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Biological properties of ligand-dependent activation of the MET receptor kinase in acute myeloid leukemia

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Aberrant kinase signaling is a hallmark of myeloid malignancies that has become the focus of targeted therapies due to its integral role in leukemia cell proliferation and survival.[1] In acute myeloid leukemia (AML) in particular, aberrant kinase signaling results from activating mutations of *FLT3*, *KIT* and *NRAS* or mutational inactivation of their negative regulators *CBL*, *PTEN* and *PTPN11*. However, recent genome-wide surveys have revealed that no more than 50% of patients possess activating genetic mutations in these kinase signaling pathways.[2] This suggests that other mechanisms of kinase activation may exist.

Ligand-dependent activation of receptor tyrosine kinases (RTKs) has long been recognized as an alternative mechanism of aberrant kinase signaling. Indeed, it is now over three decades since Sporn and Todaro[3] proposed their 'autocrine hypothesis' that postulated the essential functions of secreted factors in the promotion of autonomous cell growth required for malignant transformation. We now recognize many examples of carcinogenic autocrine signaling, involving ligands of the epidermal growth factor (EGF), insulin-like growth factor, platelet-derived growth factor, fibroblast growth factor, EPH, vascular endothelial growth factor, and Fms-like tyrosine kinase 3 (FLT3) and KIT receptors in AML

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CONFLICT OF INTEREST

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specifically.[4–6] Recently, autocrine activation of the MET receptor kinase was found in almost 40% of patients with AML, causing enhanced leukemia cell survival owing to aberrant expression of the MET ligand hepatocyte growth factor (HGF) that could be blocked by the competitive RTK inhibitor crizotinib.[7,8] However, translation of these findings into therapeutic clinical trials remains hindered by limited knowledge about the specific subtypes of AML in which MET activation occurs and by the development of resistance to MET kinase inhibitors due to the overexpression of HGF that adaptively reactivates MET signaling.[8]

Here, using reverse-phase protein arrays (RPPA) and mathematical modeling of ligand-dependent MET receptor activation, we determined signaling pathways associated with MET activation in primary AML cells and defined specific strategies to achieve durable inhibition of ligand-dependent kinase signaling in AML. Proteomic RPPA profiling of 511 AML patient specimens collected at the University of Texas M.D. Anderson Cancer Center was done using previously described methods, with experimental details and patient characteristics described in detail elsewhere.[9,10] Matched peripheral blood and bone marrow samples were available for 140 patients. Paired primary and relapse samples were available for 48 of the AML and 1 of the acute promyelocytic leukemia patients. Outcome analysis was restricted to newly diagnosed patients. The majority of AML patients received arabinofuranosyl cytidine (Ara-C)-based therapy, while acute promyelocytic leukemia patients received combination all-trans retinoic acid therapy.[9] The statistical methods used in this study are described elsewhere in detail,[9] including the use of Super-Curve algorithms, paired *t*-tests, Bonferroni multiple-hypothesis corrections, mixed-effects linear models and Pearson and Spearman correlation analysis.

Using the RPPA approach, we found that 11% of all AML, 43% of acute promyelocytic leukemia, 9% of newly diagnosed, as well as 27% of relapsed AML specimens exhibit increased levels of MET phosphorylation, as compared with CD34+ controls (Figure 1a), which did not depend on cryo-preservation status (Supplementary Figure S1a). We found that MET phosphorylation was relatively higher in the bone marrow as compared with peripheral blood ($P = 0.003$, Figure 1b), consistent with ligand-dependent signaling that would be potentiated by restricted ligand diffusion of the bone marrow microenvironment.

Analysis of MET phosphorylation with respect to the specific AML molecular subtypes revealed increased MET activation associated with the t(15;17) and t(8;21) cytogenetic subtypes in addition to specimens harboring both chromosome 7 deletion (−7) and chromosome 8 trisomy (+8) ($P<0.05$ for all comparisons, Figure 1c). Importantly, we found that increased levels of MET activation were associated with the absence of *FLT3* mutations ($P = 0.0505$; Supplementary Figure S1b). We did not identify an association between MET activation and primary chemoresistance ($P = 0.89$; Supplementary Figure S1c) but did find significantly increased MET activation in specimens collected at relapse as compared with diagnosis ($P = 0.0003$; Figure 1d). No association was observed between MET phosphorylation and overall or event-free survival, even when multivariate analysis was performed comparing MET activation with clinically relevant variables, including age, performance status, laboratory values and cytogenetics (Supplementary Figures S2a and b). Furthermore, MET phosphorylation had no apparent effect on the outcomes in the

prognostically favorable t(15;17) and t(8;21) subgroups with which it had been associated (Supplementary Figures S2c and d).

Analysis of MET phosphorylation in comparison with 210 additional RPPA protein markers revealed the expression levels of 34 and 32 proteins to be significantly positively and negatively correlated with MET phosphorylation, with the Pearson correlation coefficients of >0.2 and < − 0.2, respectively (Figure 2a). Among these proteins, we found phosphorylation of AKT1, signal transducer and activator of transcription factor 1, β-catenin, YAP1 and BAD, as well as increased expression of proto-oncogenes, such as MSI2, cyclin D1 and JUNB (Supplementary Figure S3a). These co-activated signaling pathways may constitute rational targets for combination therapy to achieve sustained MET inhibition.

To understand the basis of resistance to competitive MET kinase inhibition, we developed and analyzed a mathematical model of the activated MET signaling complex (Supplementary Figures S3b and c). The mathematical model of ligand-dependent MET receptor activation is based on the mass action equilibria of the major molecular species. We assumed that the equilibrium of monomeric (M) and dimeric (M_2) MET species is equal to its homolog EGF receptor (L_2 = 0.8 M), with M_2 being the active species.[11,12] In the case of modeling receptor activation by HGF, we used high and low HGF-binding states (β_2 = 100 pM, β_1 = 90 nM), for the dimeric (M_2) and monomeric (M) receptors, respectively.[13,14] For competitive inhibition of MET kinase by crizotinib (C_1 = 8 nM), we modeled its binding to the inactive monomeric (M) receptor.[15,16] We then calculated total MET activity based on the fractional occupancy of the dimeric MET receptor, as a function of binding of HGF and inhibitor to the monomeric and dimeric MET receptors (β_1 and β_2 and C_1 and C_2), respectively. Dose–response surfaces were plotted using Mathematica version 8 (Wolfram Research, Champaign, IL, USA).

Using this mathematical analysis, we found that residual MET kinase activity can be maintained by increasing the levels of HGF in the presence of the competitive MET tyrosine kinase inhibitor crizotinib (Figure 2b).[17] Even at supraphysiological crizotinib concentrations (1000 nM), sufficient HGF concentrations are available to maintain partial MET kinase activity. This indicates that the thermodynamic coupling between ligand-mediated receptor dimerization and inhibitor kinase binding can limit the therapeutic efficacy of competitive inhibitors, particularly those that preferentially stabilize the inactive kinase conformation.

In summary, our study establishes that autocrine MET signaling, as assessed by direct measurements of MET phosphorylation, is associated with distinct molecular abnormalities in AML such as t(8;21) and t(15;17) translocations, which trans-activate *HGF* expression by the expression of leukemogenic transcription factor fusion proteins to drive autocrine MET signaling.[8] Notably, increased MET activation was found to be associated with the absence of *FLT3* mutations in the examined patient cohort. This may indicate that autocrine MET signaling functions as an alternative pathway for leukemogenesis in cells lacking FLT3 activation or in functionally privileged subsets of cells, such as leukemia-initiating cells.[18] Consistent with this notion, we found an increased prevalence of MET activation upon

leukemia relapse, suggesting that MET signaling may contribute to enhanced cell survival or the emergence of chemotherapy-resistant cell subsets.

In the previous work, therapeutic targeting of autocrine MET activation using the competitive kinase inhibitor crizotinib led to the emergence of resistance owing to HGF upregulation.[8] Similar ligand-dependent mechanisms of resistance were found to contribute to the resistance to inhibition of EGF receptor, BRAF and FLT3 RTKs.[19] Here we found that residual MET kinase activity can be preserved in the presence of a wide range of concentrations of the competitive MET tyrosine kinase inhibitor crizotinib, even at inhibitor levels higher than those achievable clinically in patients. This resistance phenomenon may be exacerbated *in vivo*, where the dynamic and micro-environmental nature of autocrine signaling can facilitate adaptive ligand upregulation and local potentiation, respectively.

These findings suggest that therapeutic targeting of MET activation in AML, and other ligand-driven RTKs, will need to rely on strategies that can overcome the thermodynamic mass action coupling between ligand and competitive inhibitor binding. Possible approaches to overcome this barrier may include the use of ligand-neutralizing antibodies or non-competitive inhibitors, such as the recently developed covalent inhibitors of the EGF RTK.[20] Likewise, therapies targeting adaptive or cooperating pathways themselves, as identified by the studies here, may be used to potentiate the durability of competitive kinase inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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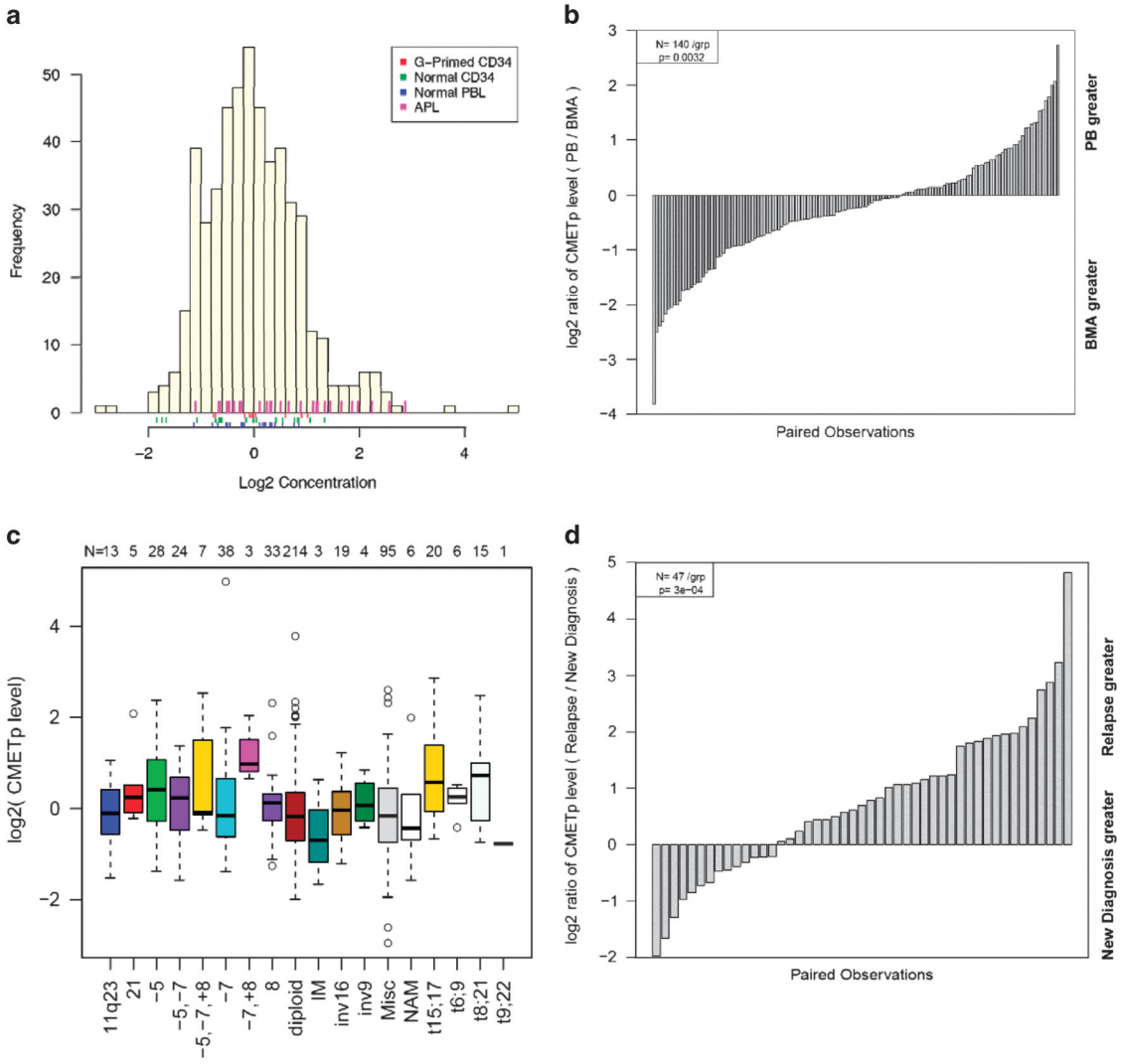

Figure 1.

(**a**) Histogram of MET phosphorylation levels detected in primary AML cells relative to normal and stimulated controls. (**b**) Distribution of MET phosphorylation levels in pairs of samples from the peripheral blood and bone marrow showing significantly higher levels in bone marrow samples. (**c**) Analysis of MET phosphorylation as a function of AML cytogenetic subtype. (**d**) Analysis of MET phosphorylation in newly diagnosed versus relapsed AML specimens, demonstrating significantly increased levels at disease relapse.

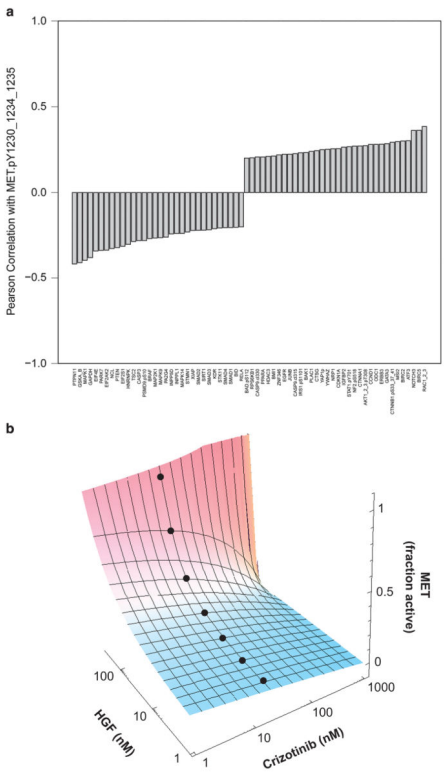

Figure 2.

(**a**) Waterfall plot of proteins significantly associated with MET phosphorylation. (**b**) Dose–response surface of ligand-dependent MET kinase activation by HGF and competitive kinase inhibition by crizotinib. Blue-to-red color gradient indicates MET kinase activation. Black dots represent crizotinib plasma trough concentrations achievable in patients.