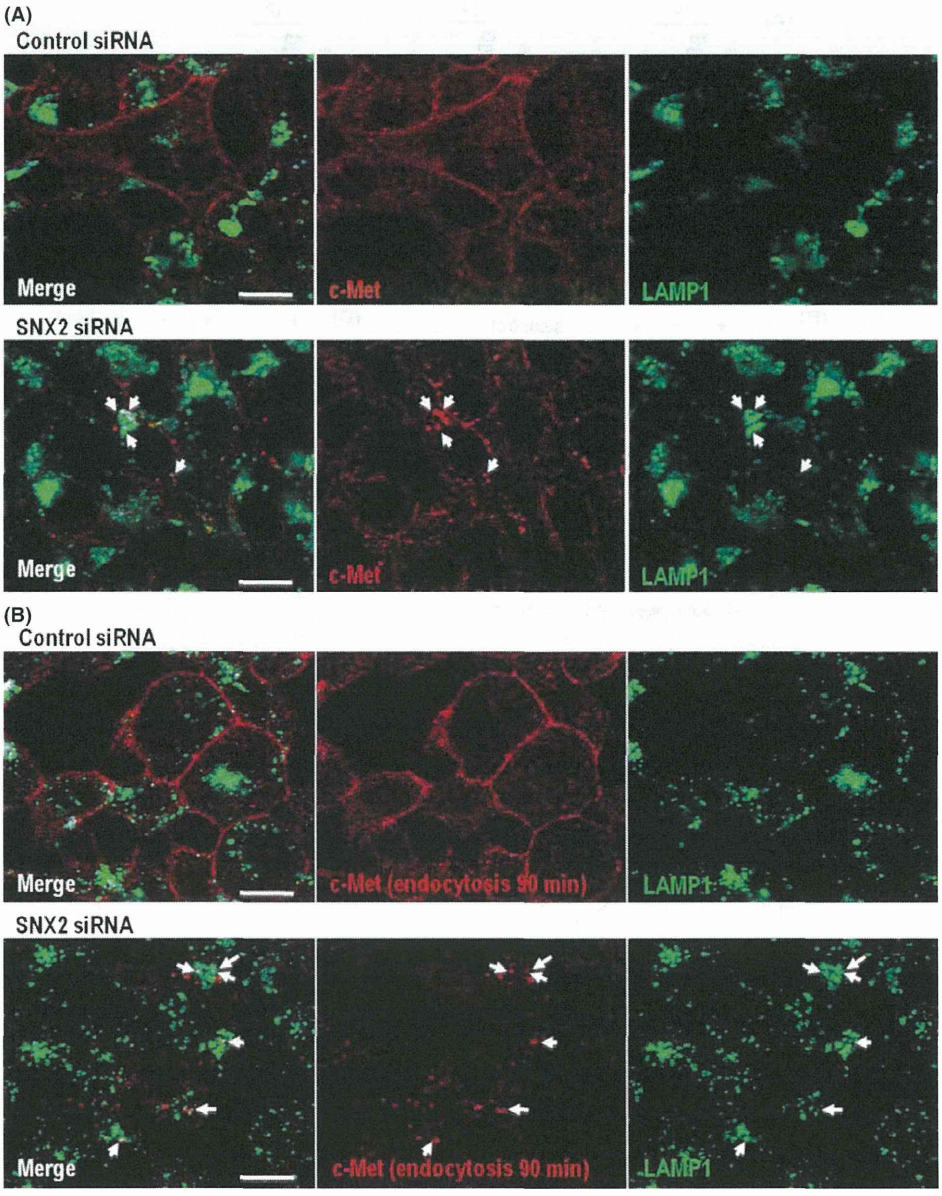

Fig. 6. Localization of c-Met protein in late endosome and/or lysosome by sorting nexin (SNX) 2 knockdown. Effect of SNX2 knockdown on c-Met expression in late endosome and/or lysosome. (A) Under SNX2 knockdown in the presence of lysosomal protease inhibitors, c-Met co-localized with lysosomal-associated membrane protein 1 (LAMP1) (arrows). (B) Under SNX2 knockdown in the presence of anti-c-Met antibody with lysosomal protease inhibitors for another 90 min, internalized antibody against c-Met also co-localized with LAMP1 (arrows).

EGFR in two cell lines, EBC-1 and H1993, which is consistent with a previous study.[17] The results from our present study also show that growth of EBC-1 or H1993 cells is closely dependent on the c-Met pathway, because they were highly susceptible to SNX2 siRNA-induced downregulation of c-Met. This also explains why EBC-1 and H1993 cells show high sensitivity to SU11274 but not to gefitinib or erlotinib (Fig. 8). In contrast, the growth of PC-9 cells was most closely dependent on the EGFR pathway, due to the presence of an activating mutation in exon 19 of the *EGFR* gene that sensitizes the cells to gefitinib.[3,17] Consistent with these previous reports, PC-9 cells showed higher sensitivity to gefitinib and lower sensitivity to SU11274 than EBC-1 or H1993 cells. Furthermore, 11–18 cells, which harbor an activating mutation in exon 21 of the *EGFR* gene, also showed higher sensitivity to gefitinib and lower sensitivity to SU11274 than EBC-1 cells. The growth of PC-9 or 11–18 cells was not affected by SNX2 siRNA, which suppressed the expression of c-Met but not that of EGFR.

C-Met amplification is one of the resistance mechanisms in EGFR-TKI. To demonstrate the importance of SNX2 in EGFR-TKI resistance, we used GR5 cells that harbor both EGFR mutation and c-Met amplification. As drug resistance to gefitinib in GR5 cells is expected to be mediated partially through c-Met amplification, SNX2 knockdown reduced expression of c-Met in GR5 cells accompanied by increased sensitivity to gefitinib. Akt phosphorylation was also suppressed by gefitinib under SNX2 knockdown (Fig. 7C). This overcoming effect of gefitinib resistance by SNX2 knockdown

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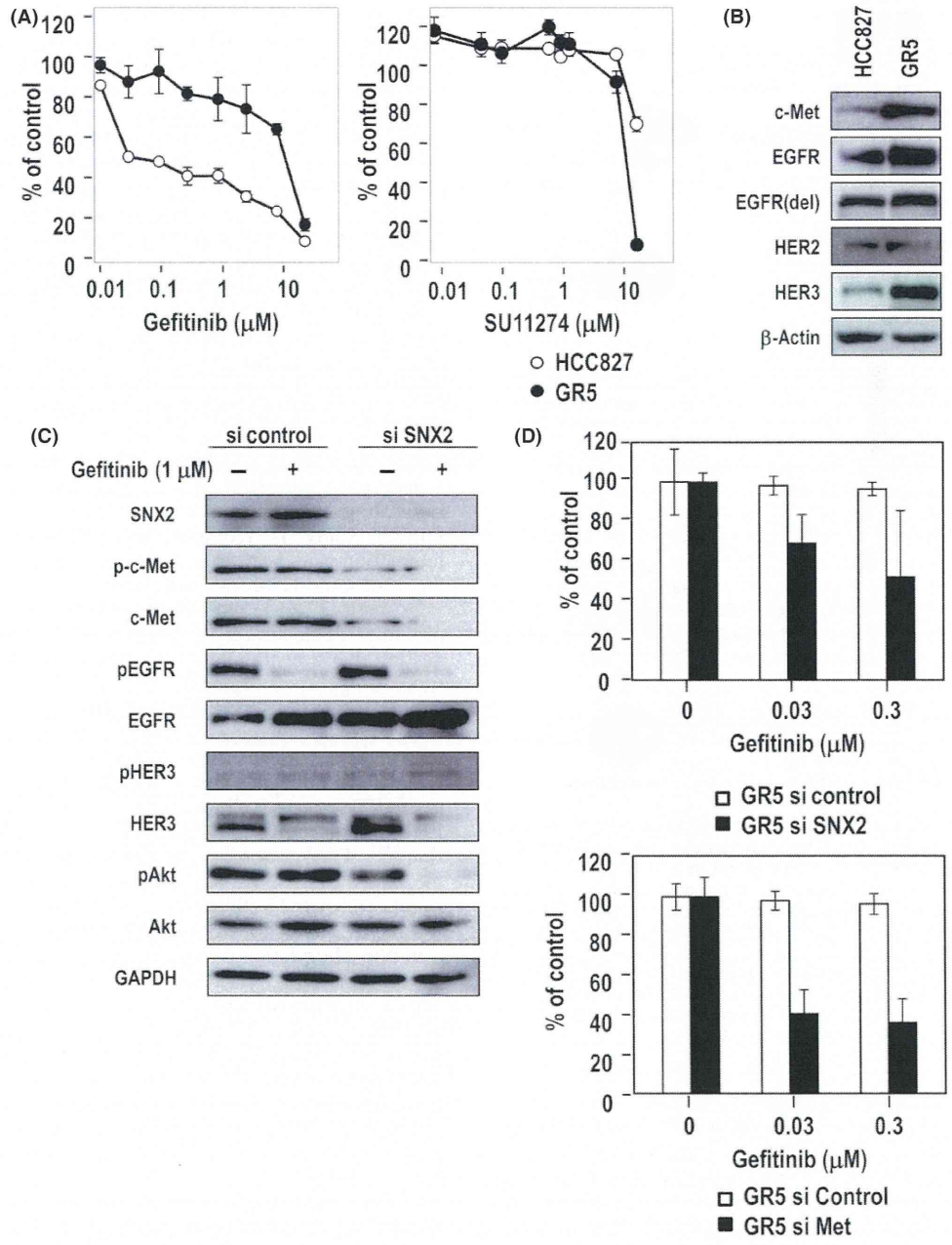

Fig. 7. Reversal effect of sorting nexin (SNX) 2 knockdown on gefitinib resistance in a lung cancer cell line harboring both activated epidermal growth factor receptor (EGFR) mutation and c-Met amplification. (A) Sensitivity of HCC827 and its gefitinib-resistant counterpart GR5 cells to gefitinib or SU11274 using a cell viability assay. (B) Expression of growth factor receptors determined using immunoblotting. Loading control, β-actin. (C) Effect of gefitinib on expression of phosphorylated c-Met (pc-Met), phosphorylated EGFR (pEGFR), phosphorylated human epidermal growth factor receptor (pHER) 2, pHER3, phosphorylated protein kinase B (pAkt) and pErk1/2 in SNX2-silenced GR5 cells determined using immunoblotting. (D) Altered drug sensitivity to gefitinib after treatment with SNX2 siRNA or c-Met siRNA, as determined using a cell viability assay.

strongly suggests an important role for SNX2 in acquiring drug resistance to EGFR-targeted drugs via c-Met amplification.

It was reported that SNX1 interacts with EGFR and EGFR degradation is enhanced in cells overexpressing SNX1,[8] suggesting its role for late endosome-to-lysosome trafficking.[8] However, in the present study we did not observe any apparent change in the expression of c-Met and EGFR or in the sensitivity to gefitinib in either EBC-1 or PC-9 cells following SNX1 knockdown (Fig. S1). This suggests that SNX1 does not play an essential role in the expression of EGFR and c-Met in our lung cancer cell lines. Gullapalli and colleagues previously reported that both SNX1 and SNX2 are involved in endosome-to-lysosome trafficking of EGFR and that EGFR degradation is also blocked by SNX2 knockdown, which enhances the expression of HER2 but not that of EGFR or HER3.[14] Together, these observations indicate that regulation of EGFR and its family proteins by SNX2 depletion is cell-type dependent and possibly involves other molecules.[9]

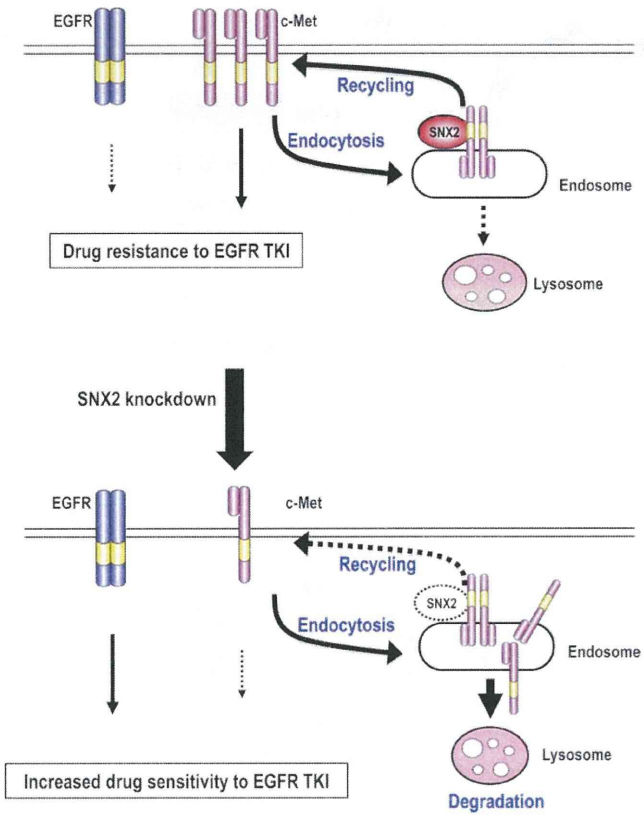

Fig. 8. Schematic model of sensitization to epidermal growth factor receptor (EGFR)-targeted drugs by sorting nexin (SNX) 2 knockdown in lung cancer cells. SNX2 promotes membrane trafficking of c-Met from recycling or early endosomes to the plasma membrane, resulting in activation of the c-Met signaling pathway and in drug resistance to EGFR tyrosine kinase inhibitors (TKI). Under SNX2 knockdown, expression of c-Met is downregulated through blockage of the SNX2-mediated recycling pathway together with promotion of the lysosome-induced degradation pathway. C-Met degradation by SNX2 knockdown is accompanied by compensatory activation of EGFR, resulting in a gain of drug sensitivity to EGFR TKI.

The specific control of c-Met by SNX2 is of particular interest. On stimulation by HGF, c-Met is rapidly internalized and transported through an early endosome followed by a late endosome and then it is degraded by the proteasome or non-proteasome pathway.[19–21] Schaaf *et al.*[16] first identified SNX2 as one of the various molecules that interact with c-Met. SNX2 is often localized in the early endosome and co-localizes with growth factor receptors such as EGFR and c-Met.[14,22] It was reported that intracellular degradation of c-Met by HGF stimulation was almost completely blocked by proteasome inhibitors such as concanamycin and lactacystin /MG132.[19,20] In the present study, downregulation of c-Met by SNX2 knockdown was not restored by MG132 (data not shown). Therefore, it seems less likely that the proteasome degradation pathway plays a key role in c-Met downregulation by SNX2 knockdown.

Western blot analysis showed that SNX2 siRNA induced downregulation of c-Met, but not EGFR, HER2, HER3 or TfR, suggesting selective downregulation of c-Met expression (Fig. 2). Although treatment with SNX2 siRNA resulted in a marked decrease in c-Met at the cell surface and degradation in late endosomes and lysosomes, localization of both EGFR and TfR was not affected (data not shown). Marked restoring of c-Met protein expression occurred with treatment by lysosomal inhibitors following SNX2 knockdown. As the SNX2 silencing conditions led to c-Met accumulation in late endosomes and lysosomes (Fig. 5A,B), the specific interaction between SNX2 and c-Met might be responsible for the rapid recycling of the c-Met protein to the cell surface from early endosomes (Fig. 8). Consistent with these biological cell findings, a cell proliferation assay demonstrated an apparent reduction of sensitivity to erlotinib when treated with a lysosomal/endosomal inhibitor (Fig. 5D), suggesting an important role for SNX2-driven c-Met trafficking in cellular sensitivity to EGFR-targeted drugs. In contrast, Casitas B lineage lymphoma (Cbl) protein was previously shown to limit the initial endocytotic step of EGFR and other growth factor receptors,[23] while the activity of the EGFR signaling complex decreased in a coordinate manner with Cbl after the exogenous addition of EGF/TGF-α.[24] Cbl might therefore be involved in the downregulation of c-Met by SNX2 knockdown. However, our previous study found no expression of Cbl protein in EBC-1 cells,[17] suggesting that Cbl is less likely to play any critical role. Further study is required to understand the SNX2/c-Met interaction and how this complex acts in close context with the endosome-lysosome pathway.

In both EBC-1 and H1993 cells, knockdown of SNX2 promotes compensatory activation of the EGFR pathway, suggesting that downregulation of c-Met results in a gain of sensitivity to gefitinib. Knockdown of c-Met also renders EBC-1 and H1993 cells sensitive to gefitinib (Fig. 8). Engelman and Jänne reported acquired resistance to gefitinib or erlotinib in lung cancer patients through gain of c-Met overexpression.[5] Bean and colleagues reported that NSCLC patients refractory to EGFR-targeted drugs harbor a representative secondary EGFR mutation of T790M, as well as c-Met gene amplification.[25] Furthermore, one could ask how the underlying mechanism of SNX2 knockdown-induced c-Met downregulation is correlated with increased sensitivity to gefitinib or erlotinib. SNX2 knockdown blocked activation of PI3K/Akt and also phosphorylation of EGFR, HER2 and HER3 in EBC-1 and H1993 cells (Fig. 2A,B). In both cell lines, cell proliferation and drug sensitivity to gefitinib, erlotinib and SU11274 were affected by SNX2 knockdown (Fig. 3). Figure 4 showed more marked inhibition of Akt and Erk1/2 phosphorylation by gefitinib when SNX2 was silenced. Taken together, SNX2 knockdown-induced c-Met downregulation might activate the alternative EGFR/HER2/HER3-driven PI3K/Akt signaling pathway resulting in sensitization of cancer cells to EGFR-targeted drugs.

Compartmentalization of signals by endocytosis of growth factors has been recently reported to determine various cell functions of normal and cancer cells.[26–28] In the concept of 'signaling endosome', the endocytic process might play a key role in cell migration, cell survival and malignant transformation.[29,30] Overexpression of a clathrin-associated protein named Huntingtin interacting protein 1 altered EGFR trafficking and also tumorigenesis by cancer cells.[31] A recent study by Joffre *et al.*[32] has first presented a close link between malignant transformation and impaired endosomal degradation. C-Met activating mutations such as M1268T and D1246N exhibited increased endocytosis or recycling activity and decreased levels of degradation. These two activating mutations in the kinase domain of c-Met lead to increased levels of c-Met endocytosis/recycling and reduced levels of c-Met degradation, resulting in malignant transformation, and the blocking of endocytotic degradation of mutant c-Met suppresses cell migration and metastasis by cancer cells.[32] Membrane trafficking of c-Met protein might therefore play an important role not only in malignant transformation and metastasis[32] but

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also in determination of drug sensitivity to molecular-targeted drugs (the present study).

In conclusion, SNX2 was shown to control expression and localization of c-Met, possibly through the endosome-lysosome pathway. Downregulation of c-Met expression by SNX2 knockdown resulted in a marked loss of sensitivity to a c-Met-targeted drug, leading to activation of alternatives to the EGFR signaling pathway. These results indicate that SNX2 could be a novel molecular target in the development of novel optimized anticancer treatments.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Spiro SG, Silvestri GA. One hundred years of lung cancer. *Am J Respir Crit Care Med* 2005; **172**: 523–9.
2 Lynch TJ, Bell DW, Sordella R *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004; **350**: 2129–39.
3 Paez JG, Jänne PA, Lee JC *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.
4 Pao W, Miller V, Zakowski M *et al.* EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A* 2004; **101**: 13306–11.
5 Engelman JA, Jänne PA. Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Clin Cancer Res* 2008; **14**: 2895–9.
6 Engelman JA, Zejnullahu K, Mitsudomi T *et al.* MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007; **316**: 1039–43.
7 Yano S, Wang W, Li Q *et al.* Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008; **68**: 9479–87.
8 Kurten RC, Cadena DL, Gill GN. Enhanced degradation of EGF receptors by a sorting nexin, SNX1. *Science* 1996; **272**: 1008–10.
9 Worby CA, Dixon JE. Sorting out the cellular functions of sorting nexins. *Nat Rev Mol Cell Biol* 2002; **3**: 919–31.
10 Haft CR, de la Luz Sierra M, Barr VA, Haft DH, Taylor SI. Identification of a family of sorting nexin molecules and characterization of their association with receptors. *Mol Cell Biol* 1998; **18**: 7278–87.
11 Griffin CT, Trejo J, Magnuson T. Genetic evidence for a mammalian retromer complex containing sorting nexins 1 and 2. *Proc Natl Acad Sci U S A* 2005; **102**: 15173–7.
12 Cozier GE, Carlton J, McGregor AH *et al.* The phox homology (PX) domain-dependent, 3-phosphoinositide-mediated association of sorting nexin-1 with an early sorting endosomal compartment is required for its ability to regulate epidermal growth factor receptor degradation. *J Biol Chem* 2002; **277**: 48730–6.
13 Carlton J, Bujny M, Peter BJ *et al.* Sorting nexin-1 mediates tubular endosome-to-TGN transport through coincidence sensing of high-curvature membranes and 3-phosphoinositides. *Curr Biol* 2004; **14**: 1791–800.
14 Gullapalli A, Garrett TA, Paing MM, Griffin CT, Yang Y, Trejo J. A role for sorting nexin 2 in epidermal growth factor receptor down-regulation: evidence for distinct functions of sorting nexin 1 and 2 in protein trafficking. *Mol Biol Cell* 2004; **15**: 2143–55.
15 Cavet ME, Pang J, Yin G, Berk BC. An epidermal growth factor (EGF)-dependent interaction between GIT1 and sorting nexin 6 promotes degradation of the EGF receptor. *FASEB J* 2008; **22**: 3607–16.
16 Schaaf CP, Benzing J, Schmitt T *et al.* Novel interaction partners of the TPR/MET tyrosine kinase. *FASEB J* 2005; **19**: 267–9.
17 Ono M, Hirata A, Kometani T *et al.* Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol Cancer Ther* 2004; **3**: 465–72.
18 Yoshida T, Okamoto I, Okamoto W *et al.* Effects of Src inhibitors on cell growth and epidermal growth factor receptor and MET signaling in gefitinib-resistant non-small cell lung cancer cells with acquired MET amplification. *Cancer Sci* 2010; **101**: 167–72.
19 Hammond DE, Urbé S, Vande Woude GF, Clague MJ. Down-regulation of MET, the receptor for hepatocyte growth factor. *Oncogene* 2001; **20**: 2761–70.
20 Kermorgant S, Zicha D, Parker PJ. Protein kinase C controls microtubule-based traffic but not proteasomal degradation of c-Met. *J Biol Chem* 2003; **278**: 28921–9.
21 Foveau B, Ancot F, Leroy C *et al.* Down-regulation of the met receptor tyrosine kinase by presenilin-dependent regulated intramembrane proteolysis. *Mol Biol Cell* 2009; **20**: 2495–507.
22 Carlton JG, Bujny MV, Peter BJ *et al.* Sorting nexin-2 is associated with tubular elements of the early endosome, but is not essential for retromer-mediated endosome-to-TGN transport. *J Cell Sci* 2005; **118**: 4527–39.
23 Yarden Y. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer* 2001; **37**: 3–8.
24 Ettenberg SA, Magnifico A, Cuello M, *et al.* Cbl-b-dependent coordinated degradation of the epidermal growth factor receptor signaling complex. *J Biol Chem* 2001; **276**: 27677–84.
25 Bean J, Brennan C, Shih JY *et al.* MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci USA* 2007; **104**: 20932–7.
26 Teis D, Wunderlich W, Huber LA. Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. *Dev Cell* 2002; **3**: 803–14.
27 Palamidessi A, Frittoli E, Garré M *et al.* Endocytic trafficking of Rac is required for the spatial restriction of signaling in cell migration. *Cell* 2008; **134**: 135–47.
28 Schenck A, Goto-Silva L, Collinet C *et al.* The endosomal protein App 11 mediates Akt substrate specificity and cell survival in vertebrate development. *Cell* 2008; **133**: 486–97.
29 Le Roy C, Wrana JL. Signaling and endocytosis: a team effort for cell migration. *Dev Cell* 2005; **9**: 167–8.
30 Polo S, Di Fiore PP. Endocytosis conducts the cell signaling orchestra. *Cell* 2006; **124**: 897–900.
31 Rao DS, Bradley SV, Kumar PD *et al.* Altered receptor trafficking in Huntingtin Interacting Protein 1-transformed cells. *Cancer Cell* 2003; **3**: 471–82.
32 Joffre C, Barrow R, Ménard L, Calleja V, Hart IR, Kermorgant S. A direct role for Met endocytosis in tumorigenesis. *Nat Cell Biol* 2011; **13**: 827–37.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Drug sensitivities to gefitinib, erlotinib and SU11274 under sorting nexin (SNX) 1 knockdown. (A) Effect of SNX1 knockdown by SNX1 siRNA, as determined using immunoblotting. (B) Effect of SNX1 knockdown on expression of c-Met and EGFR in EBC-1 and PC-9 cells, as determined using immunoblotting. (C–E) Drug sensitivity of SNX1 siRNA-treated EBC-1 and PC-9 cells to gefitinib (C), erlotinib (D) and SU11274 (E), as determined using a cell viability assay.

MET amplification as a potential therapeutic target in gastric cancer

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ABSTRACT:

Our aim was to investigate both the prevalence of MET amplification in gastric cancer as well as the potential of this genetic alteration to serve as a therapeutic target in gastric cancer. MET amplification was assessed by initial screening with a PCR-based copy number assay followed by confirmatory FISH analysis in formalin-fixed, paraffin-embedded specimens of gastric cancer obtained at surgery. The effects of MET tyrosine kinase inhibitors (MET-TKIs) in gastric cancer cells with or without MET amplification were also examined. The median MET copy number in 266 cases of gastric cancer was 1.7, with a range of 0.41 to 21.3. We performed FISH analysis for the 15 cases with the highest MET copy numbers. MET amplification was confirmed in the four assessable cases with a MET copy number of at least 4, whereas MET amplification was not detected in those with a gene copy number of <4. The prevalence of MET amplification was thus 1.5% (4 out of 266 cases). Inhibition of MET by MET-TKIs resulted in the induction of apoptosis accompanied by attenuation of downstream MET signaling in gastric cancer cell lines with MET amplification but not in those without this genetic change. MET amplification identifies a small but clinically important subgroup of gastric cancer patients who are likely to respond to MET-TKIs. Furthermore, screening with a PCR-based copy number assay is an efficient way to reduce the number of patients requiring confirmation of MET amplification by FISH analysis.

INTRODUCTION

Gastric cancer is the third most common cause of death from malignant disease in men (fifth in women) worldwide [1]. The prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor, with a median survival time of less than 1 year in individuals receiving conventional therapy [2-8]. The combination of trastuzumab, an antibody targeted to HER2, with chemotherapy has yielded a survival benefit for patients with HER2-positive gastric or gastro-esophageal junction cancer [7], with HER2-positive tumors accounting for 7 to 17% of all gastric cancers [9-11]. Further research is thus warranted to identify new therapeutic targets for gastric cancer patients.

The *MET* proto-oncogene encodes the receptor tyrosine kinase c-MET. The binding of its ligand, hepatocyte growth factor, to MET results in tyrosine phosphorylation of the receptor and activation of downstream signaling molecules. Oncogenic activation of *MET* suppresses apoptosis and promotes cell survival, proliferation, migration, and differentiation as well as gene transcription and angiogenesis [12]. In gastric cancer, such activation of *MET* has been attributed to

gene amplification [13-15]. However, the prevalence of *MET* amplification has varied among studies [13-21], possibly as a result of differences in the methods applied. This uncertainty led us to determine the prevalence of *MET* amplification in 266 formalin-fixed, paraffin-embedded (FFPE) specimens of gastric cancer obtained during surgery. To ensure the efficient detection of *MET* amplification, we adopted a sequential approach involving PCR-based determination of gene copy number followed by confirmatory FISH analysis. Moreover, to assess the potential of *MET* amplification as a therapeutic target in gastric cancer, we investigated its impact on cell survival and signal transduction.

RESULTS

MET amplification in gastric cancer cell lines

We first applied FISH (Figure 1A) and a real-time PCR–based method (Figure 1B) to examine *MET* copy number in gastric cancer cell lines whose *MET* amplification status was previously determined [22]. In gastric cancer cell lines negative for *MET* amplification, including KATO III, SNU1, SNU216, MKN1, MKN7, HSC39, MKN28, and NUGC3, the copy number of *MET* as determined by the PCR-based assay ranged between 1.3 and 3.3. In contrast, cell lines positive for *MET* amplification, including Hs746T, MKN45, and SNU5, showed *MET* copy numbers of 21.3, 21.3, and 17.9, respectively. The PCR-based assay thus revealed a high copy number for *MET* only in gastric cancer cell lines previously shown to be positive for *MET* amplification by FISH.

MET amplification in gastric cancer specimens

To determine the prevalence of *MET* amplification in advanced gastric cancer, we examined 266 FFPE specimens of surgically resected primary gastric tumors. Most of the patients were male (68.8%) and had undifferentiated-type gastric cancer (62.8%), including mucinous adenocarcinoma, signet ring cell adenocarcinoma, and poorly differentiated

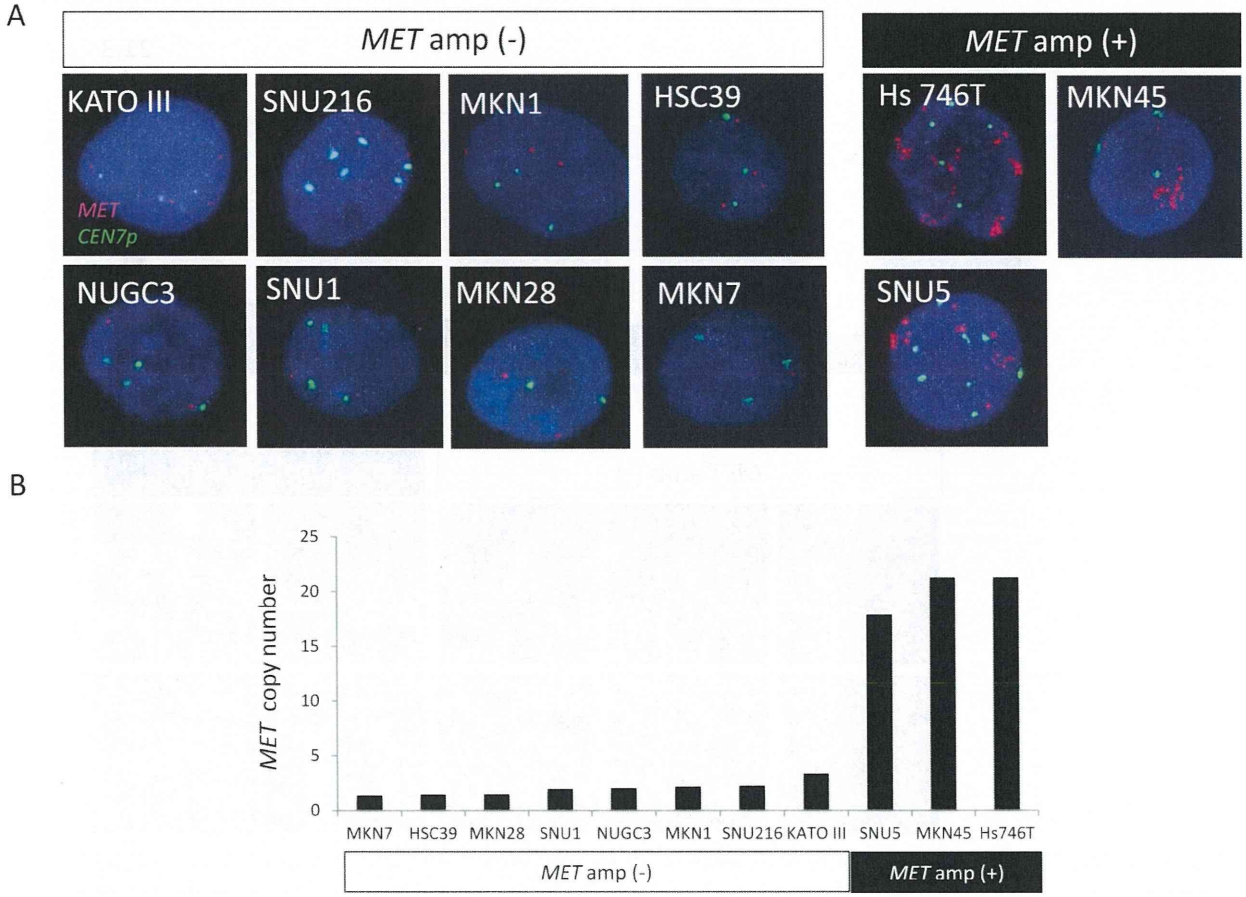

Figure 1: Amplification of *MET* in gastric cancer cell lines. *A*, FISH analysis of cell lines positive or negative for *MET* amplification (amp). Each image shows a single cancer cell, with green and red signals corresponding to CEN7p and the *MET* locus, respectively. *B*, Evaluation of *MET* copy number in gastric cancer cell lines with a PCR-based assay.

Table 1: Characteristics of the 266 study patients

Characteristic	n
Sex	
Male	183 (68.8%)
Female	83 (31.2%)
Pathological stage	
I	25 (9.4%)
II	31 (11.7%)
III	77 (28.9%)
IV	133 (50.0%)
Histology	
Differentiated type	99 (37.2%)
Undifferentiated type	167 (62.8%)

The patients had a median age of 63 years (range, 31 to 91 years).

adenocarcinoma (Table 1). The median age was 63 years, with a range of 31 to 91 years.

The PCR-based assay revealed that the median *MET* copy number for the 266 cases was 1.7, with a range of 0.41 to 21.3 copies (Figure 2A). Given that gastric cancer cell lines with *MET* amplification have been found to have a high copy number for *MET* [23], we arranged all cases in the order of *MET* copy number and performed FISH analysis for the 15 cases with the highest copy numbers (Table 2). *MET* amplification was detected by FISH in four of these cases (G72, G289, G322, and G181), which had a *MET* copy number of at least 4, whereas six cases

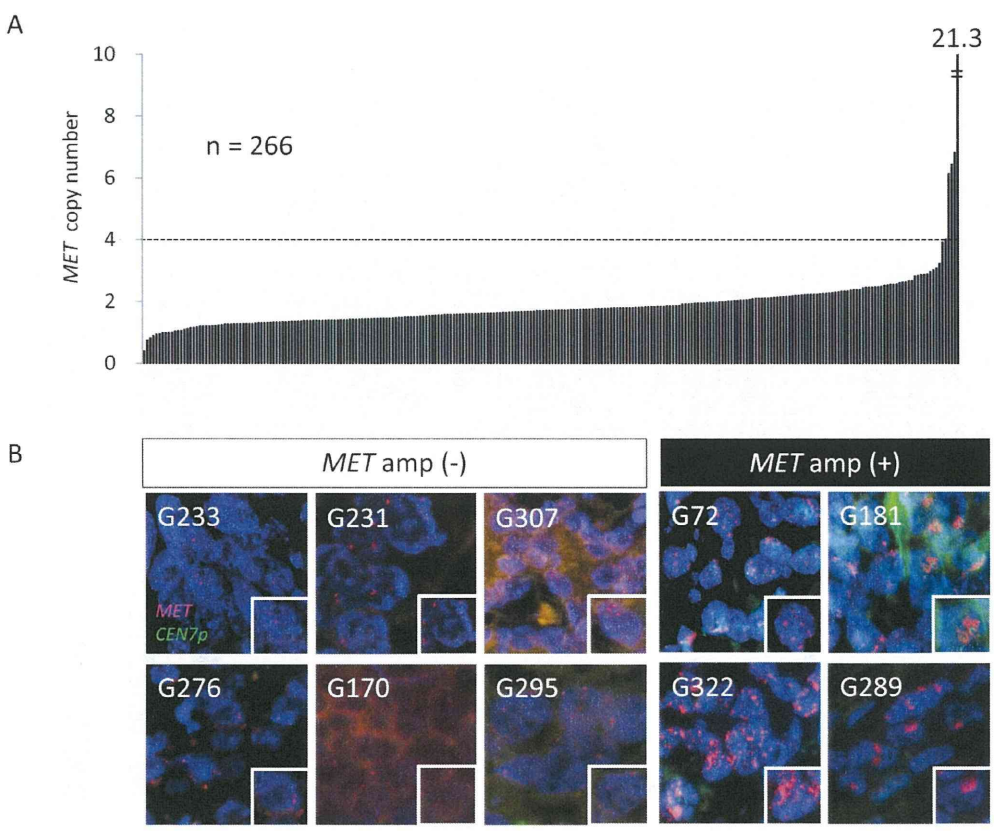

Figure 2: Amplification of *MET* in surgical specimens of gastric cancer. *A*, *MET* copy number determined with a PCR-based assay for 266 FFPE surgical specimens of gastric cancer. A *MET* copy number of >4 was observed in five cases. *B*, FISH analysis of gastric cancer specimens among the 15 samples with the highest *MET* copy numbers as determined with the PCR-based assay. Green and red signals correspond to CEN7p and the *MET* locus, respectively. Higher magnification images of individual cancer cells are shown in the insets. The specimens are grouped into those determined to be positive of negative for *MET* amplification by FISH.

(G276, G233, G295, G170, G307, and G231) with a copy number of less than 4 did not exhibit *MET* amplification (Figure 2B, Table 2). The remaining five cases (G331, G223, G217, G118, and G42) were not assessable by FISH analysis because of a lack of hybridization signals.

We thus identified four out of 266 gastric cancer patients (1.5%) as having *MET* amplification. The clinical features of patients with or without *MET* amplification are shown in Tables 2 and 3. All four patients with *MET* amplification had undifferentiated-type gastric cancer. We further examined the prognostic impact of *MET* amplification for all 266 patients but found that OS after surgery did not differ significantly between those with or without *MET* amplification (log-rank test, $P = 0.3$).

MET amplification is associated with increased sensitivity to MET-TKIs in gastric cancer cell lines

To investigate the biological impact of *MET* amplification in gastric cancer, we first examined the effects of two highly selective MET receptor tyrosine kinase inhibitors (MET-TKIs), JNJ38877605 and SGX523, on the growth of gastric cancer cell lines positive or negative for *MET* amplification. The IC_{50} values of JNJ3887605 and SGX523 for inhibition of cell growth were 0.02 to 0.05 μM and 0.06 to 0.07 μM, respectively, for cells positive for *MET* amplification, whereas they were >10 μM for *MET* amplification–negative cells (Figure 3A). An annexin V binding assay revealed that both MET-TKIs induced a substantial level of apoptosis in *MET* amplification–positive cells but were largely without

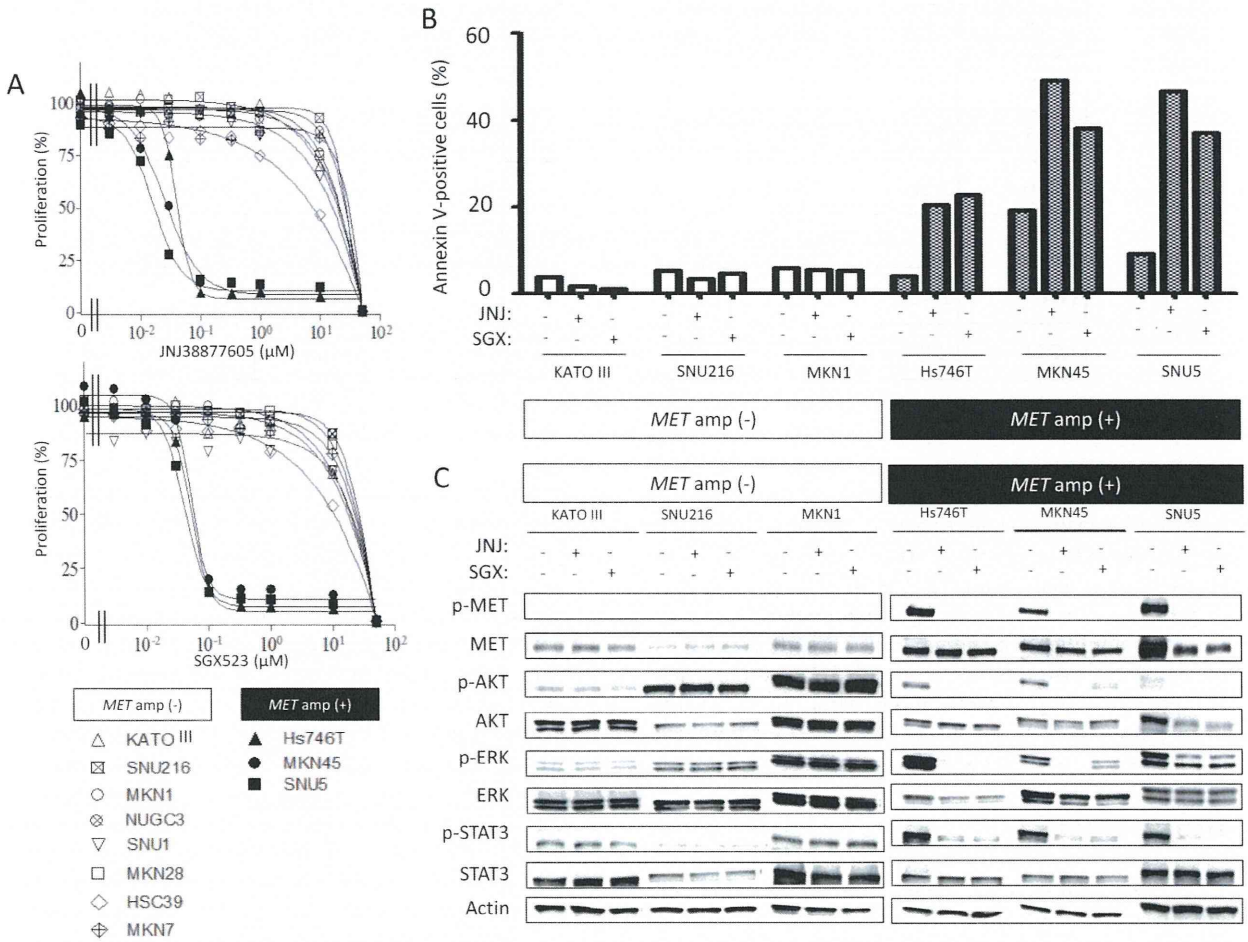

Figure 3: Effects of MET-TKIs in human gastric cancer cell lines classified according to *MET* amplification status. *A*, Effects of JNJ38877605 and SGX523 on cell growth as determined with the MTT assay. Data are means of triplicates from representative experiments. *B*, Cells were incubated in the absence or presence of 0.10 μM JNJ38877605 or 0.10 μM SGX523 for 48 h, after which the number of apoptotic cells was determined by staining with annexin V followed by flow cytometry. *C*, Cells were incubated in the absence or presence of 0.10 μM JNJ38877605 or 0.10 μM SGX523 for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of MET, AKT, ERK, or STAT3 or with those to β-actin (loading control).

Table 2: Characteristics of the 15 patients with the highest MET copy numbers

Case no.	MET copy number	MET/ CEN7p	Sex	Age (years)	Histology	Stage	OS (days)
G72	21.3	5.9 (+)	M	66	U	IV	157
G289	6.84	5.2 (+)	F	48	U	IV	483
G322	6.45	7.2 (+)	F	70	U	IV	84
G331	6.14	ND	M	76	U	II	>2764
G181	4.02	6.6 (+)	F	52	U	IIIb	>1977
G223	3.92	ND	M	62	U	IIIa	>3340
G276	3.23	1.2 (–)	M	70	U	Ib	1089
G233	3.09	1.2 (–)	F	63	U	IIIb	>4732
G217	3.02	ND	F	69	U	IIIa	>3827
G118	2.97	ND	M	66	U	IIIa	2650
G295	2.89	2.0 (–)	M	74	U	IV	539
G170	2.88	1.7 (–)	M	53	D	Ia	>2088
G42	2.87	ND	M	64	D	II	1907
G307	2.85	1.3 (–)	M	60	U	IV	824
G231	2.83	1.9 (–)	M	67	D	IIIa	>2921

Abbreviations: ND, signals not detected; U, undifferentiated type; D, differentiated type. (+) or (–) denote positive or negative for MET amplification on the basis of the MET/CEN7p ratio; > for OS indicates the patient was still alive.

effect in cell lines without *MET* amplification (Figure 3B). Immunoblot analysis showed that the MET-TKIs inhibited the phosphorylation of MET, AKT, ERK, and STAT3 in gastric cancer cells with *MET* amplification, whereas they had no effect on signaling events in those negative for *MET* amplification (Figure 3C). These findings thus indicated that gastric cancer cells with *MET* amplification are predominantly dependent on MET signaling for their growth and survival and are therefore rendered hypersensitive to MET-TKIs.

DISCUSSION

Activation of MET signaling promotes tumor cell growth, survival, migration, and invasion as well as tumor angiogenesis [24]. In gastric cancer, gain-of-function mutations of *MET* are exceedingly rare [25-27], with MET activation having been attributed mostly to gene amplification [13-15]. Previous studies based on FISH analysis have detected *MET* amplification in up to 4% of patients with gastric cancer [14, 16, 20]. On the other hand, an increase in *MET* copy number was found in 10 to 20% of gastric cancer patients by Southern blot analysis [17-19] or with a PCR-based assay [28, 29]. An increase in gene copy number in malignant tumors can result from at least two genetic mechanisms, gene amplification and polysomy. Gene amplification refers to a copy number gain for a specific gene (or group of genes) on a given chromosome arm without a change in copy number for genes located in other regions of the chromosome [30], whereas polysomy gives rise to a copy number gain for a given gene as a result of the presence of extra copies of the entire chromosome. Southern blot analysis and PCR-based copy number assays recognize a gain in gene copy number regardless of the underlying cause and are thus unable to distinguish gene amplification from polysomy, a limitation that is sometimes overlooked, with consequences for determination of the true prevalence of *MET* amplification in gastric cancer.

FISH analysis is a semiquantitative method that can be performed with two probes for determination of the number of signals for a target gene and for the centromeric

Table 3: Clinical and pathological characteristics of gastric cancer patients classified according to MET amplification status

Characteristic	MET amplification(+) (n = 4)	MET amplification(–) (n = 262)	P
Median age (range), years	59 (48–70)	63 (31–91)	0.976
Sex, n			
Male	1 (25.0%)	182 (69. 5%)	0.091
Female	3 (75.0%)	80 (30. 5%)	
Pathological stage, n			
I	0	25 (9.5%)	0.582[a]
II	0	31 (11.8%)	
III	1 (25.0%)	76 (29.0%)	
IV	3 (75.0%)	130 (49.6%)	
Histology, n			
Differentiated type	0	99 (37.8%)	0.300[b]
Undifferentiated type	4 (100%)	163 (62.2%)	

[a]Comparison between stages I + II and III + IV. [b]Comparison between intestinal-type and diffuse-type gastric cancer. P values were calculated with Student's two-tailed t test for age and the chi-square test for the other variables.

portion of the corresponding chromosome. Given that the number of centromeric signals directly reflects the copy number of the chromosome, FISH analysis yields the copy number gain for the target gene from the ratio of the copy number of the gene to that of the chromosome. FISH is thus the gold standard for detection of gene amplification. However, the identification and counting of FISH signals are hampered by many factors including cutting artifacts, nuclear overlap, and heterogeneity of tumor specimens. Moreover, FISH is expensive and time-consuming, and it requires technical expertise [31]. The efficient determination of gene amplification in a large number of tumor specimens would thus benefit from the availability of a high-throughput screening assay. In this regard, PCR-based assays for determination of gene copy number are simple to perform and rapidly yield quantitative and reproducible results. Given that, among tumors showing a gain in gene copy number, those confirmed to be positive for gene amplification show the greatest increases in gene copy number [21, 23, 32, 33], we investigated the potential of a PCR-based assay for screening in order to select cases of gastric cancer for confirmation of *MET* amplification by FISH. We thus performed PCR-based screening for *MET* copy number in 266 surgically resected specimens of gastric cancer and then applied FISH analysis to the 15 cases showing the highest gene copy numbers. *MET* amplification was confirmed by FISH in four cases among the five with a *MET* copy number of at least 4; the remaining case (G331) was not assessable by FISH because of a lack of hybridization signals. *MET* amplification was not detected in the cases with a gene copy number of <4. We therefore identified *MET* amplification at a frequency of 1.5% (4 out of 266 cases), consistent with values determined by FISH analysis in recent studies of gastric cancer [16, 20]. Our results thus suggest that screening for *MET* amplification with a PCR-based assay is an efficient means with which to reduce the number of specimens requiring evaluation by FISH analysis. As mentioned above, one specimen (G331) in the present study showed a high *MET* copy number (6.14 copies) but could not be confirmed positive for *MET* amplification because of the lack of a FISH result. The issue of how to identify *MET* amplification status in such cases remains to be resolved.

We examined the biological impact of *MET* amplification in gastric cancer cells by comparing the effects of the MET-TKIs JNJ38877605 and SGX523 between gastric cancer cell lines positive for *MET* amplification and those negative for this genetic alteration. In gastric cancer cells with *MET* amplification, the MET-TKIs markedly inhibited AKT, ERK, and STAT3 signaling and triggered apoptosis, whereas such effects were not evident in cells without *MET* amplification. To investigate whether attenuation of MET signaling by the MET-TKIs is related to the induction of apoptosis, we transfected gastric cancer cell lines with an siRNA specific for MET mRNA. Such transfection inhibited MET signal transduction as well as induced apoptosis in gastric cancer cell lines with *MET* amplification but not in those without it (data not shown). Our observations thus indicate that gastric cancer cell lines positive for *MET* amplification depend predominantly on constitutive activation of the encoded growth factor receptor for their survival and thus show high sensitivity to cell killing by MET-TKIs. Targeting of MET signaling by MET-TKIs is therefore a potentially valuable therapeutic approach for patients with *MET* amplification–positive gastric cancer. Indeed, the MET-TKI crizotinib (PF-02341066) was recently found to induce a radiographic response (partial response) and rapid clinical improvement in patients with advanced