldrich, St Louis, MO, USA). After 48 h, cytosolic extracts were made in RIPA buffer, and medium (1 ml) was mixed with 100 μl Ni–NTA Sepharose slurry (IBA, Goettingen, Germany). After a 1 h incubation at 4 °C, Ni-beads were washed with buffer (500 mM NaCl, 50 mM phosphate buffer, pH 7.4) and loaded onto a poly-prep column (Bio-Rad, Hercules, CA, USA). After washing off specifically bound proteins with 2 ml 10 mM imidazole, His-tagged an biotinylated MET extracellular domains were eluted with 0.5 ml 0.5 M imidazole, and dialysed o/n at 4 °C to 50 mM TRIS/150 mM NaCl pH 7.5.

**MET downregulation via short hairpin RNAs**

To create MET knockdown constructs, shMET1 (GTATGCATGCTTTGAA) and shMET2 (GTATGTCCATGCTTTGAA) oligonucleotide heteroduplexes were ligated in pENTR/U6 vector and subsequently Gateway-cloned into pLent6/BLOCK-iT-DEST-TagRFP according to the manufacturer’s protocols (Invitrogen). Generation of lentviruses was done in HEK293FT cells as described previously [7].

**Protein analysis**

RIPA extracts (20–40 μg protein per lane) and purified His-tagged proteins from conditioned media (equivalent of 20 μl conditioned medium) were subjected to PAGE on 8–10 % polyacrylamide gels and western blotting according to standard procedures. Nitrocellulose blots (Whatman Optitran BA-S85, GE Healthcare, Little Chalfont, UK) were blocked with PBS/Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA) (1:1), followed by overnight incubation with primary antibodies at 4 °C. Antibodies used were against MET N-terminus (clone EP1454Y, Epitomics, Cambridge, UK), MET C-terminus (clone D1C2), phosphorylated (P)-MET (Y1234/1235, clone D26), P-AKT (S473, clone D9E), P-ERK1/2 (T202/Y204, clone 20G11) (all CST), GAPDH (clone 6C5, Abcam), and α-tubulin (clone 236-10501, Molecular Probes, Life Technologies). Biotin groups and primary antibodies were visualized using, respectively, streptavidin-680 (Molecular Probes, Life Technology) and appropriate secondary antibodies [goat-anti-rabbit-IRDye800 (Rockland Immunocchemicals, Gilbertsville, PA, USA) or Alexa Fluor 680 goat-anti-mouse IgG (Molecular Probes, Life Technologies)]. Blots were scanned on the Odyssey imager (LI-COR Biosciences).

**Protein domain modeling**

A homology model for the new hybrid IPT-domain using the WHAT IF & YASARA Twinset was generated [31, 60]. We used the experimentally solved 3D structure 2uzx, which contains the human tyrosine kinase MET. The first 60 residues of the hybrid domain are identical to this structure, whereas the following 38 residues were modeled based on homology between the 2 domains.

**Confocal microscopy**

E98 and U87 cells, grown on Nunc Lab-Tek chamber slides (Sigma-Aldrich) to 40 % confluence, were fixed with 2 % PFA in 0.1 M phosphate buffer (pH 7.4) for 15 min at RT, followed by three washes with PBS, 30 min glycine incubation (100 mM in PBS) to quench PFA-induced autofluorescence, three PBS washes and permeabilization with digitonin for 10 min (100 μM in PBS). Aspecific binding was blocked by incubation with 20 % normal goat serum in PBS/1 %BSA for 20 min, and cells were incubated overnight at 4 °C in a humidified chamber with PBS/1 %BSA containing (combinations of): mouse monoclonal anti-CD44 antibody (as a surface marker, clone Hermes-1, ThermoFisher Scientific); rabbit anti-MET (clone D1C2, CST); rabbit anti-P-MET (clone D26, CST); mouse anti-EEA-1 (clone 14/EEA1, BD Biosciences, early endosome marker); anti-CLIMP-63 (clone G1/296, rough ER marker, a kind gift of J. Fransen). Primary antibodies were detected using Alexa Fluor 488- or 594-labeled secondary goat-anti-mouse and Alexa Fluor 488- or 568-labeled goat-anti-rabbit IgGs (all Life Technologies), 1:200 diluted in PBS/1 %BSA. Q-nuclear deep red (1:200, Life Technologies) was used to stain nuclei, and cells were mounted in Fluoromount G with DAPI (Southern Biotech, Birmingham, AL, USA). Cells were analyzed using a confocal laser scanning microscope (Leica SP2 CLSM) and Leica confocal software.

**Biotinylation assay**

E98 and A549 cells were allowed to adhere and grown to 80 % confluence in a 6-well plate (Cellstar, Greiner Bio-One, Kremmünster, Austria). Cells were washed 3 times with ice-cold PBS and incubated for 30 min at 4 °C with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher Scientific). This compound does not penetrate cells and biotinylation is therefore confined to exposed membrane proteins. In parallel, Tris-free RIPA extracts, prepared from equivalent numbers of E98 or A549 cells by repeated washing over Amicon Ultra K10 centrifugal filters (Merck Millipore, Billerica, MA, USA), were treated similarly to biotinylate all cellular proteins. The reaction was quenched by washing cells or cytosolic proteins three times with 100 mM glycine/PBS and two times with PBS (using K10 columns in the case of protein lysates). Intact biotinylated cells were then subjected to lysis with RIPA buffer. Protein concentrations in all lysates were determined using
the BCA protein concentration assay (ThermoFisher Scientific). MET was immunoprecipitated from 200 µg total protein in a volume of 200 µl using anti-MET (1:50, clone D1C2, CST) for 1 h at 4 °C. Immune complexes were captured by incubation for 30 min at 4 °C with 10 µl prot A agarose slurry (Roche Diagnostics, Basel, Switzerland), followed by centrifugation (14,000 rpm, 4 min) and three PBS washes. Immune complexes were solubilized by heating (5 min 95 °C) in 30 µl 2× SDS-PAGE sample buffer (0.2 % SDS, 62.5 mM Tris–HCl pH 6.8, 10 % glycerol and 0.2 µM DTT). Samples were subjected to 10 % SDS-PAGE and western blotting as described above.

RT-PCR analysis

Glioma tissues (n = 80) were obtained from the Radboud UMC and from the University of Cologne. Sarcoma (n = 25) and castration-resistant prostate cancer (CRPC) tissues (n = 43) were obtained from Radboud UMC. RNA was isolated from tissues using the mirVANA RNA isolation kit (Life Technologies) according to standard procedures. For cDNA synthesis, 1 µg RNA was reverse transcribed using the Quantitect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands) using hexanucleotides. To distinguish wtMET from METΔ7–8 transcripts, a PCR was performed in AmpliTaq Gold 360 mastermix (Applied Biosystems, Life Technologies) using primer MET1997Fw (5′-CTCCTGGAAATTGAGCAGTGG-3′, forward, located in exon 6), and primer MET2342Rv (5′-CAGTTGAAATGAGAGCTG-3′, reverse, located in exon 9). This PCR results in a 105-bp product for METΔ7–8 and a 345-bp product for wtMET. Conditions were: denaturation 95 °C, followed by 35 cycles of 95 °C denaturation; annealing at 58 °C, 30 s; elongation at 72 °C, 1 min. A final elongation step of 7 min was done at 72 °C.

Results

E98 MET protein is auto-activated in an HGF-independent fashion

Our previous experiments have shown that MET in orthotopic E98 xenografts is phosphorylated in tumor cells in a homogeneous fashion (Fig. 1a, see also [42]). In vitro, E98 cells also show high levels of phosphorylated MET when grown under serum-free conditions (Fig. 1b). HGF treatment did not further increase phosphorylation levels of MET, in contrast to A549 control cells in which HGF was required for MET activation. MET phosphorylation in E98 cells was not the result of endogenous HGF expression as revealed by RT-PCR analysis (Fig. 1c), while analysis of E98 xenograft RNA revealed the presence of mouse HGF only, as determined by Sanger sequencing (not shown). Since mouse HGF is not an activating ligand for human MET [62], we conclude that constitutive activation of MET in E98 cells and xenografts is not the result of an autocrine HGF-activation loop.

E98 cells express a truncated version of an amplified MET gene

To examine the underlying mechanism of MET auto-activation in this model, we PCR-cloned MET cDNA from E98 cells, using primers flanking the open reading frame (ORF, NM_000245.2). The MET PCR product from E98 was 240 bp smaller than that from U87 and CaCo2 cDNA (Fig. 2a), and Sanger sequencing analysis revealed an in-frame deletion of nt 2050–2289 in the coding sequence, corresponding to exons 7 and 8. The same transcript was also found in E98 xenografts (not shown). Genomic analysis of E98 cells using semi-conductor sequencing revealed high copy MET amplification (Fig. 2b). Average amplification of MET (except for exons 7/8) was about 14-fold. FISH analysis using probes specific for MET and chromosome 7 centromere confirmed the amplification (Fig. 2c). Consistent with Sanger sequencing, amplicons in exon 7 and the first part of exon 8 were absent (see insert in Fig. 2b), suggesting that the lack of exons 7 and 8 results from a genomic rearrangement (similar to the EGFR variant III

Fig. 1 MET is constitutively activated in the E98 cell line and xenograft model. a Sections of an intracranial E98 xenograft were subjected to immunohistochemistry for P-MET (upper panel, brown staining). A matching H&E staining is shown as reference. Note that tumor shows diffuse infiltration in the brain parenchyma and that the tumor cells are highly positive for this activated MET. b Western blot analysis of MET expression in serum-starved E98 and A549 cells in absence of presence of HGF. Note that the processed form of MET (arrowhead) in E98 is somewhat smaller than that of A549 while the preform is predominantly present in E98 (arrow). α-Tubulin was used as a loading control. c RT-PCR for HGF on E98, TOV-112D and HEK-293T cell line cDNA. HMBS was used as a control housekeeping gene.
(15)), instead of alternative splicing. PCR on genomic DNA using exon 6- and 9-specific primers resulted in amplification of a 910-bp fragment (predicted size from the wild-type allele, present in U87, is 3014 bp, see Fig. 2d). Sequencing of this product revealed an intronic deletion of 2114 bp (between position g.116 395 653 located in intron 6, and 116 397 766, located in exon 8). This deletion results in an intact splice donor site from exon 6, juxtaposed to the exon 9 splice acceptor site, and explains the lack of exons 7 and 8 in the resulting mRNA (Fig. 2e). Of note, a wt MET allele could not be detected in E98 cells (Fig. 2d).

In the MET protein, this rearrangement leads to loss of the C-terminus of IPT1 and the N-terminus of IPT2 (Fig. 3a). Yasara modeling, using X-ray crystallographic data of the MET ectodomain, predicts the formation of a novel IPT, composed of the remaining parts of IPT1 and

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Fig. 2 E98 MET contains an intronic deletion, resulting in a truncated transcript. a RT-PCR on cDNA from different cell lines using full-length MET primers, using HMBS as a housekeeping gene for reference. b Ion-torrent sequencing analysis exposes a high-copy amplification of MET in E98 DNA. Plotted on the x-axis are all 409 genes sequenced, in chromosomal order. The number of reads per gene was compared to blood-obtained DNA from a glioma patient as a reference. The dotted line represents the level of heterozygous losses, as can be seen for the X-chromosome (the reference blood sample was female, while the tumor DNA was male). The inset shows the relative number of reads per MET exon. Note the loss of exon 7 and part of exon 8. c FISH analysis of the number of MET (red) and chr.7 centromere (green) copies in the genome of an E98 xenograft. d Amplification of MET using exon 6- and 9-specific primers on genomic DNA of E98 and U87 cells. METΔ7-8 and wtMET amplification leads to 910- and 3041-bp products in E98 and U87, respectively. e Schematic overview of the METΔ7-8 deletion found in E98 cDNA. Splicing of exon 6 to exon 9 is indicated with the dotted orange lines. The orange solid line represents the deletion.
Interestingly, in the new IPT1/2 fusion, a small stretch of 5 extra amino acids loops out towards the Sema domain (Fig. 3b, arrow).

**METΔ7–8 is aberrantly processed**

MET is synthesized as a 190-kDa precursor protein which is proteolytically cleaved by furin between residues 307 and 308, to yield an extracellular α-chain of approximately 45 kDa, covalently linked via a disulfide bridge to the transmembrane β-chain. Reducing SDS-PAGE, followed by Western blot analysis of E98 cell extracts showed that the majority of MET protein was in a 180-kDa phosphorylated form, corresponding to the uncleaved truncated preform (Fig. 1b, arrow). In contrast, in A549 cells the matured cleaved MET protein was the predominant form (arrowhead in Fig. 1b). To investigate whether this was a specific feature of E98 cells, we analyzed the protein structure in cells after transfection with the full-length cDNAs encoding METwt or METΔ7–8. In both HEK-293T and TOV-112D cells, wtMET was properly processed to an α- and β-chain, indicating that these cells are not defective in furin-mediated processing. In contrast, METΔ7–8 was predominantly present in the uncleaved preform in both cell types (Fig. 4a, arrow). Thus, improper MET cleavage is an intrinsic property of METΔ7–8. Overexpression studies in HEK-293T and TOV-112D cells resulted in phosphorylated Y1234/1235 residues in both METwt and METΔ7–8 proteins (Fig. 4a, P-MET). Because both cell lines produce HGF (Fig. 1c), this may be a result of HGF-dependent autocrine activation.

MET is cleaved by the endoprotease furin, which is localized predominantly in the trans-Golgi network, but also in vesicles and near the plasma membrane [8, 29, 52]. To test whether the inefficient cleavage of METΔ7–8 in E98 cells is related to intracellular transport defects, we analyzed the subcellular localization of METΔ7–8 in detail via confocal microscopy. METΔ7–8 did not co-localize

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**Fig. 3** Predicted protein structure of METΔ7–8. a Schematic overview of MET and METΔ7–8. The red lined box represents the deletion, comprising parts of the IPT1 and 2 domains (represented by the red and yellow boxes, respectively, in METΔ7–8). b Yasara modeling of the remaining IPT1 and 2 domains in METΔ7–8 was performed based on wtMET IPT1. The wtMET Sema domain and IPTs are shown in blue, with the novel putative IPT domain consisting of IPT1 (red) and 2 (yellow) of METΔ7–8 superimposed on wtMET. An enlargement of the fusion part in the overview figure (arrow) is shown in the right panel. The arrow indicates the additional loop in red that is formed by the novel IPT domain.
with the cell surface marker CD44 and was confined to
the cytosol (Fig. 4b). In contrast, in U87 cells METwt did
co-localize with CD44. Additional intracellular staining
in U87 cells reflects de novo synthesized material that is
being processed for constitutive secretion. Immunostain-
ings with the early endosome marker EEA-1 and the rough
endoplasmic reticulum (RER) marker CLIMP-63 sug-

intact E98 and A549 cells or of biotinylated cell lysates. MET was
immunoprecipitated and analyzed on western blot for biotinylation,
as described in the materials and methods section. The α-chain of
MET is shown. e His-tagged ectodomains of both wild-type MET
and MET\textDelta7–8, biotinylated via a biotin-acceptor peptide at the car-
boxyterminus, were expressed in HEK-293T cells. Ni-purified culture
media and extracts of the transfected cells were used to analyze MET
localization. Both fractions were subjected to western blotting using
N-terminal MET antibodies, recognizing both the preform and the
cleaved α-portion of the ectodomain, and directly labeled streptavidin
to visualize the preform and the β-chain. GAPDH was used as a load-
ing control for the RIPA extracts (red signal at 36 KDa).
using specific antibodies, followed by SDS-PAGE/Western blot and staining for biotin and MET. Whereas METs’ N-terminal α-chain was readily biotinylated in E98 cell lysates, no detectable MET biotinylation occurred when intact cells were labeled (Fig. 4d, lane 1). In contrast, biotinylated MET was readily detected in A549 cells upon labeling of intact cells, as shown by the biotin-labeled α-chain (Fig. 4d, lane 3). Thus, these data confirm that METΔ7–8 is predominantly localized intracellular and is poorly exposed on the cell surface of E98 cells.

To further confirm a defect in intracellular trafficking of METΔ7–8, we analyzed secretion patterns of extracellular domains of wtMET or METΔ7–8 (ending with residue D929, numbering according to MET variant 2 (NP_000236.2), containing a C-terminal biotin tag. Whereas the extracellular domain of wtMET was properly processed and secreted into the culture medium (as illustrated by the presence of the 309-929 biotinylated extracellular β-chain and the MET25-308 α-chain, Fig. 4e, lane 4), no secreted MET products were found in medium of cells, transfected with the METΔ7–8 ectodomain (Fig. 4e, lane 3). Instead, all biotinylated MET products were located intracellularly (Fig. 4e, lane 1).

**185-kDa METΔ7–8 is not affected by antagonistic anti-MET antibodies but is inhibited by cabozantinib**

It was previously reported that incubation of A549 cells with VHH G2, a recombinant single-domain llama antibody against MET, results in low MET activation levels, while inhibiting the strong activation which is induced by HGF [22]. To test whether and how G2 affects phosphorylation of METΔ7–8, we treated serum-starved E98 cells with G2, either followed or not followed by HGF, using A549 cells as control. As shown in Fig. 5, HGF did not increase overall MET phosphorylation levels, although, interestingly, a slight activation was seen in the minority of processed β-fragment of MET. For G2, a similar effect was observed. Consistent with high overall MET phosphorylation levels in all samples, neither G2 nor HGF treatment resulted in altered levels of P-AKT and P-ERK1/2, both targets of MET. In A549 cells G2 and HGF induced MET phosphorylation, which increased levels of P-AKT and P-ERK1/2. Thus, in contrast to A549 cells that express METwt, E98 cells are not responsive to antibodies against or ligands of MET. However, both E98 and A549 cells responded well to the MET tyrosine kinase inhibitor cabozantinib (Fig. 5).

**Prevalence of METΔ7–8**

Data mining of the COSMIC and TCGA databases did not uncover the intronic deletion in gliomas and other tumor types (not shown). Introgene deletions spanning multiple exons are, however, in general more difficult to recognize in whole-exome sequencing (WES). Furthermore, the coverage of the exact location of the deletion is poor in the mostly used WES protocols, and as can be seen in Fig. 2b, even targeted sequencing reveals the mutation only after detailed bioinformatic analysis. We, therefore, assume that detection of METΔ7–8 requires dedicated PCR protocols.

PCR with deletion-spanning primers revealed the presence of the deletion-specific 105-bp fragment in E98 cDNA, while a number of other cell lines presented with the wt 345-bp amplicon only (Fig. 6a). Since frozen material from the patient tumor that was used to generate E98 is unavailable, we could unfortunately not obtain genomic DNA and cDNA of sufficient quality to confirm the presence of the mutation in the originating tumor. We did however perform immunohistochemistry on formalin-fixed, paraffin-embedded tumor material from this patient and observed a highly heterogeneous staining for MET, with only a small percentage of strongly positive tumor cells, apparently with intracellular staining (Fig. 6b). Of note, such immunostainings cannot discriminate between METwt and METΔ7–8. FISH analysis confirmed the presence of the MET amplification in the original tumor (Fig. 6c).

To investigate the prevalence of the METΔ7–8 mutation further, we performed the exon 6–9 PCR on cDNA, generated from a series of gliomas (n = 102) and a number of other tumor types in which MET has been suggested to play an important role (castration-resistant prostate carcinomas, n = 43; Ewing sarcoma, n = 21; rhabdomyosarcoma, n = 22) [24, 28]. METΔ7–8 was found in 6 out of 102 gliomas, both grade III and IV (5.8 %; 2 out of 5 anaplastic oligodendrogliomas, 2 out of 16 anaplastic astrocytomas and 2 out of 61 glioblastomas), and was not detected in the other tumor types tested (Table 1). All grade III tumors with METΔ7–8 were IDH1-R132H mutated. Most tumors...
that contained the mutation were heterozygous, containing also the wild-type transcript (example in Fig. 6d). Further research with higher numbers of patients is needed to investigate any correlations between MET$^{Δ7-8}$ and other molecular aberrations.

**Discussion**

A major problem in glioma treatment is diffuse growth in the neuropil, and it has been suggested that the MET oncogene is causally involved in this phenotype in subsets of tumors [16]. Several signal transduction pathways that are induced by MET activation are shared with other tyrosine kinase receptors and it is increasingly recognized that activation of MET bypasses the need for EGFR activation, generating resistance to EGFR-targeted therapies [13]. Also, there is an interesting crosstalk between MET and EGFR: the EGFR variant III (EGFRvIII) stimulates expression of both MET and its ligand HGF, possibly contributing to oncogenicity of EGFRvIII [16], but this phenomenon is counteracted by EGF-mediated activation of wild-type EGFR in the same complex [33].

We previously reported that MET is crucially implicated in proliferation, survival and migration of (EGFR-negative) E98 glioma cells in vitro. To further investigate the involvement of MET in the biological behavior of E98 cells, we Sanger sequencing of the smaller PCR products of one patient with glioma confirmed the deletion of exons 7 and 8 (not shown). Of note, due to the lack of high-quality genomic DNA from these clinical samples we could not discriminate in these samples whether MET$^{Δ7-8}$ resulted from a mutation or from exon skipping or alternative splicing, such as suggested for MET variants lacking exon 10 or 14 [37].

**Table 1** MET$^{Δ7-8}$ expression in glioma, sarcoma and CRPCs

| Tumor type | $Δ7/8$ occurrence |
|------------|-------------------|
| Glioma     | 6/102 (5.8 %)     |
| O-II       | 0/3               |
| A-II       | 0/7               |
| OA-II      | 0/2               |
| O-III      | 2/5 (40 %)        |
| A-III      | 2/16 (12.5 %)     |
| OA-III     | 0/5               |
| GBM        | 2/61 (3.3 %)      |
| Sarcoma    | 0/25              |
| Ewing tumor| 0/5               |
| Cell line  | 0/3               |
| Rhabdo tumor| 0/15             |
| Cell line  | 0/2               |
| CRPC       | 0/43              |

The incidence of MET$^{Δ7-8}$ in different subtypes and malignancy grades of diffuse glioma, sarcomas and castration-resistant prostate carcinomas (CRPC) O-II oligodendroglioma (WHO grade II), O-III oligodendroglioma (WHO grade III), A-II astrocytoma (WHO grade II), A-III anaplastic astrocytoma (WHO grade III), GBM glioblastoma

that contained the mutation were heterozygous, containing also the wild-type transcript (example in Fig. 6d). Further research with higher numbers of patients is needed to investigate any correlations between MET$^{Δ7-8}$ and other molecular aberrations.
attempted to knock down the protein by a lentiviral shRNA approach. Attempts to generate stable MET knockdowns using 2 different MET shRNAs failed since both shRNA encoding lentiviruses were lethal to E98 cells, in contrast to control viruses (data not shown). In agreement with a crucial role of MET for cell survival, we showed that treatment with the MET tyrosine kinase inhibitor cabozantinib prolongs survival of mice carrying orthotopic E98 xenografts [42]. Based on the observation that the pattern of MET phosphorylation in E98 xenografts was remarkably homogeneous in the absence of its ligand HGF, we analyzed the MET product in E98 cells in more detail and found that the protein is expressed as a truncated product, which lacks effective furin cleavage and is predominantly retained intracellular in its active, phosphorylated form. The truncation generates a novel IPT domain consisting of a fusion between the carboxyterminus of IPT1 and the amino terminus of IPT2. Modeling of the novel fusion IPT domain suggests that it adopts a similar structure as the IPT domains in wild-type MET, although a small stretch of extra amino acid residues is accommodated in a loop, extending towards the Sema domain. Since this loop approaches the furin cleavage site in the Sema domain, it may directly affect furin cleavage. Interestingly, the colorectal adenocarcinoma cell line Lovo lacks furin protease and consequently cannot properly process MET [40]. In this cell line, the unprocessed preform is still expressed on the cell surface, which lacks effective furin cleavage and is predominantly retained intracellular in its active, phosphorylated form. The truncation prevents MET from creating a novel IPT domain in METΔ7–8, which involves in defective furin cleavage, intracellular retention of the uncleaved form and auto-activation of the uncleaved preform.

Recently, effects of various activating mutations in the MET TK domain have been analyzed in detail. These mutations are expressed on the cell surface but are subject to increased rates of turnover, resulting in accumulation in early endosomes where they can still signal [27]. METΔ7–8 did not accumulate in early endosomes, suggesting that the underlying mechanism of intracellular retention is different [41]. Our confocal microscopy and biotinylation experiments strongly suggest that only the very small fraction of METΔ7–8 that is cleaved reaches the cell membrane, but this fraction is insignificant with respect to the auto-active component in the cytosol.

From a genetic perspective, the METΔ7–8 mutant resembles the auto-active EGFRvIII variant which occurs concomitantly with EGFR amplification in 25–64 % of all GBMs [51, 59]. This variant results from a genetic deletion of exons 2–7 and its activation is also independent of the ligands EGF or TNFα. Like the EGFRvIII mutation [15], the METΔ7–8 alteration appears to be restricted to only a small number of other tumor types. The mutation was detected in grade III gliomas of both oligodendrogial and astrocytic origin and GBMs. Investigations of METΔ7–8 expression in other tumor types is however warranted and is ongoing in our lab.

Another example of a tyrosine kinase receptor which often carries deletions in GBM is PDGFRA. Forty percent of the GBMs with amplified PDGFRA contain an in-frame deletion in the ectodomain, leading to constitutive activation of the TK domain [46]. Recently, also in pediatric high-grade gliomas, ligand-independent and tumorigenic in-frame PDGFRA deletion variants have been reported [47, 58]. Data on the subcellular localization of such mutated oncoreceptors are frequently lacking, and our data call for in-depth analysis of the cellular localization of receptors that are considered targetable. Interestingly, activating mutations in the RON tyrosine kinase receptor have been identified which resemble the METΔ7–8 mutation in that it also involves the first IPT domain [39]. Of note, these alterations do not lead to a loss of expression at the cell surface.

Importantly, we did not detect a METwt transcript in E98 cells, in contrast to the clinical gliomas which were analyzed in this study and which all showed abundant wtMET, also in METΔ7–8 tumors. We formally cannot exclude that the wild-type MET amplicons in our PCR derive from ‘contaminating’ non-neoplastic stromal cells in the tumor biopsies that were tested. There may, however, be another explanation for the relatively low levels of METΔ7–8 in clinical tumors: in the patient tumor from which the E98 model was generated, a low percentage of MET-expressing tumor cells was detected, although it is impossible to determine whether these cells carry the METΔ7–8 mutation because the antibodies used do not discriminate between MET and METΔ7–8. It is tempting to speculate that during the generation of the E98 model, a small subset of METΔ7–8 tumor cells in the primary tumor experienced a growth advantage, ultimately resulting in clonal outgrowth during xenograft formation. This scenario fits with the notion that clinical tumors not only show inter- but also intratumoral heterogeneity [54], and derived preclinical tumor models may only be representative for the most malignant population of tumor cells.

Expression of METΔ7–8 may have important consequences for choice of therapy. MET is increasingly recognized as an important target in multiple tumor types, including glioma, and therapeutic antibodies against HGF or the HGF binding site on MET have been developed. Since HGF is not involved in METΔ7–8 activation and METΔ7–8 is retained intracellular, MET-mutated cells will not be responsive to these therapies. Indeed, we were able to show that E98 cells do not respond to the anti-MET VHH G2. Selection of MET-mutated cells in tumors that
initially respond to antibody-based MET-directed treatment is expected to result in recurrence of treatment-resistant clones. In this respect, it will be important in future studies to assess the occurrence of the Δ7–8 mutation in paired samples of primary and recurrent tumors after MET antibody-based therapies, but also anti-EGFR therapies since cells may use METΔ7–8 to bypass EGFR signaling [3, 53]. With this in mind, the use of specific tyrosine kinase inhibitors of MET may have preference over antibody-based therapy for resistant tumors, at least in the ones that are KRAS and RAF wild type. [3, 21, 57, 61] since METΔ7–8 is sensitive to these inhibitors [42]. Such inhibitors have already shown to improve overall survival of patients with non-small cell lung carcinoma with MET amplification and renal papillary carcinoma with MET mutations [11, 45]. A recent clinical study with anti-MET antibody MetMab for lung cancer failed to meet the primary endpoint of prolonged survival [55]. A study of MET mutations and intracellular localization patterns in this patient group may be highly informative for future therapeutic directions [23].

In conclusion, we describe a highly active, non-ligand-dependent mutant of MET in 6% of gliomas, which is not exposed on the cell surface and is predicted to be non-targetable with therapeutic antibodies against MET and/or HGF.

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Conflict of interest The authors have no conflicts of interest to disclose.

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References

1. Asaoka Y, Tada M, Ikenoue T, Seto M, Imai M, Miyabayashi K, Yamamoto K, Yamamoto S, Kudo Y, Mohri D, Isomura Y, Iijichi H, Tateishi K, Kanai F, Ogawa S, Omata M, Koike K (2010) Gastric cancer cell line Hs746T harbors a splice site mutation of c-Met causing juxtamembrane domain deletion. Biochem Biophys Res Commun 394:1042–1046. doi:10.1016/j.bbrc.2010.03.120
2. Bao ZS, Chen HM, Yang MY, Zhang CB, Yu K, Ye WL, Hu BQ, Yan W, Zhang W, Akers J, Ramakrishnan V, Li J, Carter B, Liu YW, Hu HM, Wang Z, Li MY, Yao K, Qiu XG, Kang CS, You YP, Fan XL, Song WS, Li RQ, Su XD, Chen CC, Jiang T (2014) RNA-seq of 272 gliomas revealed a novel, recurrent PTPRZ1-MET fusion transcript in secondary glioblastomas. Genome Res. doi:10.1101/gr.165126.113
3. Bardelli A, Corso S, Bertotti A, Hobor S, Valtorta E, Siravegna G, Sartore-Bianchi A, Scala E, Cassingena A, Zecchin D, Apicella M, Migliardi G, Galimi F, Lauricella C, Zanon C, Perera T, Veronese S, Corti G, Amato A, Gambarcia M, Diaz LA Jr, Sausen M, Velesucale VE, Comoglio P, Trusolino L, Di Nicolantonio F, Giordano S, Siena S (2013) Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. Cancer Discov 3:658–673. doi:10.1158/2159-8290.CD-12-0558
4. Bax DA, Gaspar N, Little SE, Marshall L, Perryman L, Regairaz M, Viana-Pereira M, Vuonovirta R, Sharp SY, Reis-Filho JS, Stavale JN, Al-Sarraj S, Reis RM, Vassal G, Pearson AD, Gravde D, Ellision DW, Workman P, Jones C (2009) EGFRvIII deletion mutations in pediatric high-grade glioma and response to targeted therapy in pediatric glioma cell lines. Clin Cancer Res 15:5753–5761. doi:10.1158/1078-0432.CCR-08-3210
5. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C (1995) Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature 376:768–771. doi:10.1038/37678a0
6. Borowiak M, Garratt AN, Wustefeld T, Strehle M, Trautwein C, Birchmeier C (2004) Met provides essential signals for liver regeneration. Proc Natl Acad Sci 101:10608–10613. doi:10.1073/pnas.0403412101
7. Bourgonje AM, Navis AC, Schepens JT, Verrijp K, Hovestad L, Hilhorst R, Harroch S, Wesseling P, Leenders WP, Hendriks WJ (2014) Intracellular and extracellular domains of protein tyrosine phosphatase PTPRZ-B differentially regulate glioma cell growth and motility. Oncotarget 5:8690–8702
8. Bresnahan PA, Leduc R, Thomas L, Thorner J, Gibson HL, Brake AJ, Barr PJ, Thomas G (1990) Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. J Cell Biol 111:2851–2859
9. Cancer Genome Atlas Research N (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455:1061–1068. doi:10.1038/nature07385
10. Cancer Genome Atlas Research N (2014) Comprehensive molecular profiling of lung adenocarcinoma. Nature 511:543–550. doi:10.1038/nature13385
11. Choueiri TK, Vaishampayan U, Rosenberg JE, Logan TF, Harztark AL, Bukowski RM, Rini BI, Strinivas S, Stein MN, Adams LM, Ottesen LH, Laubscher KH, Sherman L, McDermott DF, Haas NB, Flaherty KT, Ross R, Eisenberg P, Meltzer PS, Merino MJ, Bottero DP, Linehan WM, Srinivasan R (2013) Phase II and biomarker study of the dual MET/VEGFR2 inhibitor foretinib in patients with papillary renal cell carcinoma. J Clin Oncol 31:181–186. doi:10.1200/JCO.2012.43.3383
12. Claes A, Schuring J, Boots-Sprenger S, Hendriks-Cornelissen S, Dekkers M, van der Kogel AJ, Leenders WP, Wesseling P, Jeuken JW (2008) Phenotypic and genotypic characterization of orthotopic human glioma models and its relevance for the study of anti-glioma therapy. Brain Pathol 18:423–433. doi:10.1111/j.1750-3639.2008.00141.x
13. Corso S, Giordano S (2013) Cell-autonomous and non-cell-autonomous mechanisms of HGF/MET-driven resistance to targeted therapies: from basic research to a clinical perspective. Cancer Discov 3:978–992. doi:10.1158/2159-8290.CD-13-0040
14. Ferracini R, Longati P, Naldini L, Vigna E, Comoglio PM (1991) Identification of the major autophosphorylation site of the Met/ hepatocyte growth factor receptor tyrosine kinase. J Biol Chem 266:19558–19564
15. Gan HK, Cvrljevic AN, Johns TG (2013) The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. FEBS J 280:5350–5370. doi: 10.1111/febs.12393

16. Garnett J, Chumbalkar V, Vaillant B, Gururaj AE, Hill KS, Latha K, Yao J, Priewe W, Colman H, Elferink LA, Bagler O (2013) Regulation of HGF by expression of DeltaEGFR-mediated c-Met activation in glioblastoma cells. Neoplasia 15:73–84

17. Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G (2012) Targeting MET in cancer: rationale and progress. Nat Rev Cancer 12:89–103. doi: 10.1038/nrc3205

18. Gherardi E, Youles ME, Miguel RN, Blundell TL, Iamele L, Gough J, Bandyopadhyay A, Hartmann G, Butler PJ (2003) Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. Proc Natl Acad Sci 100:12039–12044. doi: 10.1073/pnas.2034936100

19. Giordano S, Corso S, Conrotto P, Artigliani S, Gilestro G, Barberis D, Tamagnone L, Comoglio PM (2002) The sephamorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol 4:720–724. doi: 10.1038/ncb843

20. Giordano S, Di Renzo MF, Ferracini R, Chiado-Piat L, Comoglio PM (1988) p145, a protein with associated tyrosine kinase activity in a human gastric carcinoma cell line. Mol Cell Biol 8:3510–3517

21. Han CB, Ma JT, Li F, Zhao JZ, Jing W, Zhou Y, Zou HW (2012) EGFR and KRAS mutations and altered c-Met gene copy numbers in primary non-small cell lung cancer and associated stage N2 lymph node metastasis. Cancer Lett 314:63–72. doi: 10.1016/j.canlet.2011.09.012

22. Heukers R, Altintas I, Raghoenath S, De Zan E, Pepermans R, Schwall R, Amler L, Wickramasinghe D, Yauch R (2013) The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. FEBS Lett 583:25–29

23. Hirsch FR, Bunn PA Jr, Herbst RS (2014) “Companion diagnosis”: has their time come and gone? Clin Cancer Res 20:4422–4424. doi: 10.1158/1078-0432.CCR-14-0932

24. Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, Vollmer RT, Day ML (1995) Hepatocyte growth factor and its receptor (c-MET) in prostatic carcinoma. Am J Pathol 147:386–396

25. Jeffers M, Taylor GA, Weidner KM, Omtura S, Vande Woude GF (1997) Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol Cell Biol 17:799–808

26. Jo M, Stolz DB, Esplen JE, Dorko K, Michalopoulos GK, Strom SC (2000) Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem 275:8806–8811

27. Joffre C, Barrow R, Menard L, Calleja V, Hart IR, Kernogant S (2011) A direct role for Met endocytosis in tumorigenesis. Nat Cell Biol 13:827–837. doi: 10.1038/ncb2257

28. Knuedsen BS, Gmyrek GA, Inra J, Scherr DS, Vaughan ED, Nalus A, Kattan MW, Gerald WL, Vande Woude GF (2002) c-MET mutational analysis in non-small cell lung cancer. J Thorac Oncol 4:5–11. doi: 10.1097/0008-5472.JTO.0b013e3181913e0e

29. Komada M, Hatsuzawa K, Shibamoto S, Ito F, Nakayama K, Itamura N (1993) Proteolytic processing of the hepatocyte growth factor/scatter factor receptor by furin. FEBS Lett 328:25–29

30. Kong-Beltran M, Seshagiri S, Jha Z, Zhu W, Bhave K, Mendoa N, Holcomb T, Pujara K, Stinson J, Fu L, Severin C, Rangell L, Schwall R, Amler L, Wickramasinghe D, Jauch R (2006) Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res 66:283–289. doi: 10.1158/0008-5472.CAN-05-2749

31. Krieger E, Koraimann G, Vriend G (2002) Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. Proteins 47:393–402

32. Lee CC, Yamada KM (1994) Identification of a novel type of alternative splicing of a tyrosine kinase receptor. J Biol Chem 269:19457–19461

33. Li L, Puliypaddamba VT, Chakraborty S, Rehman A, Vemireddy V, Saha D, Souza RF, Hatnapa KJ, Koduru P, Burma S, Boothman DA, Habib AA (2015) EGFR wild type antagonizes EGFRVIII-mediated activation of Met in glioblastoma. Oncogene 34:129–134. doi: 10.1038/onc.2013.534

34. Longati P, Bardelli A, Ponzetto C, Naldini L, Comoglio PM (1994) Tyrosines1234–1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene 9:49–57

35. Lu KV, Chang JP, Parachonik CA, Pandika MM, Aghi MK, Meyronet D, Isachenko N, Fouse SD, Phillips JJ, Cheresu DA, Park M, Bergers G (2012) VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. Cancer Cell 22:21–35. doi: 10.1016/j.ccr.2012.05.037

36. Ma PC, Jagadeeswaran R, Jagadesh S, Tretiakova MS, Nalasura V, Fox EA, Hansen M, Schaefer E, Naoki K, Lader A, Richards W, Sugarbaker D, Husain AN, Christensen JG, Salgia R (2005) Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res 65:1479–1488. doi: 10.1158/0008-5472.CAN-04-2650

37. Ma PC, Kijima T, Mauilik G, Fox EA, Satller M, Griffin JD, Johnson BE, Salgia R (2003) c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res 63:6272–6281

38. Ma PC, Tretiakova MS, MacKinnon AC, Ramnath N, Johnson C, Dietrich S, Seiwert T, Christensen JG, Jagadeeswaran R, Krausz T, Yokes EE, Husain AN, Salgia R (2008) Expression and mutational analysis of MET in human solid cancers. Genes Chromosom Cancer 47:1025–1037. doi: 10.1002/gcc.20604

39. Ma Q, Zhang K, Guin S, Zhou YQ, Wang MH (2010) Deletion or insertion in the first immunoglobulin-plexin-transcription (IPT) domain differentially expresses expression and tumorigenic activities of RON receptor tyrosine kinase. Mol Cancer 9:507. doi: 10.1186/1476-4598-9-507

40. Mark MR, Lokker NA, Zioniacheck TF, Luis EA, Godowski PJ (1992) Expression and characterization of hepatocyte growth factor receptor-lg fusion proteins. Effects of mutations in the potential proteolytic cleavage site on processing and ligand binding. J Biol Chem 267:26166–26171

41. Mu FT, Callaghan JM, Steele-Mortimer O, Stenmark H, Par-eton RG, Campbell PL, McCluskey J, Yeo JP, Tock EP, Toh BH (1995) EGFRvIII: where wild things are contained by a self-parametrizing force field. Proteins 47:393–402

42. Muris D, Tamagnone L, Comoglio PM (1994) Tyrosines1234–1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene 9:49–57

43. Onozato R, Kosaka T, Kuwano H, Sekido Y, Yatabe Y, Mitsu-mori T (2005) EGFR and KRAS mutations and altered c-Met gene expression in lung cancers resected for stage I non-small cell lung cancer. J Thorac Oncol 4:5–11. doi: 10.1097/0008-5472.JTO.0b013e3181913e0e

44. Orhan-Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. Genes Dev 16:3074–3086. doi: 10.1101/gad.242602
45. Ou SH, Kwak EL, Siwak-Tapp C, Dy J, Bergethon K, Clark JW, Camidge DR, Solomon BJ, Maki RG, Bang YJ, Kim DW, Christensen J, Tan W, Wilner KD, Salgia R, Iafraite AJ (2011) Activity of crizotinib (PF02341066), a dual mesenchymal-epithelial transition (MET) and anaplastic lymphoma kinase (ALK) inhibitor, in a non-small cell lung cancer patient with de novo MET amplification. J Thorac Oncol 6:942–946. doi:10.1097/JTO.0b013e31821528d3
46. Ozawa T, Brennan CW, Wang L, Squatrito M, Sasayama T, Nakada M, Huse JT, Pedraza A, Utsuki S, Yasui Y, Tandon A, Fomchenko EI, Oka H, Levine RL, Fujiy K, Ladjani M, Holland EC (2010) PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas. Genes Dev 24:2205–2218. doi:10.1101/gad.197230
47. Paugh BS, Zhu X, Qi C, Endersby R, Diaz AK, Zhang J, Bax DA, Carvalho D, Reis RM, Onar-Thomas A, Broniscer A, Wetmore C, Zhang J, Jones C, Ellison DW, Baker SJ (2013) Novel oncogenic PDGFRA mutations in pediatric high-grade gliomas. Cancer Res 73:6219–6229. doi:10.1158/0008-5472.CAN-13-1491
48. Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Marioni JC, Curtis C, Watts C, Tavare S (2013) Intratumor growth-factor inhibitors. Onco Targets Ther 7:1001–1014. doi:10.2147/OTT.S44941
49. Petrelli A, Gilestro GF, Lanzardo S, Comoglio PM, Migone N, Giordano S, Graziani A, Panayotou G, Comoglio PM (1994) A multifunctional docking site mediates ligand-dependent downregulation of c-Met. Nature 416:187–190. doi:10.1038/416187a
50. Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Peschard P, Fournier TM, Lamorte L, Naujokas MA, Band H, Marioni JC, Curtis C, Watts C, Tavare S (2013) Intratumor growth-factor inhibitors. Onco Targets Ther 7:1001–1014. doi:10.2147/OTT.S44941
51. Saikali S, Avril T, Collet B, Hamlat A, Bansard JY, Drenou B, Kern HF, Klenk HD, Garten W (1995) Two independent target-sites for the ectodomain of human c-Met in human gliomas. J Neurooncol 81:139–148. doi:10.1007/s11060-006-9220-3
52. Schafer W, Stroh A, Berghofer S, Seiler J, Vey M, Kruse ML, Kern HF, Klenk HD, Garten W (1995) Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. EMBO J 14:2424–2435
53. Smyth EC, Scifani F, Cunningham D (2014) Emerging molecular targets in oncology: clinical potential of MET/hepatocyte growth-factor inhibitors. Oncotargets Ther 7:1001–1014. doi:10.2147/OTST.S44941
54. Sottomira A, Spiteri I, Piccirillo SG, Touloumis A, Collins VP, Marioni JC, Curtis C, Watts C, Tavare S (2013) Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics. Proc Natl Acad Sci 110:4009–4014. doi:10.1073/pnas.1219747110
55. Spigel DR, Ervin TJ, Ramlau RA, Daniel DB, Goldschmidt JH Jr, Blumenschein GR Jr, Krzakowski MJ, Robinet G, Godbert B, Barlesi F, Govindan R, Patel T, Orlov SV, Wertheim MS, Yu W, Zha J, Yauch RL, Patel PH, Phan SC, Peterson AC (2013) Randomized phase II trial of Onartuzumab in combination with erlotinib in patients with advanced non-small-cell lung cancer. J Clin Oncol 31:4105–4114. doi:10.1200/JCO.2012.47.4189
56. Tempest PR, Straton MR, Cooper CS (1988) Structure of the met protein and variation of met protein kinase activity among human tumour cell lines. Br J Cancer 58:3–7
57. Tian S, Simon I, Moreno V, Roepman P, Tabernero J, Snel M, van’t Veer L, Salazar R, Bernards R, Capella G (2013) A combined oncogenic pathway signature of BRAF, KRAS and PI3KCA mutation improves colorectal cancer classification and cetuximab treatment prediction. Gut 62:540–549. doi:10.1136/gutjnl-2012-302423
58. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O’Keeley M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM, Hayes DN, Cancer Genome Atlas Research N (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17:98–110. doi:10.1016/j.ccr.2009.12.020
59. Vriend G (1990) WHAT IF: a molecular modeling and drug design program. J Mol Graph 8(52–56):29
60. Viana-Pereira M, Lopes JM, Little S, Milanezi F, Basto D, Pardal F, Jones C, Reis RM (2008) Analysis of EGFR overexpression, EGFR gene amplification and the EGFRvIII mutation in Portuguese high-grade gliomas. Anticancer Res 28:913–920
61. Xia N, An J, Jiang QQ, Li M, Tan J, Hu CP (2013) Analysis of EGFR, EML4-ALK, KRAS, and c-MET mutations in Chinese lung adenocarcinoma patients. Exp Lung Res 39:328–335. doi:10.3109/01902148.2013.819535
62. Zhang YW, Su Y, Lanning N, Gustafson M, Shinomiya N, Zhao P, Cao B, Tsarfaty G, Wang LM, Hay R, Vande Woude GF (2005) EGFRvIII, IL-13Ralpha2, gp100 and TRP-2 for immunotherapy. Cancer Res 73:6219–6229. doi:10.1158/0008-5472.CAN-13-1491
63. Zhang YW, Su Y, Lanning N, Gustafson M, Shinomiya N, Zhao P, Cao B, Tsarfaty G, Wang LM, Hay R, Vande Woude GF (2005) EGFRvIII, IL-13Ralpha2, gp100 and TRP-2 for immunotherapy. Cancer Res 73:6219–6229. doi:10.1158/0008-5472.CAN-13-1491