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Enhanced expression and phosphorylation of the MET oncoprotein by glioma-specific PTPRZ1–MET fusions

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ABSTRACT

PTPRZ1–MET (ZM) proteins are a group of fusion proteins identified in human gliomas by high-throughput transcriptome sequencing. ZM fusions are associated with poor prognosis in afflicted glioma patients and mediate oncogenic effects in assays. In this study, we show that ZM-carrying patients have increased hepatocyte growth factor receptor (MET) mRNA expression levels induced by fusion with receptor-type tyrosine-protein phosphatase zeta (PTPRZ1). Furthermore, ZM fusions preserve fundamental properties of wild-type MET with respect to processing and dimerization, and enhance phosphorylation in an hepatocyte growth factor (HGF)-dependent and independent manner. Our findings suggest that ZM induces gliomas through elevated expression and phosphorylation of the MET oncoprotein.

1. Introduction

The genomic landscape features many types of human cancers with various alterations, including single nucleotide variations (SNVs), insertions and deletions (InDels), gene amplifications and translocations, which deregulate core cellular processes and signaling pathways in tumor cells [1]. Translocations, which may result in fusion transcripts and produce abnormal novel proteins, have been identified in almost every tumor type investigated, with the majority of fusion events associated with hematological malignancies [2,3]. However, with the advent of next generation sequencing (NGS) technologies, increasing numbers of recurrent fusion genes have been widely identified in solid tumors [4] as well as tumor-derived cell lines [5]. Among the translocations identified so far, many in-frame gene fusions obtain new oncogenic functions and contribute to cancer development. Alternatively, with one of the fusion partners being an oncogene, fusion leads to abnormal activation of the oncogene by several mechanisms [6], including fusing to a stronger promoter [7,8], deletion of a miRNA regulatory region [9], or escaping degradation [10].

Glioma whole genome and transcriptome sequencing has revealed a number of genomic rearrangements and fusion transcripts involving receptor tyrosine kinases (RTKs). FGFR3-TACC3 was first discovered in glioblastoma multiform (GBM) as an oncogene that induces constitutive kinase activity and triggers aneuploidy [9,11]. The most frequent in-frame fusions in GBM involve

Abbreviations: MET, hepatocyte growth factor receptor; PTPRZ1, receptor-type tyrosine-protein phosphatase zeta; ZM, PTPRZ1–MET fusion; HGF, hepatocyte growth factor; NGS, next generation sequencing; RTKs, receptor tyrosine kinases; GBM, glioblastoma multiform; FPKM, fragments per kilobase transcriptome per million fragments

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EGFR fused with several partners identified by large-scale whole genome and transcriptome sequencing [12,13]. For example, EGFR-SEPT14 is a well-studied gene fusion that constitutively activates STAT3 signaling [13].

Although extensive cancer NGS translocations have been identified for many cancers, the GBM translocation landscape is poorly understood. We examined 272 glioma patients at different pathological stages, and detected 214 fusion transcripts, including 67 in-frame fusions. Of these, previously identified fusions, including the recurrent FGFR3-TACC3 transcript, have also been detected in three primary GBMs. It is noteworthy that four novel recurrent RTK fusions of PTPRZ1–MET (ZM) have been identified in sequencing and validation cohorts, whereas the majority of fusions happen in secondary GBMs (6/40, 15%) [14].

The MET kinase, also known as hepatocyte growth factor (HGF) receptor, is a well characterized proto-oncogene that mediates invasion and metastasis in various tumor types [15]. Many carcinomas involve MET aberrance such as mutation, amplification, rearrangement, and transcriptional upregulation [16]. Data mining of The Cancer Genome Atlas (TCGA) transcriptome data revealed another ZM fusion in low-grade glioma [17]. Data suggest that ZM fusion-harboring tumors exhibit a more aggressive phenotype compared with ZM fusion-free ones. In addition, U87MG cells expressing ZM show increased migratory activity by transwell migration assay. Secondary GBM patients harboring ZM fusions have significantly compromised overall survival rates compared with those free of ZM fusions [14].

Although ZM fusions are proved to be oncogenic in cell lines, the molecular mechanisms underlining its oncogenicity motivated our further investigation. In this study, we examined patient ZM fusion RNA levels and examined dimerization and phosphorylation in transfected HEK293 cells. Our findings show that ZM fusions are activated by enhanced mRNA expression levels as well as highly phosphorylated at the kinase domain, in an HGF-independent manner.

2. Materials and methods

2.1. Expression level analysis

Quantitation of mRNA expression was analyzed using the Illumina HiSeq2000 transcriptome sequencing data of 272 grade II–IV glioma samples from the Chinese Glioma Genome Atlas (CGGA), under GEO accession number GSE48865. Expression analysis was performed using TCGA mRNA-seq pipeline for UNC data, with modifications. First, clean reads were aligned to hg19 human genome reference with MapSplice (v2.1.7) [18], followed by BAM file sorting with SAMtools (v0.1.9) [19]. RSEM (v1.2.15) [20] was then used to calculate sequencing read counts for each gene. Expression data from different samples were normalized by the Trimmed Mean of *M* values (TMM) method [21], and merged to an FPKM (fragments per kilobase transcriptome per million fragments) matrix for downstream analysis.

2.2. Expression constructs

Full-length ZM fusions were amplified from glioma cDNA using forward 5′-ATGCGAATCCTAAAGCGTTTCCTCG-3′ and reverse primers 5′-TGATGTCTCCCAGAAGGAGGCTG-3′ with Q5 DNA polymerase (New England Biolabs, Beverly, MA, USA). Wild-type MET was amplified with a different forward 5′-ATGAAGGCCCCCGCTGT GC-3′ but the same reverse primer 5′-TGATGTCTCCCAGAAGGAGG CTG-3′. PCR products were purified using QIAquick PCR purification kit (Qiagen, Germantown, MD, USA) and cloned into 3×FLAG CMV14 vector (Sigma–Aldrich, St. Louis, MO, USA) between *HindIII* and *KpnI* cloning sites.

2.3. Cell culture and transfection

HEK293 cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Foster City, CA, USA) supplemented with 10% Fetal Bovine Serum (FBS, Life Technologies) at 37 °C in a CO_2 incubator. Cells were seeded in 6-well plates and allowed to grow overnight to 60–80% confluence. ZM fusion vectors (2 μg) were individually mixed with 4-μl GenEscort II (Wisegen, Nanjing, China), incubated for 15 min at room temperature, and added to the cell medium. CMV14 vector was used as a control. Twenty-four hours after transfection, cells were collected for analysis.

2.4. Immunoprecipitation and immunoblotting

Cells were harvested with IP buffer (0.5% Triton X-100, 20 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT) together with PMSF and protease inhibitor cocktail (Sigma–Aldrich). After cell lysis and centrifugation, anti-FLAG M2-beads (Sigma–Aldrich) were added to the supernatant and incubated for 2 h. Beads were carefully washed 4 times with IP buffer, boiled in SDS gel-loading buffer, and analyzed by immunoblotting as described below.

For immunoblotting, 50-μg protein from whole cell lysates was quantified by bicinchoninic acid (BCA) assay, and subjected to SDS–PAGE separation. Resolved proteins were transferred to nitrocellulose membrane, probed with specific antibodies, and detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Primary antibodies were FLAG (M2) mouse antibody (Sigma–Aldrich), HA (F-7) mouse mAb (Santa Cruz Biotech, Dallas, TX, USA), MET (25H2) mouse mAb and phospho-MET (Tyr1234/1235) (D26) XP Rabbit mAb (both from Cell Signaling Technology, Danvers, MA, USA), and phosphotyrosine (4G10) mouse mAb (Merck Millipore, Billerica, MA, USA).

2.5. Crosslinking

Crosslinking studies were conducted as previously reported [22], with modifications. Briefly, cells were washed with cold PBS then treated with 1 mM crosslinking reagent sulfo-EGS (Pierce) for 2 h. After quenching with Tris buffer, cells were lysed and analyzed by western blotting.

2.6. Statistical analysis

Statistical analysis was performed with R (v3.0.3). A Wilcoxon rank sum test was used to evaluate expression levels between ZM fusions and ZM free subgroups. A two-tailed Student's *t*-test was used to evaluate expression and phosphorylation of WHO grade II–IV gliomas.

3. Results

3.1. Enhanced MET RNA expression by fusion with PTPRZ1

PTPRZ1 encodes the receptor-type tyrosine-protein phosphatase zeta, which is tissue-specific and highly expressed in the central nervous system (CNS) [23,24]. We performed scatter plot analysis of TMM normalized expression levels of MET and PTPRZ1 transcriptome sequencing data of 272 WHO grade II–IV gliomas (Fig. 1A). Compared with PTPRZ1, MET FPKM values appeared more diverse, ranging over nearly four orders of

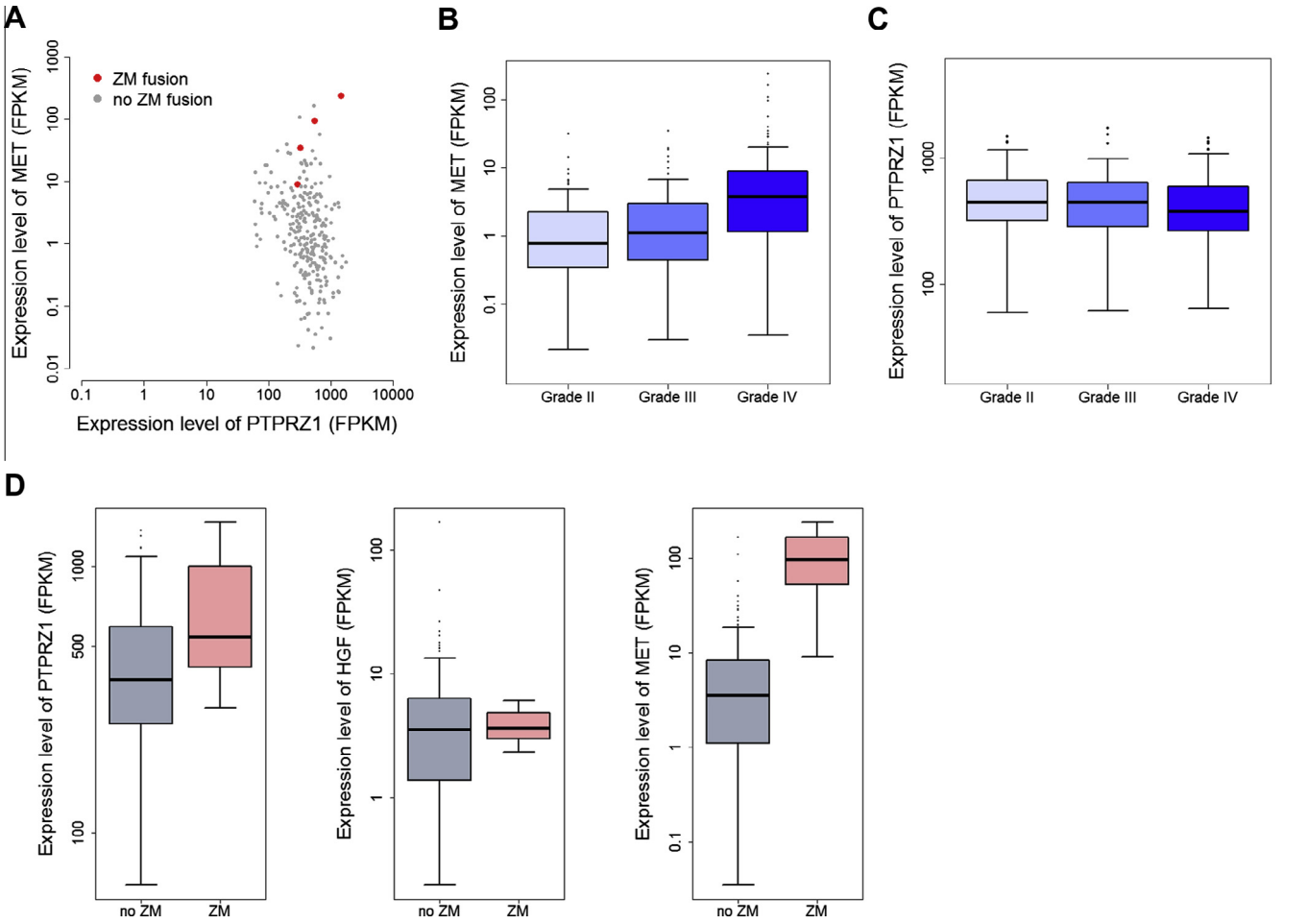

Fig. 1. MET expression levels were enhanced in ZM fusions based on transcriptome sequencing. Expression levels are presented with FPKM values on log10 scales. (A) Scatter plot of MET expression levels relative to that of PTPRZ1 from 272 WHO grade II–IV glioma samples. Red and gray dots indicate ZM-fusion and ZM-free samples, respectively. (B) MET and (C) PTPRZ1 expression levels among grade II (n = 73), III (n = 100), and IV (n = 99) gliomas shown as boxplots. (D) Boxplots of PTPRZ1, MET, and HGF expression levels between ZM-fusion (n = 3) and ZM-free (n = 96) grade IV samples.

magnitude. Previous studies showed that MET and HGF expression levels correlated with glioma grade [25,26]. In our sequencing cohort, MET mRNA expression was statistically higher in WHO grade IV gliomas (mean = 12.8, n = 99) compared with grade II (mean = 2.0, n = 73, P value <0.001, two-tailed Student's t test), and grade III gliomas (mean = 3.1, n = 100, P value <0.01, two-tailed Student's t test) (Fig. 1B). We did not observe a difference in PTPRZ1 between grades II, III, and IV (means of grade II, III, IV were equal to 505.3, 491.6 and 461.1, respectively, P value >0.1, two-tailed Student's t test) (Fig. 1C). To exclude expression variation from different grades, we analyzed MET expression of grade IV samples with and without ZM fusions (Fig. 1D). For samples afflicted with ZM fusions (n = 3, WHO grade IV), MET FPKM values were elevated compared with ZM fusion-free samples (n = 96, WHO grade IV, P-value <0.01, Wilcoxon rank sum test), whereas PTPRZ1 and HGF FPKM values did not differ (P-value = 0.2 and 0.4, respectively, Wilcoxon rank sum test). These results suggest that the PTPRZ1 promoter drives stimulation of MET mRNA, consistent with the fact that PTPRZ1 exhibits high endogenous CNS expression. Another possibility for increased MET expression is due to tandem duplication of the MET gene, which may also cause the fusion event. The enhanced MET expression among patients with ZM fusions is consistent with overexpressed MET protein based on western blot results [14].

MET expression levels were previously shown to correlate with glioma malignancy, as inhibition of MET and HGF leads to reduced tumor growth and tumorigenicity [27,28]. Enhanced MET expression in samples harboring ZM fusions might be a reason for shorter overall clinical survival of these patients. However, considering the possibility that enhanced ZM fusion is triggered by a strong PTPRZ1 promoter, it is important to test whether ZM fusions mediate oncogenic functions similar to or more deleterious than those of MET.

3.2. Expression and processing of ZM fusions in HEK293 cells

To investigate expression and processing of ZM fusion proteins, full-length ZM fusion constructs were expressed in HEK293 cells. Given the novel N-terminus amino acids introduced to MET by its fusion partner, it was not clear whether HGF binding affinity was affected. Therefore, we chose cell lines without detectable HGF mRNA or protein to help clarify fundamental characteristics of ZM proteins. HEK293 cell lysates expressing four fusion proteins (ZM_E1–E2, ZM_E2–E2, ZM_E3–E2, ZM_E8–E2), with a C-terminal FLAG-tag, were analyzed by SDS–PAGE and western blotting (Fig. 2A). Western blot analysis of controls showed two MET bands, consistent with previous studies [29]. The 170 and 140kDa bands correspond to unprocessed MET precursors and the beta-chain of mature MET, respectively. ZM fusions similarly expressed processed 140 and 170kDa products, which were likely MET signal peptide cleavage products (Fig. 2B). The ZM_E8–E2 construct contained an additional 314 amino acids relative to wild-type MET, leading to a 190kDa product corresponding to full-length fusion

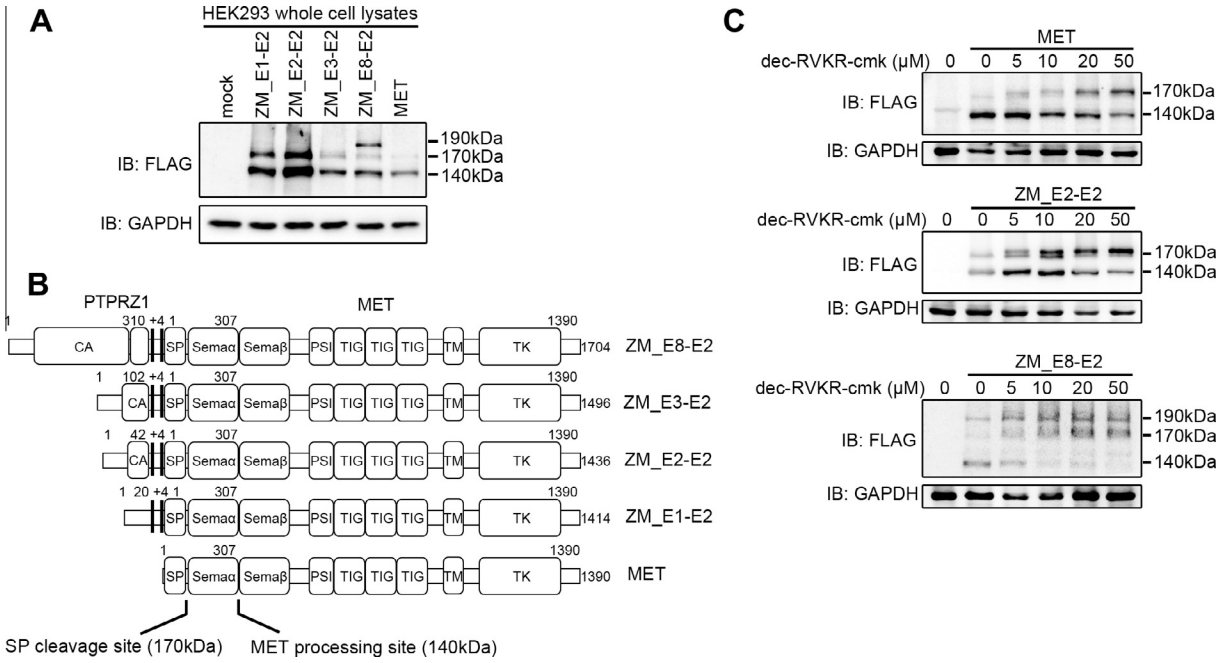

Fig. 2. ZM fusion proteins were processed by furin into mature MET. (A) Full-length ZM fusion and wild-type MET were amplified from tumor cDNA libraries, cloned into 3×FLAG CMV14 vector, and transfected into HEK293 cells. Twenty-four hours after transfection, cells were collected for western analysis. Expression of ZM fusions was detected using FLAG antibody. (B) Diagrammatic sketch of the ZM fusion constructs. The number indicates the length of the fusion protein or protein domains. Possible cleavage sites of single peptide and MET processing marked with corresponding molecular weight from cleavage site to C-terminus. CA, alpha-carbonic anhydrase; SP, signal peptide; TM, transmembrane domain; TK, tyrosine kinase. (C) After transfection, the furin inhibitor dec-RVKR-cmk was introduced into the cell medium at the indicated concentrations. Proteins were expressed for 24 h, followed by western blot analysis.

protein. Similarly, ZM_E3–E2 showed a band slightly larger than 170kDa, while ZM_E1–E2 and ZM_E2–E2 added 24 and 46 amino acids to MET, respectively, which were indistinguishable between 170kDa and full-length fusion proteins (only two bands were detected).

It is well known that the MET Sema domain is processed into alpha- and beta-subunits by the proprotein convertase furin [29]. To further confirm that the 140kDa band was processed MET, we added the furin inhibitor dec-RVKR-cmk into culture medium after transfection, at the indicated concentrations. Dec-RVKR-cmk inhibited MET and ZM-fusion processing in a dose-dependent manner. The 140kDa bands decreased as we increased the concentration of dec-RVKR-cmk from 0 to 50 μM, while full-length bands increased accordingly. Almost all processed ZM_E2–E2 and ZM_E8–E2 were inhibited at 50 μM (Fig. 2C). Inhibition assays confirmed that the 140kDa band was processed MET.

3.3. ZM fusions form homodimers and heterodimers with MET

Receptor tyrosine kinases, together with other families of cell surface receptors, are activated by dimerization or oligomerization [30]. Although it is well known that HGF binding is important for MET dimerization, recent studies reveal that in many types of carcinomas that overexpress MET protein, MET is activated in a ligand-independent manner [15,31]. The MET Sema domain is necessary for its dimerization and downstream signaling both in ligand-dependent and ligand-independent manners [22].

Since ZM fusions contain different length sequences, ranging from dozens to hundreds amino acids added to the N-terminus Sema domain, how dimerization is achieved between ZM fusions is not obvious. Sulfo-EGS is a crosslinking reagent that is cell impermeable and has been used to examine dimeric MET receptors in the cell membrane [22]. We treated cells expressing ZM-fusion

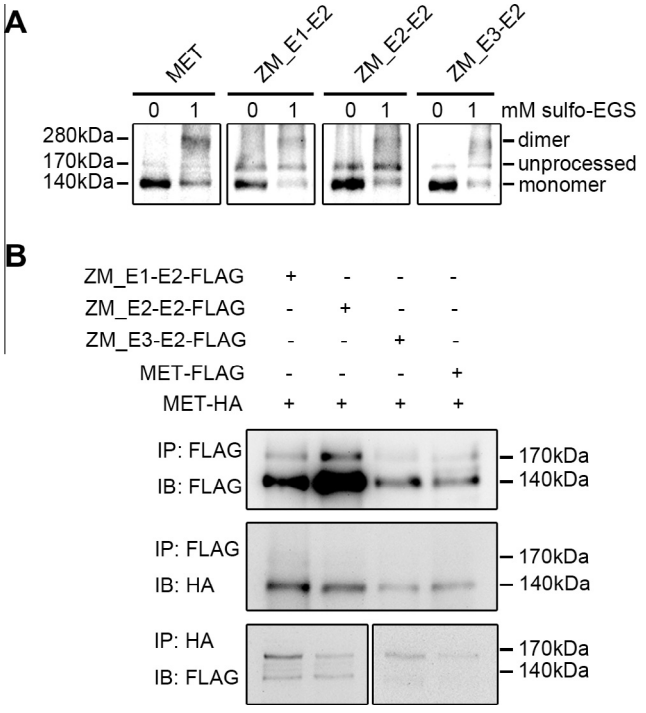

Fig. 3. Homodimer and heterodimer ZM fusions formed in HEK293 cells. (A) HEK293 expressing ZM-fusion proteins were exposed to crosslinking reagent sulfo-EGS (1 mM) for 2 h. After quenching with Tris buffer, cells were lysed and immunoblotted with FLAG antibody. (B) ZM-fusion FLAG tagged constructs were individually co-transfected with a MET-HA tagged construct into HEK293 cells. Cell lysates were immunoprecipitated and immunoblotted with either FLAG or HA antibodies.

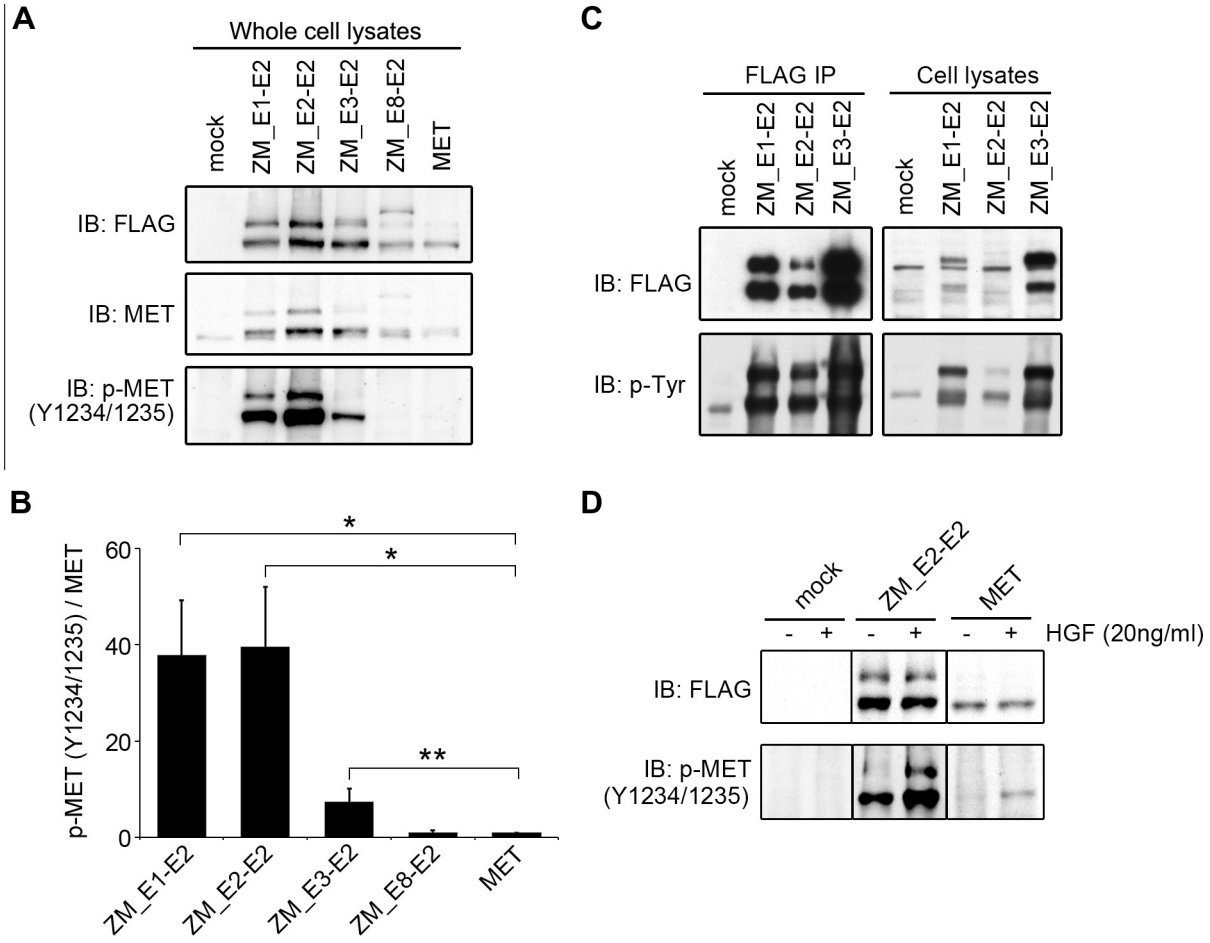

Fig. 4. ZM fusions were highly phosphorylated in transfected HEK293 cells. (A) ZM-fusion constructs were transfected into HEK293 cells and whole cell lysates analyzed with SDS–PAGE and immunoblotted with FLAG, MET, and p-MET (Y1234/Y1235) antibodies. (B) Phosphorylated-MET to MET ratio was quantified based on western blot results. Data are normalized to the ratio of MET and shown as mean ± S.E.M. ($n = 3$). $^{*}P < 0.01$, $^{**}P < 0.05$ (two-tailed Student's t test). (C) Whole cell lysates were immunoprecipitated with FLAG antibody and immunoblotted with p-Tyr antibody. (D) Cells expressing ZM fusion and wild-type MET were stimulated with HGF (20 ng/ml) for 1 h. Phosphorylation levels of ZM and MET were detected with FLAG and p-MET (Y1234/Y1235) antibodies.

and wild-type MET receptors with sulfo-EGS, followed by cell lysis and western blot analysis. Interestingly, levels of processed MET decreased in sulfo-EGS treated cells compared with those not exposed, while the unprocessed band was not affected (Fig. 3A). Meanwhile, a shifted band was detected at 280kDa in cross-linked samples, corresponding to the dimeric form of processed receptor. Both ZM fusion and wild-type MET showed similar patterns, indicating that processed ZM fusions and MET were present as membrane-bound dimers.

To test whether ZM fusions heterodimerize with wild-type MET, a FLAG-tagged ZM construct was co-transfected with a HA tagged MET construct. A 140kDa band corresponding to a ZM–MET heterodimer was detected by immunoprecipitation with anti-FLAG M2 beads and immunoblotting with anti-HA antibody, as well as by reverse immunoprecipitation with anti-HA beads and immunoblotting with anti-FLAG antibody (Fig. 3B). In addition to ZM–ZM homodimers, ZM–MET heterodimers have also been observed. Since dimerization is crucial for MET activity, this suggests that ZM fusion preserves receptor dimerization functionality, without being affected by the sequence fused to the MET N-terminus.

3.4. ZM fusions display enhanced phosphorylation levels in the absence of HGF

HGF, also known as scatter factor (SF), induces MET dimerization and activation in an autocrine and paracrine manner [15]. Activated MET, in which the core kinase domain residues Tyr1234 and Tyr1235 are phosphorylated, leads to auto-phosphorylation of Tyr1349 and Tyr1356. Subsequently, several cytoplasmic effector proteins, such as GAB1, GRB2, and PLC are recruited to phosphorylated tyrosine at the carboxyl-terminus. It is important to note that docking proteins attracted by GAB1, together with MET binding proteins, activate downstream signaling pathways [32].

It has been reported that without HGF stimulation, wild-type MET phosphorylation is low [33]. Since HEK293 cells show nearly undetectable HGF expression, it is reasonable to observe low MET phosphorylation levels at Tyr1234 and Tyr1235 residues. ZM fusion kinase domains were highly phosphorylated in HEK293 cells (Fig. 4A). To compare the phosphorylation levels of MET and ZM fusions, p-MET (Y1234/Y1235)/total-MET ratios were determined by densitometric analysis of western blots. ZM_E1–E2, ZM_E2–E2 and ZM_E3–E2 displayed elevated phosphorylation levels compared with wild-type MET ($P < 0.01$, 0.01 and 0.05, respectively, two-tailed Student's t test) (Fig. 4B), whereas ZM_E8–E2 phosphorylation was comparable with wild-type receptor. Phosphorylation

of ZM fusion proteins was confirmed by immunoprecipitation with FLAG antibody followed by immunoblotting with p-Tyr antibody (Fig. 4C). These results suggest that ZM fusions are phosphorylated in an HGF-independent manner. One possible explanation for the highly phosphorylated ZM_E1–E2, ZM_E2–E2, and ZM_E3–E2 proteins is that the peptide fused to the MET N-terminus functions as HGF.

Human recombinant HGF (20 ng/ml) was added to cell culture medium to test whether HGF activates ZM fusion proteins. After treatment, both wild-type and ZM_E2–E2 fusion proteins showed increased phosphorylation of the MET kinase domain (Fig. 4D). This suggests that HGF interacts with ZM fusions to enhance phosphorylation. To better understand the precise mechanisms of HGF and ZM interaction leading to enhanced phosphorylation, it will be important to elucidate the crystal structure of ZM bound with HGF.

4. Discussion

We have previously shown that glioma ZM fusions mediate oncogenic properties in U87MG cells, such as cell migration and invasion [14]. To better understand the mechanism of oncogenic growth, we analyzed ZM fusions at the transcription and protein levels. First, transcriptome analyses implied that enhanced MET expression is driven by the PTPRZ1 promoter, which increases transcription of fused MET in the CNS. Glioma HGF expression with and without ZM fusions suggests that enhanced MET expression levels in ZM harboring gliomas is not related to HGF regulation. Enhanced oncogene expression is mediated by its fusion partner, as also observed with NPM-ALK [8], NFASC-NTRK1, and BCAN-NTRK1 [34].

As a member of the receptor tyrosine kinase family, MET signaling has been well-studied, with receptor dimerization and phosphorylation of Tyr1234/Tyr1235 being important for MET activation [35]. Extensive analysis of MET-related gene fusions also shows that dimerization and kinase domains are preserved during fusion events. For example, TPR-MET [10] as well as several newly identified MET-related fusions [17] lose the Sema domain responsible for receptor dimerization. However, coiled–coil domains are fused to truncated MET to mediate fusion protein dimerization. We demonstrate that dimerization occurs as ZM–ZM homodimers and ZM–MET heterodimers, preserving the full-length MET sequence. In addition, we verified that ZM fusions possess characteristics similar to wild-type MET, related to furin-mediated processing. However, in the absence of HGF, ZM_E1–E2, ZM_E2–E2, and ZM_E3–E2, fusion proteins are distinguishable from MET by elevated phosphorylation levels. Therefore, our study provides evidence that ZM fusions are phosphorylated in an HGF independent manner. Together with our previous studies, this suggests that enhanced MET expression and phosphorylation is induced by fusion to PTPRZ1, which contributes to ZM oncogenic properties.

An increasing number of HGF–MET inhibitors have been developed for cancer therapy, which can be classified as extracellular inhibitors of HGF–MET and MET kinase inhibitors [16,32,36]. Pharmaceutical companies have devoted great effort to evaluating MET inhibitors in clinical studies [37]. Our previous results show that overall survival of secondary GBM patients harboring ZM fusions is significantly decreased compared with secondary GBM patients free of ZM fusions [14], suggesting that ZM fusions are a potential drug target for a subset of glioma patients. We have so far demonstrated that ZM fusions exhibit enhanced MET expression in tumor tissues and enhanced phosphorylation by cell assays. Therefore, our current results may help to develop MET kinase inhibitor therapy for secondary GBM patients harboring a ZM fusion, and this therapy may also help patients showing elevated MET kinase activity.

Acknowledgments

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