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Functional consequence of the MET-T1010I polymorphism in breast cancer

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ABSTRACT

Major breast cancer predisposition genes, only account for approximately 30% of high-risk breast cancer families and only explain 15% of breast cancer familial relative risk. The HGF growth factor receptor MET is potentially functionally altered due to an uncommon germline single nucleotide polymorphism (SNP), MET-T1010I, in many cancer lineages including breast cancer where the MET-T1010I SNP is present in 2% of patients with metastatic breast cancer. Expression of MET-T1010I in the context of mammary epithelium increases colony formation, cell migration and invasion in-vitro and tumor growth and invasion in-vivo. A selective effect of MET-T1010I as compared to wild type MET on cell invasion both in-vitro and in-vivo suggests that the MET-T1010I SNP may alter tumor pathophysiology and should be considered as a potential biomarker when implementing MET targeted clinical trials.

INTRODUCTION

A strong breast cancer family history can signal the presence of inherited risk-modifying genetic events. However, mutations in BRCA1/2, the major breast cancer predisposition genes, only account for approximately 30% of high-risk breast cancer families and only explain 15% of breast cancer familial relative risk [1-5]. Even including the estimated contributions of mutations in high penetrance (BRCA1/2, TP53, CDH1, LKB1, and PTEN), and moderate or low penetrance genes including SNPs associated with breast cancer identified through genome-wide association studies (GWAS), 50% of familial breast cancer predisposition still remains unexplained [6].

The hepatocyte growth factor (HGF) and its receptor, the transmembrane tyrosine kinase MET, promote cell proliferation, survival, motility, and invasion, as well as morphogenic changes that can stimulate repair and regeneration in normal tissues. The greatest effects of HGF, also known as scatter factor, are most clearly manifest through cellular motility and invasion. As with many growth factor receptors, the actions of MET are co-opted during tumor growth [7]. MET over-expression, with or without gene amplification has been reported in a
variety of human malignancies [7-10] where it has been suggested to provide a major resistance mechanism for targeted therapies against multiple receptors [7]. Non-synonymous germ-line mutations in the kinase domain of MET were initially described in patients with hereditary papillary renal carcinoma [11]. Sporadic and germline MET mutations have since been detected in multiple solid tumors. However, only a subset of these mutant alleles have been proven to cause malignant transformation providing a potential for therapeutic targeting [12]. In many cases, the impact of germline and somatic aberrations remains controversial and is likely context dependent with different effects based on intrinsic lineage expression programs or mutational context in particular cancer lineages.

Here we report that the MET-T1010I germline mutation occurs in approximately 2% of patients with metastatic breast cancer. Overexpression of wild type MET (MET-WT) as well as expression of MET-T1010I increases colony formation, cell migration and invasion in-vitro and tumor growth in-vivo. A selective effect of MET-T1010I on cell invasion both in-vitro and in-vivo as compared to MET-WT suggests that MET-T1010I may alter pathophysiology and should be considered for inclusion in clinical trials of MET inhibitors.

RESULTS

Matched tumor tissue and blood from 240 patients with metastatic breast cancer were submitted for targeted exome sequencing. Three somatic mutations (MET-M1192I, MET-L269V, and MET-E34K) were found in three different patients. We also found 19 germline mutations in 18 patients (one patient had two germline mutations). Figure 1A shows the MET germline mutations detected in patients with metastatic breast cancer, and their frequencies as compared with a normal population with a similar ethnic mix (1000 Genomes Project, www.1000genomes.org). Two recurrent germline mutations (as these SNPs are potentially functional) MET-N375S (n = 9) and MET-T1010I (n = 5) were observed. The frequency of MET-T1010I in patients with metastatic breast cancer was twice that in the general population (Odds Ratio [OR] = 2.09, 95% CI: 0.72−6.07). Based on the odds ratio of 2.0, we decided to assess the functional consequence of MET-T1010I in breast cancer models. Table 1 shows the patient characteristics assessed by MET

<table>
<thead>
<tr>
<th>Germline Mutation</th>
<th>1000 Genomes: Allele Frequency (N=1092)</th>
<th>95% CI for Allele Frequency in 1000 Genomes</th>
<th>Patients with Metastatic Breast Cancer: Allele Frequency (N=240)</th>
<th>95% CI for Allele Frequency in Patients with Metastatic Breast Cancer</th>
<th>Odds Ratio (95% CI) for Risk of Metastatic Breast Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET-1174H</td>
<td>0.0018</td>
<td>(0.0002, 0.0066)</td>
<td>0.004</td>
<td>(0.0001, 0.023)</td>
<td>2.28 (0.20, 25.2)</td>
</tr>
<tr>
<td>MET-N375S</td>
<td>0.02</td>
<td>(0.0127, 0.0303)</td>
<td>0.037</td>
<td>(0.0173, 0.07)</td>
<td>1.89 (0.86, 4.16)</td>
</tr>
<tr>
<td>MET-E166D</td>
<td>0.0027</td>
<td>(0.0006, 0.008)</td>
<td>0.004</td>
<td>(0.0001, 0.023)</td>
<td>1.52 (0.16, 14.66)</td>
</tr>
<tr>
<td>MET-H888Y</td>
<td>0.0032</td>
<td>(0.0001, 0.0094)</td>
<td>0.004</td>
<td>(0.0001, 0.023)</td>
<td>1.14 (0.13, 10.22)</td>
</tr>
<tr>
<td>MET-I816M</td>
<td>0.01</td>
<td>(0.005, 0.018)</td>
<td>0.004</td>
<td>(0.0001, 0.023)</td>
<td>0.41 (0.05, 3.20)</td>
</tr>
<tr>
<td>MET-M362T</td>
<td>0.01</td>
<td>(0.005, 0.018)</td>
<td>0.004</td>
<td>(0.0001, 0.023)</td>
<td>0.41 (0.05, 3.20)</td>
</tr>
<tr>
<td>MET-T1010I</td>
<td>0.01</td>
<td>(0.005, 0.018)</td>
<td>0.02</td>
<td>(0.0068, 0.0479)</td>
<td>2.09 (0.72, 6.07)</td>
</tr>
<tr>
<td>Total</td>
<td>0.0580*</td>
<td>(0.0454, 0.0742)</td>
<td>0.075**</td>
<td>(0.0451, 0.1159)</td>
<td>1.30 (0.75, 2.24)</td>
</tr>
</tbody>
</table>

*Assuming in 1000 Genomes, one mutation per patient. ** Two mutations occurred in same patient.

Figure 1: Germline mutations and survival estimates in metastatic breast cancer. Tumor and blood samples were obtained under an Institutional Review Board-approved prospective collection protocol at MD Anderson Cancer Center (MDACC). After informed consent, patients with metastatic breast cancer underwent biopsy of their metastatic disease and blood collection. For genomic DNA library preparation, and target capture, whole exome sequencing and data analysis, we called single nucleotide variants (SNVs) and small indels using an in-house developed analysis pipeline based on variant allele frequencies in the tumor and the matched normal tissues. (A) Germline MET mutations detected in patients with metastatic breast cancer, and their frequencies as compared with normal population (1000 Genome Project). (B) Overall survival outcomes of patients with MET germline mutations versus patients with wild type MET.
germline mutation status. At a median follow-up of 44 months (range, 5–384 months), there were 69 (28.7%) deaths. Five-year OS was 82% in \( \text{MET} \) mutation carriers and 71% in patients without \( \text{MET} \) mutations, \((P = 0.18)\), respectively (Figure 1B). These are not significantly different from the outcomes for patients without germline aberrations but are limited by the low number of events in the \( \text{MET} \) mutation population.

To determine if \( \text{MET}-\text{T1010I} \) alters breast cancer pathophysiology, we compared its effects to \( \text{MET}-\text{WT} \) representing \( \text{MET} \) overexpression in breast cancer and to \( \text{MET}-\text{Y1253D} \) tyrosine kinase domain mutation representing an activated \( \text{MET} \) receptor. First, to evaluate the effects of \( \text{MET} \) aberrations on cell signaling, we tested the expression and phosphorylation levels of endogenous and exogenous \( \text{MET} \), and its downstream targets, including phosphorylation of \( \text{AKT} \), \( \text{MAPK} \) and \( \text{STAT3} \) with Western blotting (Figure 2A).

\( \text{MET}-\text{WT} \) and \( \text{MET}-\text{T1010I} \) were markedly overexpressed with \( \text{MET}-\text{T1010I} \) expressed to slightly higher levels than \( \text{MET}-\text{WT} \) potentially due to decreased turnover due to interference with the effects of Y1003-mediated phosphorylation and degradation [13]. In contrast \( \text{MET}-\text{Y1253D} \) was expressed to modest levels potentially due to increased internalization and degradation of activated \( \text{MET} \) receptors [13]. Under basal conditions, all three constructs demonstrated increased \( \text{MET} \) phosphorylation (pY1234/1235) consistent with constitutive activation. Under basal conditions, \( \text{MET} \) transfected cells, regardless of the construct, demonstrated a modest but consistent increase in phosphorylation of \( \text{MAPK} \) consistent with activation of the \( \text{MET} \) receptor.

There was a slight increase in phosphorylation of \( \text{AKT} \) and \( \text{STAT3} \) with the \( \text{MET}-\text{T1010I} \) construct compared to the other constructs.

To investigate whether \( \text{MET}-\text{T1010I} \) affects cell survival, we performed clonogenic assays. In the presence of \( \text{HGF} \), \( \text{MET}-\text{WT} \) and the two mutants (T1010I, Y1253D) markedly increased colony formation and colony size compared to control cells in low serum medium in the absence of \( \text{EGF} \) and \( \text{insulin} \) which are required for optimal proliferation of \( \text{MCF-10A} \) (Figure 2B). Interestingly, both \( \text{MET} \) mutants, but not \( \text{MET}-\text{WT} \) or control, induced colony formation in the absence of \( \text{HGF} \), with \( \text{MET}-\text{T1010I} \) inducing more and larger colonies than \( \text{MET}-\text{Y1253D} \) (Figure 2C), suggesting that \( \text{MET} \) mutants are less dependent on \( \text{HGF} \) than WT \( \text{MET} \). In a three-dimensional Matrigel culture system in the presence of \( \text{HGF} \), \( \text{MCF-10A} \) cells expressing \( \text{MET}-\text{T1010I} \) formed larger acini with abnormal structures, scattering to the surrounding matrix (Figure 2D). WT \( \text{MET} \) cells showed similar but milder morphological alterations. In contrast, cells expressing \( \text{MET}-\text{Y1253D} \) did not demonstrate detectable invasion into Matrigel. In contrast, \( \text{MET}-\text{Y1253D} \) cells had normal structure but formed larger acini than control.

Table 1: Patient and tumor characteristics

<table>
<thead>
<tr>
<th></th>
<th>All Patients (N = 240)</th>
<th>( \text{MET} ) Wildtype (N = 222)</th>
<th>( \text{MET} ) Germline Mutation (N = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N %</td>
<td>N %</td>
</tr>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≤ 50</td>
<td>163 (67.9%)</td>
<td>151</td>
<td>12</td>
</tr>
<tr>
<td>Age &gt; 50</td>
<td>77 (32.1%)</td>
<td>71</td>
<td>6</td>
</tr>
<tr>
<td>Nuclear grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6 (2.6%)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>63 (27.4%)</td>
<td>59</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>161 (70%)</td>
<td>148</td>
<td>13</td>
</tr>
<tr>
<td>Subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormonal positive</td>
<td>114 (59.1%)</td>
<td>104</td>
<td>10</td>
</tr>
<tr>
<td>HER2 positive</td>
<td>25 (13%)</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Triple negative</td>
<td>54 (28%)</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>Lymphovascular Invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>114 (52.3%)</td>
<td>99</td>
<td>15</td>
</tr>
<tr>
<td>Positive</td>
<td>104 (47.7%)</td>
<td>101</td>
<td>3</td>
</tr>
<tr>
<td>Neoadjuvant therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>165 (68.8%)</td>
<td>150</td>
<td>15</td>
</tr>
<tr>
<td>Yes</td>
<td>75 (31.3%)</td>
<td>72</td>
<td>3</td>
</tr>
<tr>
<td>Adjuvant Therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>71 (29.6%)</td>
<td>67</td>
<td>4</td>
</tr>
<tr>
<td>Yes</td>
<td>169 (70.4%)</td>
<td>155</td>
<td>14</td>
</tr>
</tbody>
</table>

*Fisher’s exact p-value.
Figure 2: Transforming effects of MET aberrations in breast epithelial cells. (A) Effects of MET aberrations on cell signaling: Lysates of MCF-10A derived cells (as indicated) were used for Western blot. Effects of MET aberrations on colony formation were tested as described in Materials and Methods. MCF-10A derived cells (as indicated) were seeded in triplicate. Cells were cultured in 2.5% horse serum, lacking EGF and insulin, with HGF (40 ng/ml) (B) or without HGF (C). Photos were taken at day 11. Data are mean ± standard error of triplicates, representative of two independent experiments (*, *P < 0.0001 versus Vector). ANOVA. (D) Effects of MET aberrations on mammary acinar morphogenesis were tested as described in Materials and Methods. MCF-10A derived cells (as indicated) were resuspended in modified growth medium containing 2% Matrigel, 2% horse serum, and 5 ng/mL EGF, supplemented with HGF 40 ng/ml. Representative field images of acini were taken on day 8; original magnification, X40. Effects of MET overexpression or mutations on cell invasion were tested as described in Materials and Methods. MCF-10A derived cells (as indicated) were induced with fibronectin (5 μg/ml) alone (E) or both HGF (40 ng/mL) and fibronectin (5 μg/ml) (F). Cells were photographed at X100 magnification. Data are mean ± standard errors of triplicates, representative of two independent experiments (*, *P < 0.0001 versus Vector). ANOVA.
cells, suggesting that mutant MET (MET-T1010I) and overexpression of WT MET in the presence of HGF promote both cell proliferation and invasion in Matrigel, whereas MET-Y1253D increased cell proliferation but not invasion. However, in a Boyden chamber invasion assay, even in the absence of HGF, both MET mutations (T1010I and Y1253D) were sufficient to significantly increase invasion (P < 0.0001), with the T1010I mutant exhibiting greater invasive capacity than Y1253D (P < 0.0001) (Figure 2E). Further, MET-WT increased invasiveness in the presence of HGF (Figure 2F).

To determine whether MET overexpression or mutation was sufficient to induce tumor formation and invasion in vivo, we established tumor xenograft models in human HGF transgenic mice on a severe combined immunodeficiency (SCID) background, named hHGF Tg SCID [14]. As expected, vector-transfected MCF-10A cells did not form tumors. Furthermore, only one hHGF negative mouse of 5 injected with MET-T1010I expressing tumor cells developed a tumor and none of the MET-WT or MET-Y1253D cohorts developed tumors indicating that human HGF is required for optimal tumor development in SCID mice. Tumors grew in all mice in the MET-WT group (9/9) and most mice in the MET-T1010I (7/9) cohort with 8- and 12-day latent periods respectively, while only 3/7 mice in the MET-Y1253D cohort formed tumors which were both much smaller and had a longer latent period (20 days) (Figure 3A). Tumor weight in MET-WT group was higher than that in MET-T1010I and MET-Y1253D groups (P < 0.0001, respectively) (Figure 3B).

Xenograft tumors and all organs of each mouse were processed into hematoxylin and eosin (H&E) stained sections, which were examined and evaluated histopathologically by a veterinary pathologist blinded to the genotype of the tumors. Tumors were evaluated histologically (cellular density and morphology, mitotic rate, and invasion into surrounding tissues), and all other organs were examined for the presence of tumor metastasis. All tumors from the MET-WT and MET-T1010I groups showed dense cellularity with

![Figure 3: Effects of MET aberrations on MCF-10A xenograft in hHGF transgenic mice.](image)

(A) A total of 1x10^7 MCF-10A derived cells (as indicated) were injected into the mammary fat pad of hHGF/SCID females. Each group consisted of 7-9 mice. Tumor volume was calculated with the formula \(V = \frac{l \times w^2}{2}\) (Mean ± SEM; ANOVA, *, P < 0.05 MET-WT versus T1010I, Y1253D and Vector groups, respectively). (B) Tumor weight. (*, P < 0.05 compared with T1010I, Y1253D and Vector groups, respectively). ANOVA. (C) H&E for histological images from representative tumor from MET-WT, MET-T1010I and MET-Y1253D. All tumors from the MET-WT and MET-T1010I groups showed dense cellularity with undifferentiated and markedly pleomorphic tumor cells (ptc), and high mitotic rate. In contrast, tumors from MET-Y1253D had significantly lower cellularity with less pleomorphic and well-differentiated tumor cells forming glandular and acinar structures (green arrows). Compared to tumors from the MET-WT group, MET-T1010I tumors exhibited more aggressive invasion (black arrows) into surrounding adipose tissue and skeletal muscle (sm). (D) Data are presented as grade of tumor invasion (*, P < 0.05; **, P < 0.01; *** P < 0.0001 versus Vector). ANOVA.
undifferentiated and markedly pleomorphic tumor cells (ptc), and high mitotic rate. In contrast, tumors from MET-Y1253D had significantly lower cellularity with less pleomorphic and more well-differentiated tumor cells forming glandular or acinar structures (green arrows). Compared to tumors from the MET-WT group, MET-T1010I tumors exhibited more aggressive tumor invasion (black arrows) into surrounding skeletal muscle (sm) (Figure 3C). Local or peripheral infiltration of tumor cells into surrounding adipose, skeletal muscle or mammary gland tissues were graded into 5 levels (0−4) by a veterinary pathologist (Figure 3D). Consistent with the in-vitro data, MET-T1010I tumors exhibited aggressive invasion into surrounding tissues. No metastasis was detected at the 28-day time-point in any group.

DISCUSSION

We demonstrate that MET germline mutations occur in 7% of patients with metastatic breast cancer with MET-T1010I found in 2% of patients. This was not detected in the Collaborative Oncological Gene-environment Study (COGS) potentially due to our study being restricted to patients with metastatic breast cancer [4,6]. More recently, Kurian et al. performed targeted exon sequencing of 42 genes including MET in the germline DNA of 198 women referred for BRCA testing, 174 with breast cancer. This study of limited size also failed to detect germline mutations in MET [15]. In contrast, the MET-T1010I SNP has been reported to be associated with hereditary renal papillary cancer, non-small cell lung cancer, colon cancer, and gastric cancer and has been found in a patient with breast cancer [12,16-18]. In our study, germline MET-T1010I mutations are in excess of the numbers predicted based on allele frequency in the 1000 Genomes Project in metastatic breast cancer, however, due to the low number of cases, this did not reach significance.

We did not detect survival differences between MET mutation carriers and non-carriers in this small sample set. In survival studies looking at the prognostic effect
of BRCA1/2 mutation in breast cancer, no OS differences have been demonstrated. However, BRCA mutations have been associated with longer recurrence-free survival in triple-negative disease [19]. However, larger studies will be required to determine whether these SNPs in MET are associated with altered patient outcomes.

Somatic and germline mutations in MET are predominantly located in the non-kinase domain, mainly in regions encoding the extracellular semaphorin domain (E168D, L229F, S323G, and N375S) and the intracellular juxtamembrane domain (R988C, T1010I, S1058P, and exon-14 deletions) [20]. However, only some of these mutant alleles have been proven to cause malignant transformation as a result of constitutive receptor activation positing a potential for therapeutic targeting or altering response to therapy targeting other signaling molecules [12]. The MET juxtamembrane domain regulates ligand-dependent MET internalization as a consequence of Y1003 phosphorylation in response to HGF binding leading to MET ubiquitination and degradation [13]. Mutations in the juxtamembrane domain such as T1010I have been proposed to result in MET accumulation at the cell surface and persistent signaling leading to tumorigenic activity [13]. Indeed in our studies when expressed in MCF-10A as compared to a constitutively active MET variant, MET-T1010I is expressed at an elevated level potentially contributing to its selective effect on invasion in-vitro and in-vivo. Though some previous reports suggested that MET-T1010I could have oncogenic properties, the results of functional studies are contradictory. Schmidt et al. proposed that the T1010I mutation is a SNP as it lacked the ability to transform NIH-3T3 cells [11]. Tyner et al. found similar results in the BA/F3 murine myeloid cell model [21]. In contrast, Lee et al. found tumorigenic effects in their NIH-3T3 model [17], and Tengs et al. reported the transforming potential of MET-T1010I in non-small cell lung cancer [22]. We demonstrate that MET-T1010I has marked functional consequences in the MCF-10A normal breast epithelial cell line, which is more likely to reflect the appropriate context for breast cancer than previous studies with BA/F3 and NIH-3T3. Interestingly, our data showed differences between effects of the somatic activating MET mutation, MET-Y1253D, and the germline MET-T1010I. Despite a low level of expression in MCF-10A compared to MET-WT, MET-Y1253D increased cell survival independent of exogenous HGF in vitro, suggesting that increased kinase activity is sufficient for these processes. However, in contradistinction to MET-WT and MET-Y1253D, MET-T1010I selectively increases invasion both in-vitro and in-vivo. This effect of MET-T1010I is not fully recapitulated by an activating mutation in the tyrosine kinase domain or by overexpression of MET-WT suggesting that the effects of MET-T1010I are not solely due to overexpression or due to increased kinase activity. Whether this selective effect relates to increased MET-T1010I stability [13] will require further exploration.

Exogenous expression of MET-T1010I resulted in a modest increase in expression of exogenous MET-Flag and endogenous MET compared to the other constructs. This was associated with a slight increase in basal phosphorylation of MET, MAPK, AKT and STAT3. However, these differences compared to MET-WT or MET-Y1253D were modest. As indicated above, decreased levels of MET-Y1253D in MCF-10A cells are consistent with phosphorylation-mediated degradation [23]. Since the juxtamembrane domain regulates ligand-dependent MET internalization as a consequence Y1003 phosphorylation leading to MET ubiquitination and degradation [13], the MET-T1010I SNP in the juxtamembrane domains may contribute to MET accumulation at the cell surface and persistent pathway activation contributing to effects on tumor pathophysiology [13].

In conclusion, we found MET germline mutations in 7% of patients with metastatic breast cancer, with at least one of the SNPs contributing to aggressive behavior when expressed in the MCF-10A cell model. Our findings constitute evidence justifying further studies of the frequency of MET germline mutations in high-risk populations, the role of MET inhibitors in cancer prevention, and the use of MET-T1010I as biomarker for inclusion of patients in therapeutic studies of MET inhibitors.

METHODS

Human tissues

Clinical information

Tumor and blood samples were obtained under a prospective collection protocol at MD Anderson Cancer Center (MDACC). After informed consent, patients with metastatic breast cancer underwent biopsy of their metastatic disease and blood collection. Patients and tumor characteristics were collected by chart review. The Institutional Review Board of MDACC approved the laboratory study. Patients with metastatic breast cancer were categorized according to MET germline mutations (mutant versus wild type). Patients’ characteristics including age, race, tumor size, lymph nodes, pathologic stage, histology, grade, lymphovascular invasion, and (neo)adjuvant chemotherapy were tabulated and compared between groups using Fisher’s exact test. Overall survival (OS) was measured from the date of diagnosis to the date of death or lost to follow-up. The Kaplan-Meier product limit method was used to estimate the survival outcomes of all patients by groups and was compared using the log-rank statistic.
Genomic DNA and library preparation

Genomic DNA was quantified by Qubit (Invitrogen) and quality was accessed using Genomic DNA Tape for the 2200 Tapestation (Agilent). DNA from each sample (200-500 ng of genomic DNA) was sheared by sonication with the following conditions: Peak Incident Power 175, Duty Cycle 20%, Intensity 5, Cycles per Burst 200, and 120 seconds using Covaris E220 instrument (Covaris). To ensure the proper fragment size, samples were checked on TapeStation using the DNA High Sensitivity kit (Agilent). The sheared DNA proceeded to library prep using KAPA library prep kit (KAPA) following the “with beads” manufacturer protocol. Briefly, this protocol consists of 3 enzymatic reactions for end repair, A-tailing and BioO adaptor ligation, followed by barcode (BioO) insertion by PCR using KAPA HiFi polymerase (6 cycles). PCR primers were removed by using 1.8x volume of Agencourt AMPure PCR Purification kit (Agencourt Bioscience Corporation). At the end of the library prep, samples were analyzed on TapeStation to verify correct fragment size and to ensure the absence of extra bands. Samples were quantified using KAPA qPCR quantification kit. Equimolar amounts of DNA were pooled for capture (8-12 samples per pool).

Targeted capture

202 genes including MET that are clinically relevant in cancer, based on mutational data in the Catalogue of Somatic Mutations in Cancer (COSMIC) and The Cancer Genome Atlas (TCGA) with a minimum of 3% frequency across disease sites or 5% disease specific were selected for capture. We designed biotin labeled probes with Roche Nimblegen for capturing target regions (all exons in those 202 genes) and followed manufacture’s protocol for the capture step. Briefly, DNA was pooled (8-16 samples), dried out and after addition of the capture reagents and probes; samples were incubated at 47°C on thermocycler with heated lid (57°C) for 64–74 hours. The targeted regions were recovered using streptavidin beads and the streptavidin-biotin-probe-target complex was washed and another round of PCR amplification was performed according to manufacturer’s protocol. The quality of each captured sample was analyzed on TapeStation using the DNA High Sensitivity kit and the enrichment was accessed by qPCR using specific primers designed by Roche Nimblegen. The cutoff for the enrichment was 50-fold minimum.

Sequencing

The captured libraries were sequenced on a HiSeq 2000 (Illumina Inc., San Diego, CA, USA) on a version 3 TruSeq paired end flowcell according to manufacturer’s instructions at a cluster density between 700 –1000 K clusters/mm². Sequencing was performed on a HiSeq 2000 for 2 × 100 paired end reads with a 7 nt read for indexes using Cycle Sequencing v3 reagents (Illumina). The resulting BCL files containing the sequence data were converted into “.fastq.gz” files and individual libraries within the samples were demultiplexed using CASAVA 1.8.2 with no mismatches. All regions were covered by >20 reads.

Data Analysis

We aligned the T200 target-capture deep-sequencing data to human reference assembly GRCh37 using BWA [24] and removed duplicated reads using Picard [25]. We called single nucleotide variants (SNVs) and small indels using an in-house developed analysis pipeline [26], which classified variants into 3 categories: somatic, germline, and loss of heterozygosity based on variant allele frequencies in the tumor and the matched normal tissues. We called copy number alterations using a previously published algorithm [27], which reports gain or loss status of each exon. To understand the potential driver identity and functional consequence of detected variants, we compared them with dbSNP, COSMIC [28], and TCGA databases, and annotated them using VEP [29], ANNOVAR [30] and CanDrA [31].

Cell lines and animal models

Construction of recombinant lentiviruses expressing wild type and mutant MET

GeneART synthesized constructs expressing human wild type (WT) or mutant METs (MET-WT-Flag, MET-T1010I-Flag, and MET-Y1253D-Flag) were designed by us. pMA vector 2 was used as the backbone in these plasmids. The constructs were sequenced to ensure the validity of the sequence and the orientation. Then the human full-length cDNAs for wild type MET and the two mutants, with Kozak sequence before ATG and Flag-tag after MET, were sub-cloned into pLVX-tdTomato-N1 vectors (Clontech, Mountain View, CA) with XhoI/Xmal enzymes. The constructs (pLVX-MET-WT-Flag-tdTomato, pLVX-MET-T1010I-Flag-tdTomato, pLVX-MET-Flag-Y1253D and the empty vector pLVX-tdTomato-N1) were sequenced by GeneART to confirm the sequence. We confirmed that the orientation of the constructs was correct with restriction enzymes.
Generation of Lentiviruses expressing wild type and mutant METs

To generate the lentiviruses expressing wild type MET and its mutants, we used two expression systems. One of them was the ViraPower Lentiviral Expression System (Invitrogen). In addition, we used the Lenti-X™ Lentiviral Expression System (Clontech). A total amount of 7 µg of pLVX-tdTomato-N1 vector, pLVX-MET WT-Flag-tdTomato, pLVX-MET T1010T-Flag-tdTomato, or pLVX-MET-Y1253D-Flag-tdTomato were co-transfected into the Lenti-X 293T cells, with 36 µg of the Lenti-X HTX packaging Mix, using 7.5 µl Xfect Polymer (Clontech, CA, USA). Lentiviruses containing supernatants were collected after 48 hours, followed by a brief centrifugation (500 g for 10 minutes) and passed through a 0.45 µm filter to remove cellular debris. Then they were used to infect the mammary epithelial cells. Selection began 48 hours after infection in growth medium with 1 µg/ml puromycin for two weeks. Both lentiviral expression systems allowed similar specific expression levels.

Cell culture

MCF-10A, non-transformed mammary epithelial cell lines obtained from our Characterized Cell Line Core, MDACC and grown at 37°C in humidified 5% CO₂. MCF-10A cells were maintained in DMEM/F12 (Thermo Scientific, South Logan, Utah) supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF (Peprotech), 10 µg/ml insulin (Sigma), 100 ng/ml cholera toxin (Sigma), 0.5 µg/ml hydrocortisone (Sigma), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were frozen at early passages and used for less than 4 weeks in continuous culture.

Western Analyses

Cells were washed twice with cold phosphate-buffered saline and lysed in ice-cold lysis buffer [1% Triton X-100, 50mM HEPES, pH 7.4, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaF, 10mM Na pyrophosphate, 1mM Na₂VO₄, 10% glycerol, protease inhibitor cocktail (Roche Applied Science), and phosphatase inhibitors, PhosSTOP (Roche Applied Science)]. Western blot was performed as described previously [32].

Morphogenesis Assay

Three-dimensional culture of cells was carried out on a Matrigel basement membrane [33]. Briefly, 4 x10⁵ cells were resuspended in a modified growth medium, containing 2% growth factor-reduced Matrigel (BD Biosciences), and subsequently seeded onto the Matrigel matrix in 8-well chamber slides (BD Bioscience). Medium was replaced every 3 days. Photographs of representative fields were taken as indicated. Acini were photographed and counted in 10 randomly chosen fields and expressed as means of triplicates, representative of three independent experiments.

In vitro Invasion Assay

Cell in-vitro invasion was analyzed with 24-well Biocoat Matrigel invasion chambers with 8 µm polycarbonate filters (Becton Dickinson). Cells were starved for 20 hours in serum-free DMEM F12 lacking growth factors. A total of 1x10⁵ cells in 0.6 ml DMEM F12 were inoculated into the upper chamber, and 0.75 ml DMEM F12 containing fibronectin (5 µg/ml) with or without HGF (40 ng/ml) was added to the lower chamber. The cells were allowed to pass through the Matrigel at 37°C, 5% CO₂, for 22 hours. Non-invasive cells on the upper surface of the filter were removed by wiping with a cotton swab. The cells that penetrated through the pores of the Matrigel to the underside of the filter were stained with 0.25% crystal violet in 20% methanol for 30 minutes. Invasive cells were photographed and counted in 10 random fields.

Clonogenic Assay

For clonogenic assays, 1,000 cells were seeded in a 60 mm dish, in growth medium, for 11 days. For inhibitory assay, after attaching on the dish, cells were treated for 2 days with drugs as designed. The cells were rinsed with PBS, followed by staining with 0.25% crystal violet / 20% ethanol. Quantitative analysis of the total number and size of clones was performed with AlphaVIEW SA software (Cell Biosciences).

Tumor Xenografts studies

All animal studies were carried out under ACUF-approved protocols. hHGF Tg SCID females or hHGF negative littersmates at 6-8 weeks of age were used. Exponentially growing MCF-10A derived cells, including MET-WT, MET-T1010I, MET-Y1253D, and control cells (vector), were harvested. After being washed, 1x10⁵ cells in growth factor reduced Matrigel and PBS (1:1), in total 200 µl, were injected into the mammary fat pads of mice. Each group consisted of 7-9 mice. Tumor sizes were determined by measuring the length (l) and the width (w) with calipers twice weekly. Tumor volume was calculated with the formula \(V = \frac{lw^2}{2}\). Differences in tumor volume among groups at each time point were
analyzed using ANOVA. At the end of the experiment (28 days after tumor cell injection), mice were sacrificed. Tumors were harvested, followed by measurement of tumor size and weight. Tumors were cut and flash-frozen in liquid nitrogen for Western blot or fixed in 10% neutral-buffered formalin for histopathological analysis. Fixed tissues were processed, embedded in paraffin, and cut in 4 µm thick tissue sections that were stained with H&E for microscopic examination. Xenograft tumors and all the organs, of each mouse, were subjected to double-blind histopathological analysis by a veterinary pathologist.

Statistical Analyses

Statistical analyses were carried out using the ANOVA test (for multiple groups) and the Student t test (for two groups). Differences with P-values < 0.05 were considered statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest other than GBM being on the advisory board of Symphogen and AstraZeneca and receiving research support from GSK and AstraZeneca.

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