

Aberrations of c-MET drive breast cancer metastasis in an HGF-dominant environment

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Abstract

Background

Metastases are a key cause of mortality in breast cancer patients. Heterogeneity of breast cancer makes treatment challenging and emphasizes the need to define drivers of metastasis that can be selectively targeted. We previously reported upregulation and phosphorylation of c-MET (MET) in breast cancer, with both being correlated with poor prognosis. We also detected MET-T1010I, a germline mutation in patients with metastatic breast cancer, and demonstrated that MET aberrations promoted tumor growth and invasion in an HGF-dominant environment in vitro and in vivo. However, direct evidence of MET aberrations driving breast cancer metastasis is lacking. Because mouse HGF does not bind human MET effectively, commonly used mouse models are unsuitable for studying the HGF-MET axis.

Methods

We created and used human HGF/MET-paired spontaneous metastatic breast cancer mouse models where human HGF was expressed in HGF-transgenic mice with a SCID background and different MET variants were expressed in mammary xenografts. Snap-frozen breast tumor tissues were analyzed by protein array, and the data were further analyzed for pathway scores. Phosphor protein arrays assessed the effect of the MET aberrations on the phosphorylation of receptor tyrosine kinases and protein kinases. Serum HGF was quantified by ELISA assay. Cytokine and chemokine chips assessed 60 serum cytokines and chemokines.

Results

The aberrations of MET drive breast cancer metastasis in a human HGF-dominant environment, with a higher rate and shorter latency in MET-T1010I than with MET overexpression. Circulating HGF is increased in breast cancer patients, compared to age-matched healthy women. The enhanced circulating HGF was strongly correlated with circulating IL-16 and eotaxin-2/CCL-24, which are implicated in cancer metastasis. Interestingly, functional proteomics analysis of tumor tissues revealed previously unrecognized crosstalk between the HGF-MET axis and FGFR3, accompanied by activation of the EMT pathway and immunosuppression. These results deepen our understanding of the mechanisms underlying breast cancer metastasis and highlight the potential of combination strategies targeting FGFR3 and MET.

Conclusion

Aberrations of the HGF-MET axis drive metastasis in breast cancer and crosstalk with FGFR3, suggesting combined targeting of FGFR3 and MET as a potential novel approach to treat MET-activated

cancers.

Background

Breast cancer is the most prevalent malignancy in women and the leading cause of cancer deaths in women worldwide, with 670,000 associated deaths [1]. The American Cancer Society reports that an estimated 319,750 new cases and 42,680 deaths are expected to occur from breast cancer in the United States in 2025 [2]. Metastases are responsible for about 90% of all cancer-related deaths and are the primary cause of mortality in breast cancer patients [3]. Breast cancer is a heterogeneous disease with intratumoral and intertumoral heterogeneity, making treatment challenging. Comprehension of mechanisms involved in breast cancer progression and discovery of critical and addressable drivers of metastasis is crucial to guide personalized therapy and aim for curability.

The cellular-mesenchymal epithelial transition factor (c-MET, MET) is a receptor tyrosine kinase (RTK) encoded by the *MET* proto-oncogene that is expressed in epithelial cells across diverse tissue lineages. Hepatocyte growth factor/scatter factor (HGF/SF) is a unique high-affinity ligand for MET [4, 5]. It is produced and secreted mainly by stromal cells and acts primarily on cells of epithelial origin, playing a role in tissue regeneration and normal mammary gland development physiologically. However, the HGF-MET axis can be dysregulated in malignancy. MET is highly expressed in different types of cancer [6–10], including breast cancer [11–16]. The FDA has approved the MET antibody-drug conjugate telisotuzumab vedotin for patients who have high MET protein overexpression with metastatic non-small cell lung cancer (NSCLC) [17], and the MET inhibitors capmatinib and tepotinib for NSCLC patients with MET exon 14 skipping mutations [18]. Breast cancer, derived from epithelial cells, express both HGF and MET [19, 20]. Increased HGF levels are detected in the serum of breast cancer patients, with higher levels in patients with metastatic diseases compared to primary disease [21, 22]. In our previous study of 257 patients across various breast cancer subtypes, elevated MET expression and its phosphorylation were significantly associated with poorer clinical outcomes [16]. Additionally, enhanced phosphorylation of MET in primary breast cancer was associated with a higher risk of recurrence [16]. To investigate whether HGF is a key factor that determines activation of MET in breast cancer, we established a set of PDX-derived breast cancer cell lines. Eight lines with different levels of MET expression were treated with or without HGF, followed by assessing MET activation. MET signaling was not activated in the absence of HGF stimulation in all MET-positive lines. Incubation with HGF induced significant phosphorylation of MET for each line ($P < 0.0001$, respectively) except in the MET-negative parent line. Thus, HGF is a key factor in the activation of MET signaling in the MET-positive breast cancer lines [23]. In another study, we detected MET-T1010I in a subset of metastatic breast cancers [24]. MET-T1010I is a germline mutation in exon 14, juxtamembrane (JM) domain that regulates MET degradation [25]. Despite compelling evidence that the HGF-MET axis plays a crucial role in breast cancer pathophysiology, clinical trials of MET or HGF inhibitors have not yielded the expected benefits, with short-lived responses in only about 10% of patients treated with the VEGFR2/MET inhibitor, cabozantinib [26, 27]. The mechanisms underlying the lack of prolonged benefit need to be further elucidated. However, a lack of appropriate animal models limits fully understanding the mechanism of HGF-MET axis in breast cancer because

mouse HGF does not effectively activate human MET [28, 29] and commonly used mouse models are thus unsuitable for studying the HGF-MET axis.

In our pre-clinical studies, we created and used a human HGF/human MET-paired breast cancer mouse model, in which human HGF is expressed in HGF transgenic mice with SCID backgrounds (termed hHGFTg/SCID) [30], while wild-type MET or MET-T1010I genes are expressed in mammary xenografts. Using these breast cancer models, we demonstrated that MET-T1010I or overexpression of wild-type MET transforms mammary epithelial cells, inducing tumor formation and invasion in a human HGF dominant environment [23, 24]. Despite clinical studies suggesting that aberration of MET is potentially involved in the process of metastasis [31], direct evidence for a role for the HGF-MET axis as a driver of metastasis, the primary cause of mortality in breast cancer patients, remains lacking.

In the present study, we created and used a human HGF/MET -paired spontaneous metastatic breast cancer mouse model, comprehensively explored the role of the HGF-MET axis aberrations in metastasis in breast cancer, and provide direct evidence that MET overexpression or MET-T1010I drives metastasis in an HGF-dominant environment, with a higher rate and shorter latency in MET-T1010I than in wildtype MET overexpression. Mechanism analysis revealed previously unrecognized crosstalk between the HGF-MET axis and FGFR3 signaling, which activates epithelial-mesenchymal transition (EMT) signaling and drives metastasis. The findings may explain why monotherapy with a MET inhibitor did not provide meaningful clinical benefit. We also found that the higher circulating HGF was strongly correlated with circulating IL-16 and eotaxin-2/CCL-24, which are implicated in cancer metastasis. Together, these extend our understanding of the mechanisms underlying breast cancer metastasis and have implications for cancer treatment, highlighting the potential of combination strategies targeting FGFR3 and MET to generate benefit in breast cancer patients.

Methods

Further information can be found in Supplemental Methods.

Cells and cell culture

AU565, HCC1937, HCC1569, HCC1954, MDA MB157, ZR75.1, BT474, and SKBR3 cell lines were obtained from the American Type Culture Collection (Manassas, VA). All other cell lines were from the MDACC Characterized Cell Line Core at The University of Texas MD Anderson Cancer Center. Mycoplasma contamination and identity verification with short tandem repeat were performed regularly by the Core.

Generation of lentiviruses expressing wild-type MET or MET-T1010I

Lentiviruses expressing wild-type (WT) MET or MET-T1010I were generated as previously described [24], and in the Supplementary Methods.

Plasmid construction and gene knockdown

pGIPZ–short hairpin RNA (shRNA) constructs targeting *MET* and a nonsilencing control were purchased from the Functional Genomics Core at MD Anderson Cancer Center. Six different shRNAs targeting *MET* were tested (Table S1). Lentivirus was produced by co-transfecting the lentiviral shRNA plasmid, the viral packaging plasmid psPAX2 and the envelope plasmid pMD2.G into HEK293T cells, and 48 hours later, was used to infect the target cells. The infected cells were selected with 1 µg/ml puromycin (Gibco) for 10 days. The shRNA construct with the highest knockdown efficiency, as determined by immunoblot, was selected for further experiments.

Drug and reagents

MET tyrosine kinase inhibitor (TKI) tepotinib (Selleck Chemicals, Houston, TX) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and stored at – 20°C. The stock solution was further diluted to an appropriate final concentration upon use. Human recombinant HGF was purchased from R&D Systems (Minneapolis, MN).

Migration or migration inhibition assays

Cell migration assays or inhibition assays were performed with 24-well BD BioCoat migration chambers. In detail, see Supplementary Methods.

Cell growth and morphogenesis assays

Cell growth and morphogenesis assays were performed in three-dimensional (3D) culture in Matrigel as described [23] and in the Supplementary Methods.

Western blot analyses

Western blot analysis was performed as described previously [23], and in the Supplementary Methods.

Reverse phase protein array (RPPA)

The cell lysis was prepared in the same way for Western blot analysis. Mouse snap-frozen breast tumor tissues stored at – 80°C underwent lysis in the cold lysis buffer with homogenization and were subsequently analyzed by RPPA, as previously described [33]. The data were analyzed using R statistical software as previously described [33, 34] and in the Supplementary Methods. Antibodies utilized in the study are shown in Table S2.

Pathway score analysis

RPPA data from snap-frozen tumor samples were further analyzed for pathway scores based on a panel of pathways published previously [35]. These pathways were predefined based on a PubMed literature search of review and original articles describing the various pathways in detail. For details, see Supplementary Methods.

Phospho-receptor tyrosine kinase (p-RTK) and phosphor-protein kinase (p-PK) arrays

Phosphorylation of 49 human RTKs and 42 PKs in MCF10A cells transduced with WT MET or MET-T1010I was assessed using the human phospho-RTK Array kit and the human phosphor-PK kit (R&D Systems, Minneapolis, MN) following the manufacturer's directions. In detail, see Supplementary Methods.

Establishment of human HGF-MET-paired breast cancer spontaneous metastasis mouse model

All animal studies were carried out under Animal Care and Use Form-approved protocols. The hHGF Tg/SCID mice were bred in a room specifically for SCID mice. After testing serum human HGF, 6-week-old female mice were used for experiments. For details, see Supplementary Methods.

Human samples

Blood samples from breast cancer patients and healthy volunteers, along with clinical data, were obtained under protocols approved by the institutional review board at the University of Texas MD Anderson Cancer Center. Patients and tumor characteristics were collected by chart review [36]. The Institutional Review Board of MD Anderson approved the laboratory study. Sample preparations see Supplementary Methods.

Circulating HGF analysis

Serum HGF was quantified by Enzyme-linked immunosorbent assay (ELISA) using the Human HGF ELISA Kit (R&D Systems) following the manufacturer's instructions. Concentrations were calculated by comparing the sample absorbance to standard curves.

Cytokine and chemokine chip detection

In total, 60 serum cytokines (Table S3) were measured using the kits the Bio-Plex Pro™ Human Chemokine Panel 40-plex, Bio-Plex Pro Human Cytokine Group I, and Bio-Plex Pro Human Cytokine Group II (Bio-Rad Laboratories, Inc., CA) according to the manufacturer's instructions. In detail see Supplementary Methods.

Statistical analyses

One-way ANOVA was used for multiple groups, and the *t*-test was used for two groups. RPPA data were analyzed as previously described [23, 33]. For details, see Supplementary Methods.

Results

Inhibition or knockdown of MET restrains TNBC and HER2 + breast cancer with MET dysregulation in an HGF-dominant environment

We previously examined MET protein expression and its phosphorylation (p-MET) in 257 breast cancers using RPPA. Upregulation of MET and p-MET were found in hormone receptor-positive (HR+), human epidermal growth factor receptor 2-positive (HER2+) and triple negative breast cancer (TNBC), and both were correlated with poor prognosis in all receptor subtypes [16]. Conflicting results question whether MET overexpression contributes to breast cancer and is an adverse prognostic marker in HER2 + breast cancer [37]. Therefore, we examined MET expression in a panel of breast cancer cell lines, including all three subtypes. MET expression was detected in all three subtypes, with higher levels in the more clinically aggressive subtypes of TNBC and HER2 + breast cancer than in HR+ breast cancer in the panel assessed (Fig. 1A,1B).

Cell migration plays critical roles at multiple stages of metastasis. We therefore tested migration ability induced by recombinant human HGF (HGF) in MET positive HCC1937 (TNBC) and HCC1569 (HER2 + BC). HGF induced migration in both these subtypes (Fig. 1C, 1F), while in the absence of HGF, the cells did not migrate. Tepotinib, a MET inhibitor FDA-approved for lung cancer, restrained this cell migration (Fig. 1C,1D, 1F,1G). Consistent with its effects on migration, tepotinib blocked MET downstream signaling activated by HGF (Fig. 1E,1H) in both subtypes of breast cancer tested.

To further confirm the contribution of MET to cell motility, we silenced *MET* using shRNA in HCC1937 (triple-negative) and HCC1954 (HER2+) breast cancer cells (see “Methods” section). Reduction of MET expression in the puromycin-resistant stable cell lines was confirmed with Western blot (Fig. 1I, 1J; Fig. S1A, S1B, respectively). The cells transduced with the most efficient shRNA (shMET-6) were used for further studies. Compared to the no silencing control, knockdown of MET significantly reduced HGF-induced migration in HCC1937 cells ($P < 0.000$; Fig. 1K, 1L) and HCC1954 cells ($P < 0.000$; Fig. S1C, S1D).

Overexpression of MET increases HGF-induced migration and scatter in vitro and drives metastasis of breast cancer in an HGF-dominant environment

We first designed constructs expressing Flag epitope-tagged full-length wild-type MET. The gene cassette (Fig. 2A) [24] was transferred into a lentiviral vector and used to transduce HCC1954 cells, a HER2+/HR- breast cancer cell line established from a primary breast carcinoma with no metastases. This line did not form histopathologic metastatic lesions after subcutaneous injection in severe combined immunodeficiency (SCID) mice, although cells have been detected in the lung by PCR [38]. The HCC1954 cells were transduced with human WT MET or the empty vector (Vector), used as control. The expression of tdTomato fluorescent protein was confirmed, as indicated by the tdTomato-MET-Flag fusion protein located in the cell membrane and cytoplasm, as the expected localization for MET (Fig. 2B, right). In contrast, tdTomato protein was expressed diffusely in the control cells (Fig. 2B, left). Expression of exogenous Flag-tagged MET was further confirmed with Western blotting (Fig. 2C).

We then examined the effect of MET overexpression on cellular behaviors. Compared to empty vector-transduced cells, MET-transduced cells exhibited significantly higher HGF-induced migration (Fig. 2D, 2E, $P < 0.0001$). Tepotinib significantly and dose-dependently inhibited the HGF induced migration ($P < 0.0001$, $P < 0.05$, respectively, Fig. 2F, 2G). Similarly, overexpression of MET significantly increased cell proliferation, scatter, and invasion in HGF-supplemented 3D Matrigel ($P < 0.0001$, Fig. 2H, 2I). Targeting MET with tepotinib significantly inhibited these processes ($P < 0.0001$, Fig. 2J, 2K). Consistent with the effects on cellular functions, proteomic analysis with RPPA shows that tepotinib inhibited phosphorylation of MET and blocked HGF-MET downstream signaling, including the PI3K-AKT and MAPK pathways (Fig. 2L, 2M). Effects on several critical signaling intermediaries were verified with Western blotting (Fig. 2N).

To investigate whether overexpression of MET drives metastasis of breast cancer in an HGF-dominant environment, we established an orthotopic surgical resection model of breast cancer as illustrated in Fig. 3A to mimic clinical post-resection micro-metastasis in human patients. In the human HGF/MET-paired breast cancer metastasis mouse model, human HGF was expressed in the host, and human MET was expressed in the xenografts. When the primary xenograft tumor reached approximately 1000 mm³, the tumors were resected following a survival surgery protocol, and mice were carefully monitored. When exhibiting abnormal health conditions or aged 40 weeks, the mice were sacrificed with CO₂. The main organs (brain, lung, and liver) were subjected to double-blind histopathological analysis by a veterinary pathologist. No metastatic disease was found in the control group, which was transduced with empty vector. Intriguingly, metastatic dissemination was detected in the lungs, brain, or liver in the mice from WT MET overexpression group with the rate of metastasis 15% and a latency of 26 weeks. Figure 3B represents metastatic lesions in the lungs (a), brain (b), and liver (c), respectively, concordant with breast cancer metastatic spread patterns for the subtype represented by the model in patients [39, 40].

HGF-MET axis drives metastasis through the EMT signaling

To elucidate the mechanism underlying MET overexpression-driven metastasis, we performed RPPA on snap-frozen xenograft tumors isolated from human HGF transgenic mice. Compared to the control group (empty vector), MET overexpressing xenografts exhibited a notably different distributions of proteins that have been shown to contribute to metastasis via epithelial-mesenchymal transition (EMT) signaling, including significantly increased TROP2 ($P < 0.0001$, Fig. 3C), IGF-1R- β ($P < 0.0001$, Fig. 3D), PAI-1 ($P < 0.01$, Fig. 3E), collagen VI ($P < 0.05$, Fig. 3F), mildly increased N-cadherin (Fig. 3G), and decreased claudin 7 (Fig. 3H). In addition, MET overexpression decreased PD-L1 (Fig. S2).

MET-T1010I induces aggressive behavior in vitro in an HGF-dominant environment

We previously identified MET-T1010I, a germline mutation in exon 14 JM domain that regulates MET degradation, in a subtype of metastatic breast cancer [24], a germline mutation in exon 14, JM domain that regulates MET degradation [25], as illustrated in Fig. 4A. We also demonstrated that this mutation transduced immortal breast epithelial cells and induced tumor formation and invasion [24]. To investigate whether MET-T1010I drives metastasis of breast cancer, we designed tdTomato-MET T1010I-Flag construct and prepared lentiviruses and then established a stable cell line expressing tdTomato-MET-T1010I-Flag fusion protein in HCC1954 cells, using the same method that was used to establish overexpression of wild-type MET. Similar to the pattern of expression of exogenous WT MET, the tdTomato-MET-T1010I-Flag fusion protein localized to cytoplasm and cell membrane (Fig. 4B). Expression of Flag-tagged MET-T1010I was confirmed with Western blotting (Fig. 4C).

We examined the effect of MET-T1010I on cell migration and scatter in vitro. Compared to empty vector-transduced cells, MET-T1010I-transduced cells exhibited significantly higher HGF-induced migration ($P < 0.0001$, Fig. 4D, 4E). The MET inhibitor tepotinib significantly restrained migration at a low dose ($2.5 \mu\text{M}$) ($P < 0.0001$ vs. vehicle) with a high dose ($5 \mu\text{M}$) almost completely blocking HGF-induced migration (Fig. 4F, 4G). MET-T1010I significantly increased cell growth, scatter, and invasion in HGF-supplemented 3D Matrigel ($P < 0.0001$, Fig. 4H, 4I). The pattern of MET-T1010I-induced effects was similar to that of overexpression of wild-type MET, but more obvious. Tepotinib restrained these phenotypes ($P < 0.0001$, Fig. 4J, 4K). Consistent with receptor activation, RPPA showed that tepotinib inhibited phosphorylation of MET and its downstream signaling mediators, including PI3K-AKT, MAPK pathways (Fig. 4L), similar to the effects observed in WT MET overexpression cells. Figure S3 shows the quantitation. Effects on several important phosphorylated signaling transduction proteins were verified with Western blotting (Fig. 4M).

MET-T1010I drives metastasis of breast cancer in HGF-dominant environment with a higher rate and shorter latency than that induced by MET overexpression

To investigate whether MET-T1010I drives metastasis of breast cancer in an HGF-dominant environment, we used the orthotopic surgical resection model described earlier. Again, no metastatic disease was found in the group transduced with the empty vector. In the HGF expressing mice, MET-T1010I induced metastatic lesions in the lung, brain, or liver at a higher rate of 23% (Fig. 5A), and shorter incubation period (17 weeks) than that induced by MET overexpression. Figure 5B represents metastatic lesions in lung (a), brain (b), and liver (c), respectively.

Effects of MET-T1010I on cellular signaling pathways in vivo in an HGF-dominant environment

To elucidate the effects of MET-T1010I on signaling processes that could contribute to tumor progression, we performed RPPA on the xenograft tumors. Based on unsupervised hierarchical clustering, the tumors expressing MET-T1010I formed a cluster at the top of the dendrogram. The tumors from the control group (empty vector-transduced) formed the bottom cluster (Fig. 5C).

Quantitative analysis of the RPPA data revealed that multiple oncogenic pathway members were significantly enhanced, including PI3K-AKT (Fig. S4A), TSC-mTOR (Fig. S4B), and the Cell Cycle (Fig. S4C). Similar to the MET overexpression model, expression of MET-T1010I significantly decreased the expression of PD-L1 and Syk proteins (Fig. S4D). The RPPA data were further assessed for pathway scores using a previously published approach [35], as described in Methods. Compared with the control group, several signaling scores were significantly increased, including EMT ($P < 0.0001$, Fig. 5D), PI3K-AKT ($P < 0.0001$, Fig. 5E), TSC-mTOR ($P < 0.0001$, Fig. 5F), DNA Damage Response ($P < 0.0001$, Fig. 5G), Cell Cycle ($P < 0.0001$, Fig. 5H), Apoptosis ($P < 0.0001$, Fig. 5I), and Core Reactive ($P < 0.0001$, Fig. 5J). MET-T1010I expression was associated with immunosuppression, as indicated by a decreased Immune Signature ($P < 0.0001$, Fig. 5K).

HGF-MET axis activates FGFR3 in breast cancer

Expression of MET-T1010I protein or overexpression of wild-type MET exhibited similar patterns of biological effects, with MET-T1010I showing greater activity than WT MET. To compare effects of the MET aberrations on cell signaling, we analyzed WT MET or MET-T1010I-transduced MCF-10A cells side by side, using phospho-kinase arrays. An empty vector-transduced cell line was used as a control [24]. Overexpression of WT MET or expression of MET-T1010I exhibited similar patterns, significantly activating multiple downstream oncogenic pathways that contribute to tumor growth, invasion, and metastasis (Fig. 6A, 6B). Consistent with the biological behavior, MET-T1010I induced higher signaling activities than WT MET.

To explore why MET inhibitors have not resulted in the expected clinical effects, we hypothesized that bypass survival mechanisms might be involved. Thus, we examined activation of 50 receptor tyrosine kinases (RTKs) using phosphorylated RTK antibody arrays. On stimulation with HGF, WT MET or MET-T1010I-transduced MCF-10A cells show phospho-MET (p-MET) to be significantly increased compared to empty vector-transduced cells, with much higher levels in MET-T1010I-transduced cells (Fig. 6C, 6D). Several phospho-RTKs were increased in overexpression of WT MET or MET-T1010I-transduced cells (Fig. 6C), including increased activation of EGFR (WT MET > MET-T1010I), FGFR3 (MET-T1010I > WT MET), ALK (MET-T1010I > WT MET), c-Ret (MET-T1010I > WT-MET), RYK (MET-T1010I > WT MET), and insulin receptor (InsulinR, WT MET > MET-T1010I). Intriguingly, phosphorylated FGFR3 (p-FGFR3) emerged with the highest signal in MET-T1010I-transduced cells, 26.8-fold higher than in empty-vector-transduced cells. Overexpression of WT MET also increased p-FGFR3 by 3.6-fold higher than in empty vector-transduced cells (Fig. 6E). Pearson correlation coefficient analysis showed a very high positive correlation between p-MET and p-FGFR3 ($r = 0.997$, $P < 0.0001$, Fig. 6F), suggesting the HGF-MET axis preferentially activates FGFR3.

Elevated circulating HGF levels in breast cancer patients are positively correlated with metastasis-related cytokines

In our studies, the biological phenotypes induced by MET were observed in an HGF-dominant environment. To explore whether HGF is dysregulated in breast cancer patients, we measured circulating HGF levels in 55 patients with breast cancer, including all three main subtypes and a set of healthy women as controls side by side. Serum HGF levels in similarly aged healthy women averaged 922.8pg/ml (range, 255–2805 pg/ml). However, HGF levels were much higher in breast cancer patients, with an average of 2476 pg/ml (1005–6005 pg/ml) ($P < 0.0001$, Fig. 7A). We further examined the serum samples from the breast cancer patients for a total of 60 cytokines (Table S3). Among them, 28 documented pro-metastasis cytokines were significantly increased (Table 1), including PDGF-BB, SCF, β -NGF, INF- γ , VEGF, TNF- α , RANTES, IL-1b, IL-4, IL-6, IL-8, IL-16, IL-2Ra, CCL1, CCL2, CCL3, CCL8, CCL11, CCL17, CCL20, CCL21, CCL22, CCL24, CCL27, CXCL5, CXCL12, CXCL13, and CXCL16. Intriguingly, Pearson analysis showed a strongly positive correlation between HGF and eotaxin-2/CCL-24 levels ($r = 0.8563$, $P < 0.0001$, Fig. 7B) and IL-16 ($r = 0.9103$, $P < 0.0001$, Fig. 7C).

Table 1
Serum cytokines in breast cancer patients or age-matched healthy women

Cytokine in serum	Breast cancer Average (Range) (pg/ml)	Normal Average/Range (pg/ml)	P Value
IL-16	1013.9 (440.3–1298.7)	716.4 (440.3 – 1010.7)	< 0.0001
PDGF-BB	2320.4 (763.9–4839.2)	694.57 (394.4–1066.5)	< 0.0001
SCF	62.0 (24.8–115.9)	28.6 (22.8–30)	< 0.0001
β-NGF	1.5 (0.6–3.1)	0.6 (0.4–0.9)	< 0.0001
CCL1/I-309	520.3 (427.1–631.7)	404 (366.1–446.4)	< 0.0001
IL-4	171.6 (97.5–256.4)	83.6 (0–162.4)	< 0.0001
SCYB16/CXCL16	722.3 (364.2–950.2)	460 (359.1–595.3)	< 0.0001
MCP-1/CCL2	192.2 (109.2–382.9)	77.8 (53.54–100.09)	< 0.0001
MCP-2/CCL8	169.6 (69.3–311.7)	88.7 (75.88–102.8)	< 0.0001
CTACK/CCL27	2473.9 (1451.6–3729.9)	1194.6 (943–1531.4)	< 0.0001
Eotaxin/CCL11	237.4 (155–310.5)	185.25 (164.1–204.67)	0.0001
CXCL13	124.8 (77.1–304.1)	59.7 (35.7–81.23)	0.0001
MIP-1α/CCL3	59.3 (38–92.1)	36.1 (28.2–42.8)	0.0002
INF-γ	320.5 (198.2–518)	192.7 (120.9–255.3)	0.0003
IL-1β	28.1 (13.6–57.6)	15.8 (12–19.3)	0.0008
CCL21	11453.4 (8492.2–15858.2)	9124.4 (7905.9–11028.9)	0.0009
MDC-4/CCL22	1579.9 (749.4–2846.7)	934.9 (749.4–1170.7)	0.001
IL-6	42.9 (0–96.6)	5.2 (0–21.4)	0.0012
IL-8/CXCL8	72.9 (33–186.9)	27.1 (16.8–37.6)	0.0018
TARC/CCL17	610.4 (282.5–1365.6)	304.5 (211.7–404.6)	0.0019
IL-2Rα	49.5 (15.2 – 123.6)	11.51 (7.85–15.17)	0.0021
VEGF	119.1 (9.8–339)	23.2 (13.69–30.73)	0.0059
TNF-α	158.5 (88.87–288.5)	125.1 (90.69–145.2)	0.0186
SDF-1/CXCL12	5994.3 (3026.5–7878.1)	4933 (4371.3–5660.5)	0.0252
Eotaxin-2/CCL24	959.8 (312.8–1880.4)	593.8 (435.1–975.3)	0.0264

Cytokine in serum	Breast cancer Average (Range) (pg/ml)	Normal Average/Range (pg/ml)	P Value
RANTES	19066.5 (4369.9–58020.8)	12634.3 (8532.1–17581.1)	0.0266
MIP-3 α /CCL20	80.8 (30.1–282.3)	42.4 (36.4–52)	0.0465
CXCL5/ENA-78)	3987.5 (2230.6–7321.4)	3007.4 (1092.3–4364.2)	0.049

Discussion

In this study, by creating and using human HGF/MET-paired spontaneous metastatic breast cancer mouse models, we provided direct evidence that overexpression of wild-type MET or MET-T1010I drives breast cancer metastasis in an HGF-dominant environment, with MET-T1010I being a stronger driver. Furthermore, we comprehensively analyzed the underlying mechanisms and revealed previously unrecognized crosstalk between the HGF-MET and FGFR3 axes. FGFR3 contributes to metastasis by driving epithelial-to-mesenchymal transition (EMT) [41–43]. EMT signaling was activated in tumors from the human HGF/MET-paired mouse model. Crosstalk between the HGF-MET axis and FGFR3 may strengthen the effect on tumor metastasis. This discovery deepens our understanding of the mechanisms of breast cancer metastasis and highlights the limitations of targeting MET alone and the potential of combination strategies. Future studies are needed to test the effects of co-targeting FGFR3 and MET signaling in vitro and in vivo. In addition, how aberrations in the HGF-MET axis activate FGFR3 signaling needs to be better elucidated, including through heteroreceptor complex formation, ligand-dependent transactivation, increased FGF production, or other mechanisms.

Our in vivo models demonstrated that MET overexpression or MET-T1010I decreased PD-L1 levels. This may explain why inhibition of MET in lung cancer increased PD-L1 levels [44], providing a rationale for combination therapy with dual blockade of MET and PD-L1. The exact underlying MET-driven mechanism remains unknown. Recent studies reported that GSK3 β phosphorylates PD-L1, leading to its degradation [45, 46]. However, this differs from our results, wherein aberrant activation of MET signaling inhibits GSK3 β via Ser9 phosphorylation, and MET inhibition decreases phosphorylation of the inhibitory site.

The MET receptor in the cell membrane is internalized upon activation by its ligand HGF and is then either recycled to the cell surface or trafficked through internal compartments for degradation via the lysosomal or proteasomal pathways, thereby representing a critical mechanism for preventing excessive receptor signaling. Exon14, which locates the JM domain of the receptor, is essential for its degradation. MET-T1010I mutation is in exon 14. After stimulation with HGF, the cells with MET-T1010I expression exhibited markedly increased and prolonged MET activity, 7-fold that of empty vector-transduced cells, and over 3.5-fold that of the cells with MET overexpression, suggesting the mutation might impair MET degradation, leading to its accumulation. However, how MET-T1010I impairs MET degradation remains to be determined.

HGF is the only known ligand that activates MET. The side-by-side assay in this study determined that circulating HGF is significantly increased in breast cancer patients compared with healthy controls, consistent with previous studies [21, 22]. Intriguingly, the enhanced HGF levels exhibit a strong positive correlation with IL-16 and eotaxin-2 (CCL-24). Eotaxin-2 is implicated in metastasis in colon cancer and hepatocellular carcinoma through its role in promoting tumor cell growth, invasion, and the formation of new blood vessels via M2 macrophage polarization, angiogenesis, invasion, migration, and recruitment of eosinophils [47–49]. We are the first to show that eotaxin-2 is significantly elevated in breast cancer patients and highly correlated with HGF levels. Research on the role of IL-16 in cancer metastasis is still in its early stages. It has been reported that breast cancer tumor cells could induce neutrophils to secrete IL-16 in distant sites, such as the lungs, potentially promoting a premetastatic niche [50]. The role of IL-16 in breast cancer and the mechanism connecting IL-16 and the HGF-MET axis remains to be elucidated.

Conclusions

In this study, we created a human HGF/ MET-paired spontaneous metastatic breast cancer mouse model. Using this physiologically appropriate tool, we provided direct evidence that MET overexpression or MET-T1010I drives breast cancer metastasis in an HGF-dominant environment. Mechanism analysis revealed that HGF-MET axis crosstalk with FGFR3 activates EMT signaling and contributes to breast cancer metastasis. This novel finding highlights the potential of combination strategies that co-target FGFR3 and MET. We also found that MET aberrations reduce PD-L1, which may explain why inhibition of MET in lung cancer increases PD-L1 levels and provides a rationale for combination therapy with MET inhibitors and PD-L1 inhibitors. In addition, the discovery that circulating HGF in breast cancer patients strongly correlates with IL-16 and eotaxin-2, which are implicated in metastasis, extends our understanding of the mechanisms of breast cancer metastasis.

Abbreviations

3D Three dimensional
AKT Protein kinase B (PKB)/Akt
ALK Anaplastic lymphoma kinase
ANOVA Analysis of variance
CCL
24 C-C motif chemokine ligand 24/ eotaxin-2
CXCL Chemokine (C
X-C motif) ligand
DMSO Dimethyl sulfoxide
ELISA Enzyme-linked immunosorbent assay
EMT Epithelial
mesenchymal transition

FGFR3 Fibroblast growth factor receptor 3
HER2 Human epidermal growth factor receptor 2
HGF Hepatocyte growth factor
HR Hormone receptor
INF Interferon
IL Interleukin
JM Juxtamembrane
MAPK Mitogen
activated protein kinase
MET Mesenchymal
epithelial transition factor /HGF receptor
mTOR Mammalian target of rapamycin
NGF Nerve growth factor
PCR Polymerase Chain Reaction
PDGF Platelet
derived growth factor
PD
L1 Programmed cell death ligand 1
PK Protein Kinase
PI3K Phosphoinositide 3
kinase
p
PK Phospho-protein kinase
p
RTK Phospho-receptor tyrosine kinase
RANTES Regulated upon activation, normal T cell expressed and secreted/ CCL5
RPPA Reverse
phase protein array
RTK Receptor Tyrosine Kinases
RYK Receptor like tyrosine kinase
SCF Stem cell factor
SCID Severe combined immunodeficiency
Syk Spleen tyrosine kinase
TKI Tyrosine kinase inhibitor
Tg Transgenic
TNBC Triple negative breast cancer
TNF Tumor necrosis factor
TSC Tuberous sclerosis complex
VEGFR Vascular endothelial growth factor receptor
WT Wild type

Declarations

Ethics approval and consent to participate

All animal experiments were performed according to a UT MD Anderson Cancer Center Animal Care and Use Committee (IACUC) approved protocol, #00001044. Human samples were obtained under protocol approved by the institutional review board at The University of Texas MD Anderson Cancer Center. The Institutional Review Board of MD Anderson approved the laboratory study # 2020 – 0732.

Consent for publication

All authors have read and approved the final version of the manuscript and consent to its publication in this journal.

Competing interests

The authors declare no competing financial interest.

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Author Contribution

S. L. : Conceptualization; Investigation; Methodology; Visualization; Data acquisition; Data analysis; Interpretation; Writing original draft; Writing-review & editing; Funding acquisition. M. G.: Histopathological analysis on animal samples. Z. J.: RPPA data analysis. H. D.: Data acquisition. Y. L.: Supervised RPPA analysis. R. A.: Supervised Z. Ju. S. H.: Provided samples. G. B. M.: Review & editing; Discussions. SY. L.: Supervised H. Dai; Discussion; Review & editing. D. T.: Funding acquisition; Supervision; Review & editing. All authors reviewed the manuscript.

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Data Availability

All data relevant to the study are included in the article or uploaded as supplementary information. Data are available upon reasonable request.

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Figures

Figure 1

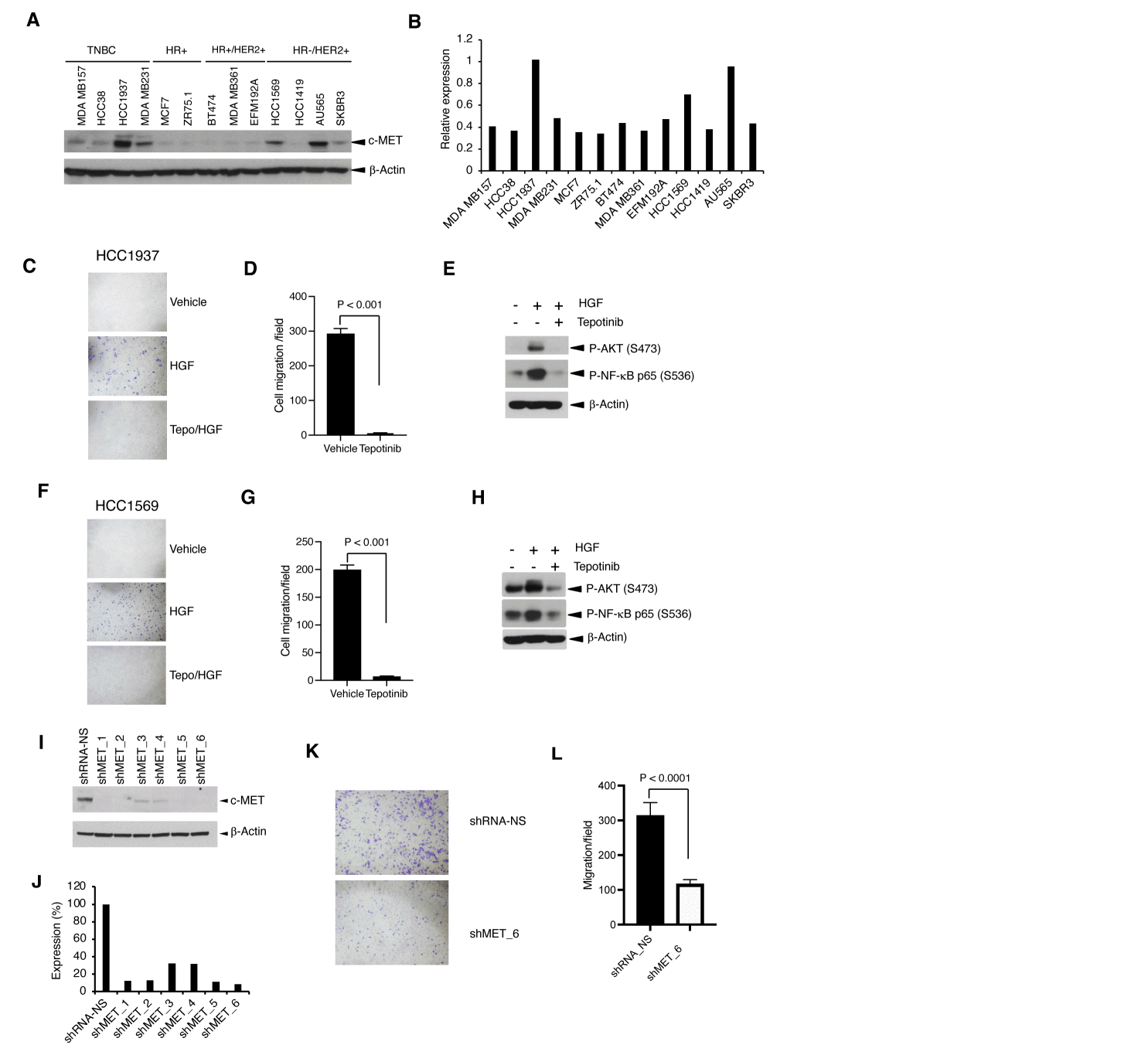


Figure 1

Expression of MET and associated biological behavior in breast cancer subtypes.

(A) Different subtypes of breast cancer cells were examined for MET expression using Western blot analysis. (B) The density of the bands was quantitatively analyzed. Cell migration was analyzed as described in Methods. TNBC cells HCC1937 (C) and HER2+ cells HCC1569 (F) with high MET expression

were induced to migrate with HGF (50 ng/ml) and fibronectin (5 µg/ml) or vehicle for 22 hours. The migration inhibition effect was tested using the MET inhibitor tepotinib (Tepo, 5 µM). Migrated cells were photographed at ×100 magnification and were quantitatively analyzed. Data are mean ± standard deviation of triplicates, representative of two independent experiments (*t*-test) (**D**, **G**). Inhibitory effect of tepotinib (5 µM) on MET downstream signaling was tested in HCC1937 cells (**E**) or HCC1569 cells (**H**) using Western blot analysis. MET was silenced in HCC1937 cells by shRNAs. The puromycin-resistant stable colonies were pooled together and designated as shMET. A pool of cells infected with the lentivirus containing a non-effective vector (shRNA-NS) was selected and used as the control. Western blot analyses were used to confirm the reduction in MET expression (**I**). The density of the bands was quantitatively analyzed (**J**). The cells transduced with shMET-6 were used for migration assays (**K**). Data were analyzed using *t*-test analysis of variance and are reported as the mean ± SD of triplicates (**L**)

Figure 2

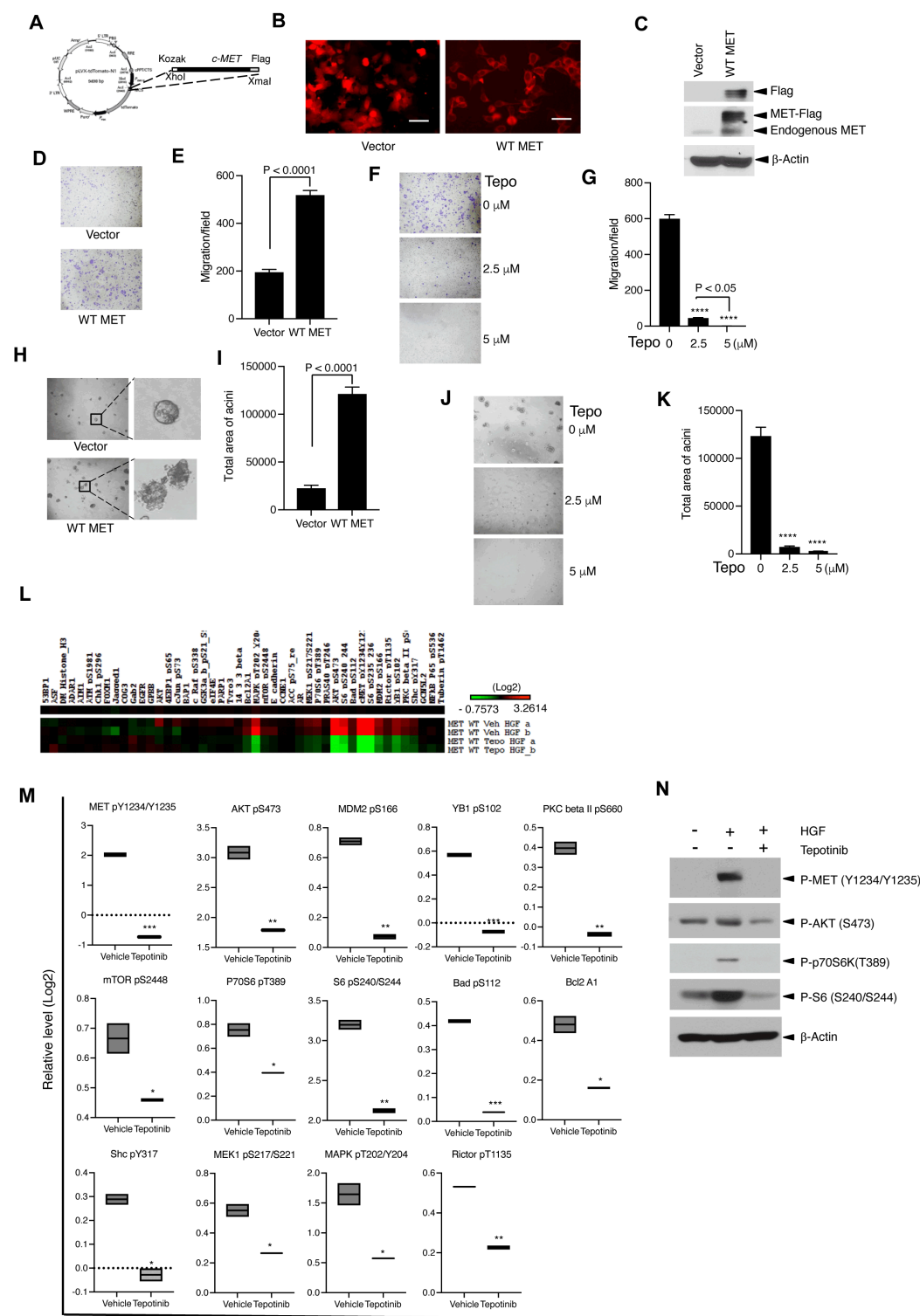


Figure 2

Overexpression of MET increased cell migration, proliferation, and scatter induced by HGF, which were blocked by tepotinib.

(A) Constructs expressing Flag epitope-tagged full-length human MET. (B) Lentiviruses expressing wild-type (WT) MET were used to transduce HER2+ breast cancer HCC1954 cells. The expression of

tdTomato fluorescent protein was examined (scale: 50 μ m). **(C)** The expression of exogenous flag-tagged WT MET was confirmed by Western blotting analysis. **(D)** Cell migration induced by HGF (50 ng/ml) and fibronectin (5 μ g/ml). **(E)** Migrated cells were quantitatively analyzed. **(F)** The inhibitory effect of tepotinib (Tepo) on cell migration was tested with the different doses. **(G)** Migrated cells per field were quantitatively analyzed (****, $P < 0.0001$ vs. Vehicle, ANOVA). **(H)** The effect of MET overexpression on acini formation and growth was tested in low FBS (2.5%) medium supplemented with 20 ng/ml HGF containing 2% in growth factor reduced Matrigel. Representative field images of acini were taken on day 8; original magnification, $\times 40$. Acini were photographed and counted in 10 randomly chosen fields. **(I)** The total area of the acini was quantitatively analyzed using AlphaView SA software (Cell Biosciences) and expressed as means $\pm$ standard deviation (SD), representing three independent experiments. **(J)** The inhibitory effect of tepotinib (Tepo) on acini growth was examined with the different doses. **(K)** The total area of the acini was quantitatively analyzed. **(L)** HCC1954-derived cells with overexpression of MET were treated with tepotinib (Tepo, 5 mM) or vehicle in serum-free medium overnight, followed by stimulation with HGF (40 ng/ml) for 30 minutes. Cell lysates were subject to RPPA. Data are presented in a matrix format: each column represents an antibody target and each row a sample. In each sample, the ratio of the abundance of the molecule to its median abundance across all samples is represented by the color of the corresponding cell in the matrix (see the scale for expression levels). **(M)** The RPPA data were further analyzed (mean $\pm$ standard deviation; P value based on the log2 data). **(N)** A portion of the RPPA data was validated by Western blot. Data were expressed as mean $\pm$ SD, and analyzed with one-way ANOVA for multiple groups, and the t -test was used for two groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ vs. vehicle).

Figure 3

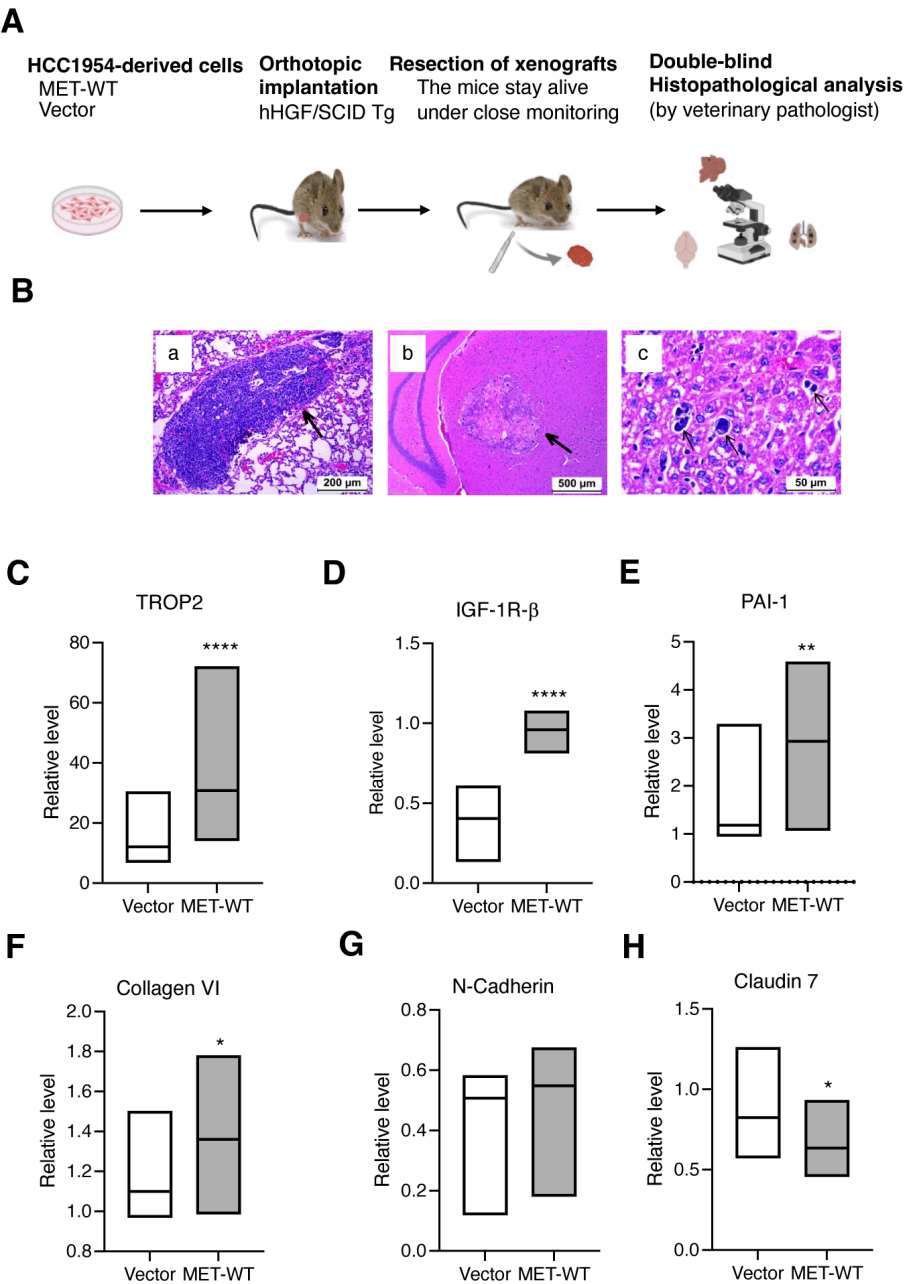


Figure 3

Effects of MET overexpression on metastasis in breast cancer in an HGF-dominant environment.

(A) Human HGF/MET-paired metastasis mouse models were established as illustrated and described in Methods. (B) Represent metastatic disease in lung (a), brain (b), and liver (c), (C-H) snap-frozen xenograft tumor lysates were subjected to RPPA. EMT signaling proteins were further analyzed for the

(A) Molecular structure of MET with T1010I mutation. (B) Lentiviruses expressing MET-T1010I were used to transduce HER2+ breast cancer HCC1954 cells. The expression of tdTomato fluorescent protein was examined (scale: 50 μ m). (C) The expression of exogenous Flag-tagged MET-T1010I was confirmed by Western blotting analysis. (D) Cell migration induced by HGF (50 ng/ml) and fibronectin (5 μ g/ml). (E) Migrated cells were quantitatively analyzed. (F) The inhibitory effect of tepotinib (Tepo) on cell migration was tested with the different doses. (G) Migrated cells per field were quantitatively analyzed (****, $P < 0.0001$ vs. Vehicle) (H) The effect of MET-T1010I on acini formation and growth was tested as described in Fig. 2H. Representative field images of acini were taken on day 8; original magnification, $\times 40$. Acini were photographed and counted in 10 randomly chosen fields. (I) The total area of the acini was quantitatively analyzed using AlphaView SA software and expressed as mean $\pm$ standard deviation (SD), representing three independent experiments. (J) The inhibitory effect of tepotinib (Tepo) on acini growth was examined with the different doses as described in Fig. 2J. (K) The total area of the acini was quantitatively analyzed. (L) HCC1954-derived cells with MET-T1010I expression were treated with tepotinib (Tepo, 5 mM) or vehicle in serum-free medium overnight, followed by stimulation with HGF (40 ng/ml) for 30 minutes. Cell lysates were subjected to RPPA as described in Fig. 2L. (M) A portion of the RPPA data was validated by Western blot. Data were expressed as mean $\pm$ SD, and analyzed with one-way ANOVA for multiple groups, and the t -test was used for two groups (****, $p < 0.0001$ vs. vehicle).

Figure 5

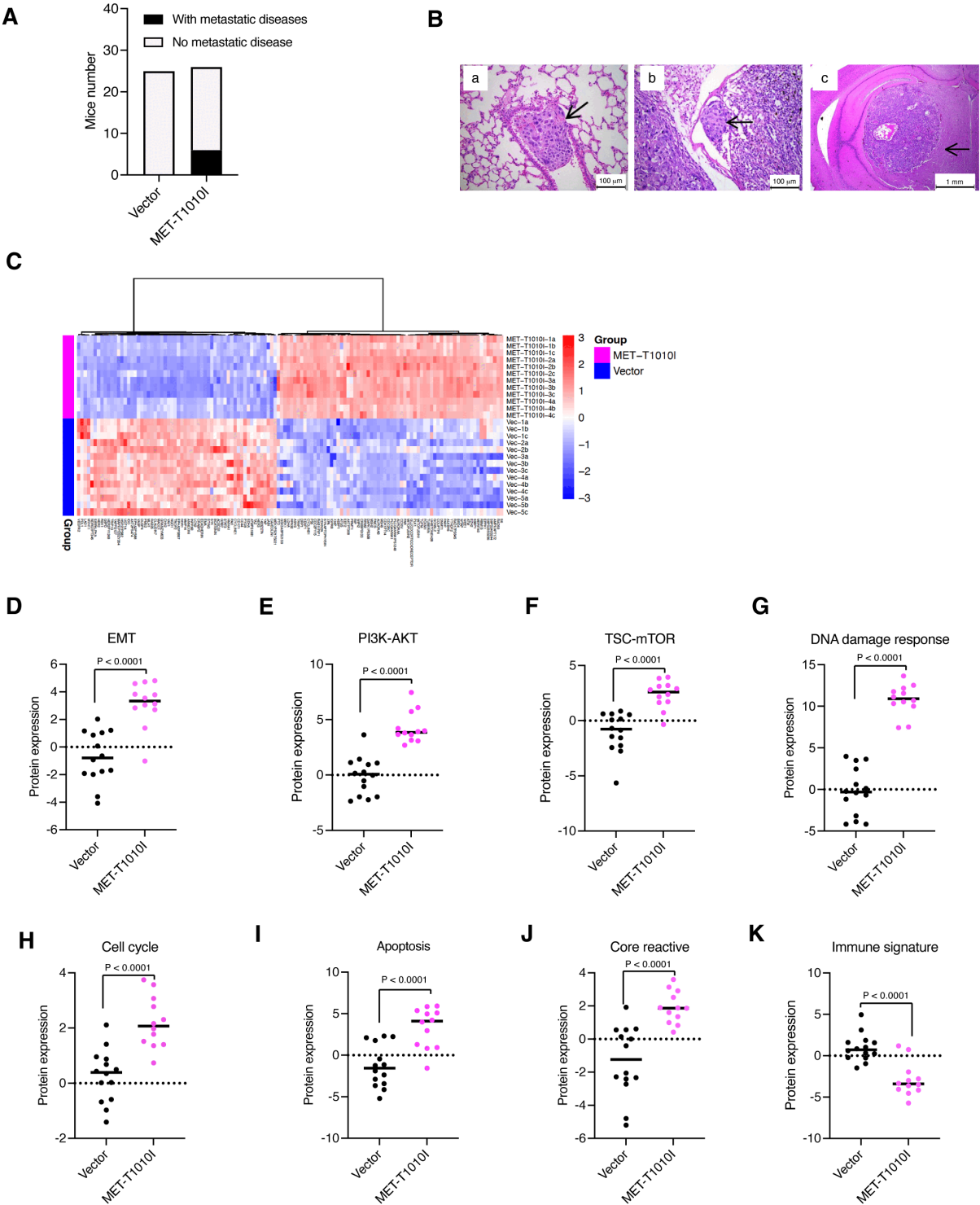


Figure 5

Effects of MET-T1010I on metastasis in breast cancer in an HGF-dominant environment.

The human HGF/MET-T1010I-paired metastasis mouse model was established as described in Methods. (A) Metastatic diseases were analyzed. (B) Representative metastatic disease in the lung (a), mammary lymph node (b), and brain (c). (C) Snap-frozen xenograft tumor lysates were subjected to RPPA. Data are

presented in a matrix format: each column represents an antibody target and each row a sample. In each sample, the ratio of a molecule's abundance to its median abundance across all samples is represented by the color of the corresponding cell in the matrix (see the scale for expression levels). The RPPA data were further analyzed for pathway scores based on the predefined [35] scheme as described in Methods. Mesenchymal Transition (EMT) (D), PI3K-AKT (E), TSC-mTOR (F), DNA damage response (G), Cell cycle (H), Apoptosis (I), Core reactive (J), and Immune Signature (K). The *t*-test was used for two groups.

Figure 6

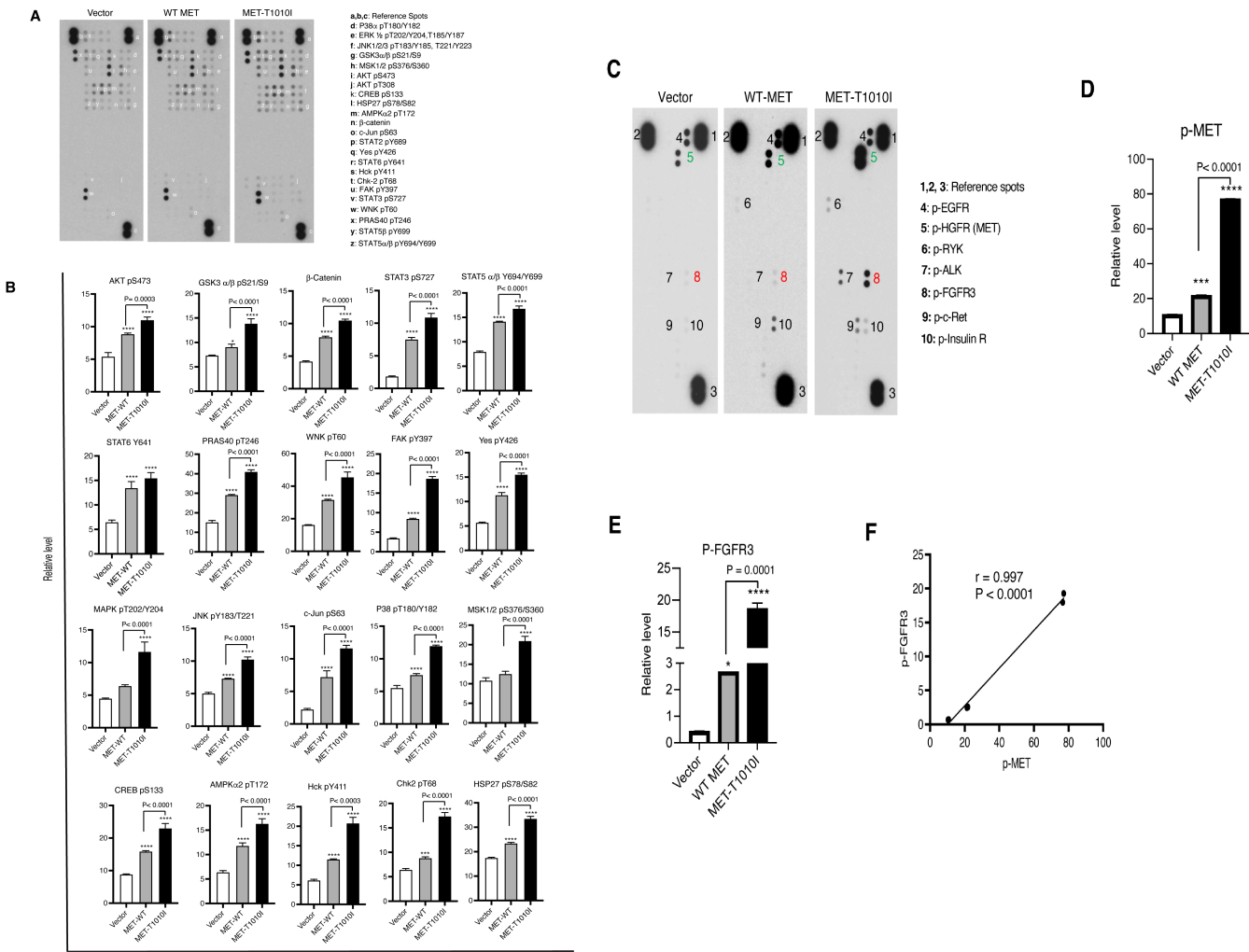


Figure 6

Activation of protein kinases (PK) and receptor tyrosine kinases (RTK) induced by aberrant HGF-MET axis in breast cancer.

The effects of MET aberrations on cell signaling were examined by phospho-kinase array and phospho-RTK array. The cell lysates from WT MET or MET-T1010I-transduced MCF-10A cells were assessed using the human phospho-RTK Array kit or the human phospho-PK kit. After serum-starvation overnight, the cells were stimulated with HGF (40 ng/ml) for 30 minutes. Cell lysates were prepared and assessed for phospho-protein kinase array (A) or Phospho-RTK array (C). Quantitative analysis was performed with AlphaView SA software. Some key kinases were further analyzed to determine the difference between

the groups, from p-PK array (**B**), and p-MET (**D**), p-FGFR3 (**E**) from p-RTK array (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ vs. Vector Group). (**F**) Correlation between phospho-MET (p-MET) and phospho-FGFR3 (P-FGFR3) was analyzed with the Pearson r coefficient.

Figure 7

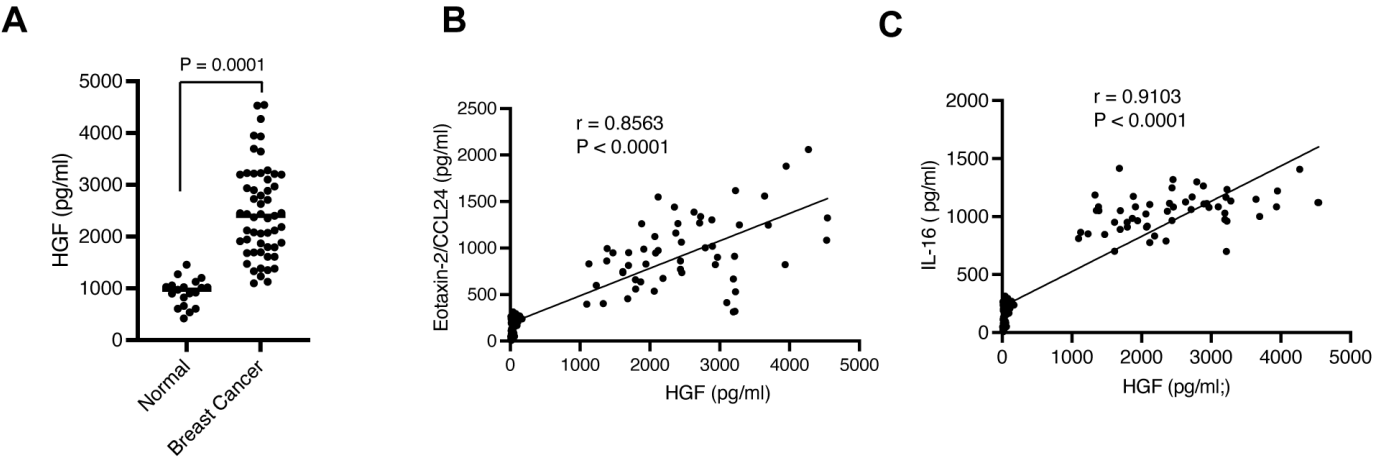


Figure 7

Upregulation of circulating HGF and correlation with metastasis-related cytokines in breast cancer patients.

(**A**) The serum samples from breast cancer patients or healthy women were measured for HGF with ELISA. The patient samples were also assessed for 60 cytokines as described in Methods. Correlations between HGF and eotaxin-2/CCL24 (**B**) or IL-16 (**C**) were analyzed with the Pearson r coefficient.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryfile.pdf](#)