c-Myc Alterations Confer Therapeutic Response and Acquired Resistance to c-Met Inhibitors in MET-Addicted Cancers

Aijun Shen, Lu Wang, Min Huang, Jingya Sun, Yi Chen, Yan-Yan Shen, Xinying Yang, Xin Wang, Jian Ding, and Meiyu Geng

Abstract

Use of kinase inhibitors in cancer therapy leads invariably to acquired resistance stemming from kinase reprogramming. To overcome the dynamic nature of kinase adaptation, we asked whether a signal-integrating downstream effector might exist that provides a more applicable therapeutic target. In this study, we reported that the transcriptional factor c-Myc functions as a downstream effector to dictate the therapeutic response to c-Met inhibitors in c-Met–addicted cancer and derived resistance. Dissociation of c-Myc from c-Met control, likely overtaken by a variety of reprogrammed kinases, led to acquisition of drug resistance. Notably, c-Myc blockade by RNA interference or pharmacologic inhibition circumvented the acquired resistance to c-Met inhibition. Combining c-Myc blockade and c-Met inhibition in MET-amplified patient-derived xenograft mouse models heightened therapeutic activity. Our findings offer a preclinical proof of concept for the application of c-Myc–blocking agents as a tactic to thwart resistance to kinase inhibitors. Cancer Res; 75(21): 1–12. ©2015 AACR.

Introduction

Despite the remarkable clinical success, the major challenge for targeted therapy lies in quick acquisition of acquired resistance (1–4). Various molecular mechanisms of resistance have been revealed, including gatekeeper mutations, amplification or overexpression of target genes, or modification of signaling pathways. Among them, activation of compensatory kinase(s), which allows cancer cells overriding the response to target inhibition, has been frequently observed (5–7). For example, resistance to the EGFR inhibitor gefitinib can result from MET amplification, HER2 amplification, or upregulation of kinases like IGFR, FGFR, or AXL (6, 8). The arising compensatory kinases seem to be highly variable, suggesting kinome-wide changes in tumor cells following pharmacologic or genetic pressures. Indeed, human kinome sets that feature inherent plasticity and robustness undergoes rapid and dynamic remodeling known as ‘kinome reprogramming,’ which allows circumventing the effects of selective kinases inhibitors (9). For example, MEK inhibition caused acute ERK activity loss but induced a dynamic reprogramming of kinome in triple-negative breast cancer cells (10). As such, although combination therapy to concurrently diminish provoked kinases has been accepted as a therapeutic strategy to overcome kinase resistance (11, 12), the application of this strategy is indeed more complicated than it appears.

Recently, in-depth analysis of kinases signaling network has revealed that divergent kinases signaling pathways often converge on common downstream effectors to modulate cell survival (13–15). For example, EGFR-mutant non–small cell lung cancer (NSCLC), Ber-Abl–translocated chronic myeloid leukemia, HER2-amplified breast cancer, BRAF-mutant melanoma, and EMLA-ALK–translocated NSCLC all require proapoptotic BH3-only protein Bim to mediate apoptosis resulted from kinases inhibition (16–20). Elevation of Bim indicates the therapeutic outcomes of target inhibition, whereas Bim silence is associated with loss of therapeutic efficacy (21–24). These evidence triggers an increasing appreciation of a ‘kinase downstream effector’ paradigm in kinase-addicted cancer, where these downstream effectors integrate the complexity of upstream kinase signaling network to regulate cell growth and provide “fragile points” of kinase-addicted cancer (25). To date, it remains unknown whether the same paradigm exists in kinase-addiction–derived resistance. To identify fragile points of acquired resistance, if any, will gain valuable therapeutic opportunities for acquired resistance and bring a conceptual breakthrough in resistant management in kinase-targeted therapy.

To address this question, we herein take c-Met, a receptor tyrosine kinase aberrantly overactivated in many solid tumors and associated with poor prognosis (26, 27), as a representative target to discover whether there exists a “fragile point” in acquired resistance of c-Met–addicted cancer. Indeed, this study has discovered that c-Myc serves as a downstream effector that critically converges upstream signaling to drive cell growth in both c-Met–addicted cancer and derived resistance. Transitionally, while disturbing c-Myc will provide therapeutic options for overcoming...
acquired resistance to c-Met inhibition, c-Myc also serves as a functional molecular probe to inspect the response to c-Met inhibitors, monitor the emergence of acquired resistance, and assess the effectiveness of alternative therapies. Our study has proved the fundamental role of c-Myc in directing clinical decision-making in c-Met–targeted cancer therapy.

**Materials and Methods**

**Cell lines and reagents**

EBC-1, BGC-823, MKN-1, MKN-28, and MKN-74 cells were obtained from Japanese Research Resources Bank. MKN-45 and BaF3 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. HCC827 and HCC827/GR6 were kindly gifted by Dr. Pasi A. Jänne (Dana-Farber Cancer Institute, Boston, MA). Other cell lines used in this study were obtained from the ATCC and authenticated by short tandem repeat (STR) testing. All the cell lines were maintained in appropriate medium as suppliers suggested.

(+)JQ1 and (−)JQ1 were kindly provided by Dr. James E. Bradner (Dana-Farber Cancer Institute), and the other inhibitors were obtained from Selleck Chemicals. SGX-523 and PF-2341066 for *in vivo* studies were obtained from Haoyuan Chemexpress Co., Ltd. 17-DMAG for *in vivo* studies was obtained from LC Laboratories. All these reagents were dissolved in DMSO for *in vitro* studies and in 0.5% sodium carboxymethyl cellulose (SGX-523) or sterile PBS (PF-2341066 and 17-DMAG) for *in vitro* studies.

**DNA construction, virus production, and infection**

The retroviral constructs MSCV-Myc, pBABE-TPR-MET, and empty vectors were obtained from Addgene. pBABE-TPR-MET (L290T) was constructed with a site-directed mutagenesis kit (Sbsbio). To generate cells with stable expression, the plasmids were transfected into amphotropic phoenix 293T packaging cells with Lipofectamine 2000 (Invitrogen). After 48 hours, virus-containing medium was collected, filtered, and used to infect host cells in the presence of 6 mg/mL of polybrene. The stable transfectants were obtained by selection with 1 mg/mL puromycin (Sigma) for 2 weeks followed by immunoblotting validation.

**Generation of c-Met–resistant cells**

To generate cells resistant to c-Met inhibition, EBC-1 cells were exposed to SGX-523 at concentrations increased stepwise from 10 nmol/L to 1 μmol/L when the cells resumed growth kinetics similar to the untreated parental cells. After about 6 months, a resistant subpopulation called EBC-1/SR were obtained and the resistant cells were maintained in the presence of SGX-523 at 1 μmol/L for another 2 months. After several passages in drug-free conditions, the resistant cells retained their resistant phenotype as determined by cell viability assay. Parental cells were maintained concomitantly without SGX-523, and no significant change in the sensitivity was noted during the period.

**Animal studies**

Four- to 6-week-old nu/nu athymic BALB/c mice were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All studies were done in compliance with Institutional Animal Care and Use Committee guidelines of Shanghai Institute of Materia Medica. Tumors were generated by transplanting 5 × 10⁶ tumor cells resuspended in PBS (200 μL/mouse) into the right flank. Prior to initiation of treatment, mice were randomized among control and treated groups (*n* = 6 per group). For efficacy studies, mice were treated with drugs using the indicated doses. For combination treatment, both drugs were given concurrently. The average tumor diameter (two perpendicular axes of the tumor were measured) was measured in control and treated groups with Vernier calipers every 3 days. For statistical analysis, data were analyzed by the unpaired two-tailed Student *t* test and *P* < 0.05 was considered statistically significant. To prepare lysates, mice were sacrificed and tumor tissues were resected and homogenized in cold RIPA lysis buffer (Beyotime) supplemented with protease and phosphatase inhibitors (Merck) and then processed for immunoblotting.

Animal studies using patient-derived xenograft models were conducted by Crown Bioscience and in strict accordance with the Guide for the Care and Use of Laboratory Animals of the NIH (Bethesda, MD).

**Results**

**Multiple kinases are involved in resistance acquisition to c-Met inhibition**

Accumulated evidence has suggested the role of MET amplification in rendering c-Met addicted cell growth (28–31). We firstly confirmed this by interrogating a panel of NSCLC and gastric cancer cells with SGX-523, a highly selective c-Met kinase inhibitor (32). Indeed, cell lines harboring MET amplification showed constitutively activated c-Met and accordingly showed exquisite sensitivity to c-Met inhibition (Supplementary Fig. S1A–S1C). c-Met–addicted EBC-1 cells were then selected to generate acquired resistance. The resultant cells, designated as EBC-1/SR, showed at least 500-fold reduced sensitivity to SGX-523 and cross-resistance to other putative c-Met inhibitors PHA-665752, PF-2341066, and XL-880 as well (Fig. 1A). The copy number of MET proto-oncogene in EBC-1/SR cells was identical to that of parental cells (Fig. 1B); meanwhile, c-Met phosphorylation was effectively inhibited by SGX-523 (Fig. 1C). These data ruled out the possibilities of resistance resulted from loss of MET amplification or occurrence of c-Met mutations that decreased the affinity for kinase inhibitors.

Obviously, SGX-523 failed to block AKT and ERK signaling in EBC-1/SR cells (Fig. 1C), suggesting that alternative kinase(s) should be engaged in the resistance acquisition. To identify compensatory kinases in resistant cells, we conducted phospho-receptor tyrosine kinases arrays to compare the phosphorylation status in the parental and resistant cells. The phosphorylation of multiple kinases, including PDGFR, TYRO10, Tyk2, CSK, VEGFR, etc., was increased in the resistant cells, suggesting the activation of multiple kinases upon resistance occurrence (Fig. 1D). Next-generation sequencing and genome-wide copy number analyses revealed that none of driving point mutation, amplification, or fusion was detected in the genome of resistant cells, largely excluding the self-activation of these kinases (data not shown). We then asked whether ligand-mediated activation of kinases contributed to the resistant phenotype. Indeed, profiling of the secreted proteins with high-density antibody arrays showed increased level of multiple cytokines and growth factors, such as platelet-derived growth factor (PDGF), VEGF, nerve growth factor, placental growth factor, etc., in the culture medium of resistant cells (Fig. 1E; Supplementary Table S1). These data suggested that the acquired resistance to c-Met inhibition may arise from ligand-mediated activation of multiple kinases in the resistant cells.
Figure 1. Multiple kinases are coactivated in c-Met-resistant cells. A, IC50 values of SGX-523, PHA665752, PF-2341066, and XL-880 against EBC-1 and EBC-1/SR cells were assessed using CCK-8 assay. Bars, means ± SD. B, MET gene copy number of EBC-1 and EBC-1/SR cells was examined using quantitative real-time PCR and normalized to that of TOP3A. Bars, means ± SD. C, EBC-1 and EBC-1/SR cells were treated with SGX-523 at 1 μmol/L for 3 hours, and cells lysates were immunoblotted with the indicated antibodies. D, tyrosine phosphorylation of 71 RTKs in EBC-1 and EBC-1/SR cells was assessed using phospho-RTK arrays. Up- and downregulated kinases (fold change > 2) are listed. E, secreted proteins in the culture medium of EBC-1 and EBC-1/SR cells were profiled using high-density antibody arrays. Upregulated cytokines and growth factors (fold change > 2) are listed. F, viability of EBC-1/SR cells upon treatment with a panel of kinase inhibitors was assessed using CCK-8 assay. Bars, means ± SD.
For further confirmation, we probed sensitivity of resistant cells to a panel of kinase inhibitors, alone or in combination with SGX-523 (Supplementary Table S2). As expected, kinase inhibitors specifically targeting PDGFR, FGFR, EGFR, etc., failed to suppress the growth of resistant cells even when combined with SGX-523. Instead, multitarget kinase inhibitors, such as dasatinib and PP-121, were able to inhibit cell growth. Likewise, simultaneous disruption of multiple kinases by targeting Hsp90 using 17DMAG and ganetespib circumvented the resistance in EBC-1/SR cells (Fig. 1F). Similar results were obtained in another generated resistant MKN-45/SR cells (Supplementary Fig. S1D).

Together, our data suggest that concomitant inhibition of kinase complements responsible for resistance acquisition to c-Met inhibition is largely challenged by such dynamic reprogrammed nature of kinome, particularly considering the lack of clinically applicable inhibitors of some aroused kinases.

c-Myc determines the biologic response of c-Met inhibition in MET-addicted cancer cells

Inspired by the evidence that kinase inhibition only achieves therapeutic outcomes when the signaling attenuation is delivered to downstream effectors, which regulates ultimate cell survival, we asked whether it is possible to identify such downstream molecules of the c-Met signaling pathway, namely a "fragile point" of both c-Met–addicted cancer and its derived resistance. To this end, we first probed c-Met–driven cellular processes in c-Met–addicted EBC-1 and MKN-45 cells by global transcriptional profiling, using HCC827 cells with activated c-Met but lacking MET amplification as a negative control (Supplementary Fig. S2A). Bioinformatic annotations classified the substantially modulated genes by SGX-523 treatment into three major biological processes, namely cell cycle regulation, DNA replication, and cell metabolism (Supplementary Fig. S2B), which were confirmed by flow cytometry detected G1–S arrest, reduced in situ EdU incorporation, and impaired lactate secretion, respectively (Supplementary Fig. S2C–S2E). These data suggested that c-Met blockade in c-Met–addicted cells led to a state of proliferation arrest.

Stalled cell-cycle progression at G1–S phase is likely an integrated outcome resulting from impaired cell proliferation and metabolism. Thus, the major cell-cycle regulators involved in G1–S transition were probed upon c-Met inhibition in four MET-amplified cell lines (EBC-1, NCI-H1993, SNU-5, and MKN-45), with HCC827 cells as a negative control. After treatment with SGX-523 for 24 and 48 hours, c-Met phosphorylation was equally abolished in all cell lines, but the G1–S regulators were affected only in cells sensitive to c-Met inhibition. Intriguingly, we found that the alteration of G1–S transition regulators, such as CDK4, CDK6, cyclin D1, and P27, all varied among the cell lines. Only the transcription factor c-Myc was unanimously abolished across all MET-amplified cell lines (Fig. 2A). This finding was further confirmed by expanding to other c-Met inhibitors (Supplementary Fig. S2F) or disrupting c-Met expression using siRNAs (Fig. 2B). Moreover, the decreased c-Myc level upon c-Met inhibition was found to be a coordination of transcriptional suppression and compromised protein stability (Supplementary Fig. S2G–S2I), suggesting a role in integrating upstream signaling. We hypothesized that c-Myc is a candidate molecule that possibly dictates the blockade of G1–S transition upon c-Met inhibition in MET-amplified cancer cells.

To further test this possibility, we treated EBC-1 cells with JQ1, which suppress c-Myc transcription by targeting BET bromodomain (33, 34). As expected, the treatment of JQ1 dramatically downregulated c-Myc expression and inhibited the survival of EBC-1 cells despite the activated c-Met signaling (Fig. 2C). Likewise, c-Myc siRNA largely inhibited cell growth and induced G1 phase arrest (Fig. 2D; Supplementary Fig. S2K), suggesting that solely c-Myc inhibition could override active c-Met–driven cell growth. Moreover, ectopic expression of c-Myc into EBC-1 cells largely rescued the growth delay caused by c-Met inhibition (Fig. 2E). In vivo, mice bearing EBC-1 xenograft models were treated with SGX-523 at 10 or 20 mg/kg twice a day for 15 consecutive days. Along with the strikingly inhibited tumor growth, intratumoral c-Myc level was consistently and profoundly decreased after SGX-523 treatment, and c-Myc downregulation was detectable as earlier as on day 3 (Fig. 2F and G).

Together, these results indicated that c-Myc downregulation is essential for determining the biologic response of c-Met inhibition in MET-addicted cancer cells. c-Myc serves as a downstream effector that critically converges c-Met signaling to drive c-Met–dependent cell growth.

c-Myc is coupled to c-Met to control cell growth in MET-addicted cells

It appeared that the activated c-Met signaling in c-Met–addicted cells establishes an association with c-Myc for controlling cell growth. For confirmation, we generated "gain-of-MET addiction" using BaF3 cells, whose survival dependence will switch from IL3 to established oncogenic kinase (35). Once TPR-MET fusion was introduced into BaF3 cells, the resultant BaF3/TPR-MET cells were no longer dependent on IL3 for cell survival (Fig. 3A) and exhibited a profound sensitivity to SGX-523 (Fig. 3B), indicative of gain-of-MET addiction. Notably, IL3 deprivation that caused c-Myc reduction in BaF3 cells was not observed following the introduction of TPR-MET (Fig. 3C). In contrast, inactivation of c-Met by SGX-523 in BaF3/TPR-MET cells led to a dramatic reduction in the c-Myc level (Fig. 3D). These data suggest that c-Myc is incorporated into c-Met signaling to govern cell growth upon gain-of-MET addiction.

To further confirm the observed association between c-Met and c-Myc in BaF3/TPR-MET cells, a strategy using gatekeeper mutation of TPR-MET kinase domain plus inhibitors with different modes of action was used to "switch off" or "turn on" c-Met signaling. The gatekeeper residue was identified by aligning the kinase domain of c-Met and that of ALK, EGFR, and ABL (36–38). Leucine 1157 (L1157) in the kinase domain of c-Met was identified as a candidate gatekeeper residue (Fig. 3E). As expected, substitution of the same residue leucine 290 in TPR-MET with threonine (T), termed TPR-MET (L290T), conferred more than 100-fold resistance to SGX-523 compared with BaF3/TPR-MET cells (Fig. 3F). Along with loss of c-Met inhibition by SGX-523, c-Myc level was restored in the mutant cells (Fig. 3G). In contrast, ARQ-197, a non–ATP-competitive c-Met inhibitor (39), effectively inactivated c-Met signaling and inhibited cell growth of TPR-MET (L290T) cells (Fig. 3F). Upon the switch off of c-Met signaling, c-Myc expression was largely diminished by ARQ-197 in TPR-MET (L290T) cells (Fig. 3G). These data together demonstrated that c-Myc is stringently coupled to c-Met to control cell growth in c-Met–addicted cells.

Loss of c-Myc regulation by c-Met leads to resistance to c-Met inhibition

Our results so far have demonstrated the critical role of c-Myc in mediating c-Met–addicted cell growth. It will be critical to know...
whether c-Myc retains growth control in cells with developed resistance to c-Met inhibition. Intriguingly, c-Myc disruption using siRNA resulted in effective proliferation inhibition in EBC-1/SR cells (Fig. 4A), suggesting that resistant cells remained highly dependent on c-Myc. Similar results were recapitulated by blocking c-Myc using JQ1 (Fig. 4B). Meanwhile, c-Myc protein level remained intact when exposing EBC-1/SR cells to SGX-523 (Fig. 4B and C) or c-Met siRNA (Fig. 4A), indicating the dissociation of c-Myc and c-Met in cells with acquired resistance to c-Met inhibition. Nevertheless, these cells remained dependent on c-Myc for cell survival.

c-Myc is a growth-regulatory oncoprotein itself, and there is a possibility that c-Myc reactivation accounts for cell growth independent of c-Met activity. In particular, we observed an elevated c-Myc level in EBC-1/SR cells (Fig. 4C). We measured MYC gene locus in the resistant cells. No alteration of MYC gene copy was observed (Fig. 4D), which largely ruled out the self-activation of c-Myc. Instead, we found an increased mRNA level in the resistant

Figure 2.
c-Myc downregulation is essential for c-Met inhibition caused by growth arrest in MET-addicted cells. A, EBC-1, NCI-H1993, SNU-5, MKN-45, and HCC827 cells were treated with SGX-523 at 1 µmol/L for 24 or 48 hours followed by immunoblotting analysis of cell-cycle regulators. B, EBC-1 and HCC827 cells were treated with scramble or c-Met siRNAs for 72 hours followed by immunoblotting analysis. C and D, c-Myc was disrupted using (+)-JQ1 at 1 µmol/L (C) or c-Myc siRNAs (D) in EBC-1 cells before subject to immunoblotting. Cell viability and cell-cycle distribution were analyzed. Bars, means ± SD. E, sensitivity of EBC-1 cells stably transfected with empty vector or MSCV-Myc was measured using CCK-8 assay. Values represent means ± SD. F, sensitivity of EBC-1 cells stably transfected with empty vector or MSCV-Myc was measured using CCK-8 assay. Values represent means ± SD. G, tumor lysates after last dose or on day 3 were subjected to immunoblotting with indicated antibodies.
cells (Fig. 4E) and reactivation of signaling molecules like AKT and ERK (Fig. 4C). Together with the multiple activated kinase in EBC-1/SR cells (shown in Fig. 1), we speculated that c-Myc regulation in EBC-1/SR cells was overtaken by other compensatory kinases, which in turn drove cell growth independent of c-Met activity. Consistently, Hsp90 inhibitors were able to effectively diminish the expression of c-Myc alone or in combination with SGX-523 (Fig. 4F). Furthermore, in the EBC-1/SR xenograft model, mice barely responded to SGX-523 treatment as expected. In agreement, the intratumoral level of c-Myc following treatment was marginally affected by SGX-523, whereas combination of SGX-523 with the Hsp90 inhibitor 17-DMAG largely decreased the c-Myc level along with strikingly suppressed tumor growth (Fig. 4G and H). These data together suggested that c-Myc retains growth control in c-Met–resistant cells, regardless of the switched growth dependency from c-Met to other compensatory kinases.

Then, we proceeded to examine whether c-Myc is dynamically governed in the process of resistance acquisition in c-Met–addicted cells. To mimic the dynamics of resistance acquisition in clinical treatment, EBC-1 cells were treated with SGX-523 at 1 μmol/L every 3 days for up to 4 months. The sensitivity of cells to SGX-523 and JQ1 and the status of phosphorylated c-Met and c-Myc were simultaneously monitored on days 0, 3, 15, 30, 60, 90, and 120, respectively (Fig. 4I). As expected, cells exhibited increasingly declined sensitivity to SGX-523. The IC_{50} increased more than 300-fold from 10 nmol/L to more than 30 μmol/L after 4-month treatment, although the copy number of MET proto-oncogene remained the same and phosphorylation of c-Met was constantly abolished (Fig. 4J and K). Despite the gradually lost growth control of c-Met kinase, c-Myc dominated the cell growth throughout the whole process, as suggested by a constant sensitivity to JQ1 (Fig. 4I). Importantly, there appeared a dynamic transition of c-Myc regulation along with the development of resistance.
resistance. The diminished c-Myc expression by SGX-523 was gradually restored regardless of deprival of c-Met signaling (Fig. 4K). These data indicated that resistance development in c-Met–addicted cancer cells is indeed a process that c-Met kinase gradually loses control of c-Myc, which is instead overtaken by the activation of multiple alternative kinases.

Figure 4.
c-Myc is required for cell growth of c-Met–resistant cells. A and B, c-Myc in EBC-1/SR cells was inhibited using c-Myc siRNA (A) or (+)-JQ1 (B). Cell lysates were immunoblotted with the indicated antibodies and cell survival was assessed by CCK-8 assay. Bars, means ± SD. C, EBC-1 and EBC-1/SR cells were treated with SGX-523 at 1 μmol/L for 24 hours, and cell lysates were immunoblotted with the indicated antibodies. D, MYC gene copy number in EBC-1 and EBC-1/SR cells was examined. Bars, means ± SD. E, c-Myc mRNA levels of EBC-1 and EBC-1/SR cells were examined by quantitative real-time PCR and normalized to that of RPL13A. Bars, means ± SD. F, EBC-1/SR cells were treated with indicated inhibitors for 24 hours and then subjected to immunoblotting analysis using indicated antibodies. G and H, mice bearing EBC-1/SR xenografts were administered with vehicle control, SGX-523 at 20 mg/kg twice per day (BID), 17-DMAG at 50 mg/kg 4 times per week (2 days/2 days off schedule), and their combination for 15 consecutive days. Tumor volume was measured every 3 days. Intratumoral c-Myc was examined on last dose using immunoblotting. I–K, EBC-1 cells were treated with SGX-523 at 1 μmol/L every 3 days for 120 days, during which cell sensitivity was measured, and cell lysates were collected at indicated time points (I). IC50 of EBC-1 cells to SGX-523 and (+)-JQ1 were measured at indicated time points following the regimen (J). Values represent means ± SD. Cell lysates were collected at indicated time points and subjected to immunoblotting (K).
c-Myc is dispensable for cell survival of other kinase addiction

It is important to know whether c-Myc is selectively coupled to c-Met or generally serves as an effector for oncogenic kinases in controlling cell growth. To address this question, we treated HCC827 cells that are solely addicted to EGFR with both gefitinib and EGFR siRNA. We found that EGFR inhibition by either gefitinib or EGFR siRNA caused a dramatic decrease in the c-Myc level (Fig. 5A and B). Importantly, knockdown of EGFR per se significantly inhibited cell growth of HCC827 cells. However, knockdown of c-Myc alone failed to impact cell growth of HCC827 cells (Fig. 5B).

Similar results were obtained in B-RAF V600E mutation–addicted A375 cells. Inhibition of B-RAF signaling in A375 cells by either PLX-4032 or B-RAF siRNA resulted in c-Myc reduction. However, disruption of c-Myc using siRNA only slightly affected cell viability of B-RAF–addicted cancer cells, in contrast to B-RAF siRNA, which led to a dramatic inhibition of cell growth (Fig. 5C).

For further confirmation, we extended to HCC827/GR6 cells, a cell derived from HCC827 cells, and simultaneously addicted to amplified MET and mutant EGFR (40, 41). We found that although combined treatment with SGX-523 and gefitinib largely inhibited cell growth and decreased the c-Myc level in HCC827/GR6 cells (Fig. 5D), depletion of c-Myc also failed to substantially...
inhibit cell growth (Fig. 5E). All these indicated that c-Myc dependency on cell survival is restricted to c-Met–addicted cancer cells.

c-Myc directs clinical decision-making in MET-oriented clinical treatment

Our data thus far implied the important clinical applications of c-Myc in c-Met–targeted cancer therapy. Given the strictly limited accessibility to clinical test of c-Met inhibitors, we alternatively resorted to patient-derived xenograft (PDX) models, which faithfully recapitulate tumor heterogeneity and histologic characteristics of primary tumors, to mimic the clinical settings (42–44).

We started with screening Hubase, a database composed of genetic background of more than 300 patient tumors covering a variety of cancer types (Supplementary Table S3), to seek PDX models with MET amplification (copy number higher than 5). As shown, NSCLC represents the major cancer type harboring MET amplification, and we also found that other tumor types such as gastric cancer, liver cancer, and esophageal cancer bear MET amplification (Table 1). A cohort of 7 MET-amplified PDX models, which represented 7 individual patients eligible for c-Met orientated one-arm trial, was selected for the treatment of the c-Met inhibitor. Mice were treated with PF-2341066, a clinically available c-Met inhibitor, at 12.5 or 25 mg/kg once a day for 21 consecutive days and tumor volume was examined every 3 days. Intriguingly, only 4 of 7 PDX models responded to PF-2341066, including three NSCLC models (LU-2503, LU1901, and LU0858) and one liver cancer model (LI0612; Table 1). Accordingly, intratumoral c-Myc expression in responders was reduced under treatment, and downregulation was detectable as early as on day 3 (Fig. 6A and B; Supplementary Fig. S3A). In contrast, the c-Myc level remained constant in the nonresponders, although c-Met phosphorylation was similarly decreased (Fig. 6C; Supplementary Fig. S3B). These data suggested that MET amplification, although accepted as a predictive biomarker and currently used as criteria for patient enrollment clinically, is not able to ensure the responsiveness to c-Met–targeted therapy. Inspection of the c-Myc level enabled us to distinguish nonresponders from c-Met inhibitors at the earliest stage of the treatment.

To further mirror the clinical scenario, the responder LU2503 was continuously treated until loss of responsiveness. As often occurs clinically, this model developed resistance after consecutive treatment with PF-2341066 at 12.5 mg/kg once a day for about 2 months, and the treatment was terminated thereafter. It was observed that the reduced c-Myc level was restored again by the treatment (Fig. 6D). Consistently, in an LU1901-derived resistant model called LU1901-R1, combined treatment with PF-2341066 and the Hsp90 inhibitor 17-DMAG effectively suppressed tumor growth and decreased c-Myc expression. These data suggested that c-Myc expression is able to predict resistance occurrence and monitor the efficacy of alternative therapies (Supplementary Fig. S3C and S3D).

Collectively, such a PDX-based study that mirrors clinical management has proved that to inspect the c-Myc level will assist clinical decision-making in c-Met–targeted therapy in determining ultimate response, indicating the occurrence of acquired resistance, and predicting the effectiveness of alternative therapies.

Discussion

In the current study, we discovered that multiple kinases were simultaneously implicated in the acquisition of resistance to c-Met inhibition. Moreover, the kinases arising in our study were not overlapped with those reported by others, namely HER family and BRAF fusion (45, 46). As such, the identification of kinase signaling complements responsible for resistance acquisition in each cellular context seems difficult and nearly impossible. Alternatively, we found that c-Myc functions as a common downstream effector to sustain growth advantage in both c-Met–addicted cancers and acquired resistance. Congruent with this notion, targeting c-Met– and Hsp90-associated multiple kinases can modulate c-Myc expression and ultimately cell survival in c-Met–addicted and –resistant cells, respectively. Notably, depletion of c-Myc pharmacologically (siRNA) or genetically (shRNA) provided a powerful approach to overcome resistance to c-Met inhibition. Although inhibitors directly targeting c-Myc remain unavailable, our findings suggested that disrupting a "c-Myc–like" kinase-associated downstream effector, rather than identifying all possible compensatory kinases, may give rise to a successful proof of concept of circumventing acquired resistance in a broader range of kinase-addicted cancers.

Being a fragile point in both c-Met–addicted and acquired resistant cells enables c-Myc as a probe to indicate response and resistance for c-Met inhibitors. Indeed, our PDX-based co-clinical trial witnessed the clinical power of c-Myc for c-Met–targeted therapy. After enrollment of MET amplified "patients," the alteration of c-Myc level allowed us to stratify the nonresponder at the beginning of treatment (on the third day), thereby avoiding delay in starting alternative treatment. For the responders proceeding to the treatment, the serial detection of c-Myc predicted the emerging resistance before tumor relapse and further dictated response to alternative therapies. These suggested that c-Myc can serve as a functional response biomarker to guide the clinical trial of c-Met–targeted therapy (Fig. 6E).

The coming challenge of our findings lies in how to facilitate detecting c-Myc in clinic. While collection of patient biopsies serially seems unreasonable, it is of urgent need to develop

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Abbreviations: ES, esophageal cancer; GA, gastric cancer; LI, liver cancer.
Figure 6.
c-Myc determines clinical response and resistance for c-Met-targeted therapy. A–C, mice bearing patient-derived LU2503 (A), LU0858 (B), and ES0026 (C) xenografts were administered with vehicle control or PF-2341066 at 12.5 or 25 mg/kg once a day (QD) for 21 consecutive days. Tumor volume was measured every 3 days. Intratumoral c-Myc was examined on last dose using immunoblotting. D, mice bearing LU2503 xenografts were administered with vehicle control or PF-2341066 at 12.5 mg/kg once a day for about 2 months. Tumor volume was measured at indicated time points. Intratumoral c-Myc was examined at indicated time points using immunoblotting. E, a proposed scheme showing the application of c-Myc as an indicator to guide in clinical trials of c-Met inhibitors.
clinically applicable tools to image c-Myc in a noninvasive manner. Molecular imaging probes that directly binding to c-Myc is not available to date; however, a recent report has shown significant progress in this regard. It demonstrated that using 89Zr-transferrin PET scans to track TFRC expression, a downstream reporter of c-Myc expression, can quantitatively annotate c-Myc status, which makes the molecular imaging of c-Myc possible, although indirect, for the benefit of assessing response and resistance for c-Met–targeted therapy in patients (47).

Taken together, the recognition of a new paradigm as “kinase-downstream effector” can help us understand both kinase addiction and resistance acquisition. Interrupting these key effectors will give rise to powerful approaches to overcome acquired resistance. Moreover, integrating downstream effectors into clinic enables a more advanced and precise decision-making to benefit the patients by optimizing treatment design and increases the success of kinase inhibitors in clinical practice.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Shen, J. Ding, M. Geng
Development of methodology: A. Shen, X. Wang, J. Ding, M. Geng

References


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