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Met receptor sequence variants R970C and T992I lack transforming capacity

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Abstract

High-throughput sequencing promises to accelerate the discovery of sequence variants, but distinguishing oncogenic mutations from irrelevant "passenger" mutations remains a major challenge. Here we present an analysis of two sequence variants of the MET receptor (hepatocyte growth factor receptor) R970C and T992I (also designated R988C and T1010I). Previous reports indicated these sequence variants are transforming and contribute to oncogenesis. We screened patients with chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML), colorectal cancer, endometrial cancer, thyroid cancer, or melanoma as well as individuals without cancer and found these variants at low frequencies in most cohorts, including normal individuals. No evidence of increased phosphorylation or transformative capacity by either sequence variant was found. Since small-molecule inhibitors for MET are currently in development, it will be important to distinguish between oncogenic sequence variants and rare single-nucleotide polymorphisms to avoid the use of unnecessary and potentially toxic cancer therapy agents.

Keywords

cancer genetics; cancer genomics; targeted therapy

Introduction

Tyrosine kinases play a critical role in numerous cellular processes(1). Therapies targeted to dysreglated kinases have proven more successful than conventional approaches(2–5). One emerging kinase target is the hepatocyte growth factor receptor, MET, identified as part of

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the fusion oncogene, TPR-MET(6,7). Subsequently, point mutations in MET were identified in renal papillary carcinoma(8–10). More recently, MET variants, R970C and T992I (also designated R988C and T1010I), have been found in lung cancer cell lines as well as individuals with lung, thyroid, renal, breast cancer, CLL, and lymphoma(11–18). These variants have been characterized by Ba/F3 transformation and phospho-tyrosine immunoblots among other assay systems and were concluded to be transforming(13,14).

To better understand the cancer genetics of these MET variants we determined R970 and T992 genotypes for patients with CLL, AML, CMML, colorectal cancer, endometrial cancer, thyroid cancer, or melanoma as well as normal individuals. This process revealed low frequency MET^{R970C} and MET^{T992I} in most of the cohorts, including the normal individuals. Since these sequence variants were identified in a wide variety of different malignancies including individuals without clinically detectable cancer, we hypothesized that these were rare single nucleotide polymorphisms not relevant to oncogenesis. In support of this, we examined the human and murine versions of each of these sequence variants in Ba/F3 transformation assays as well as for tyrosine phosphorylation status and found no evidence of a transforming phenotype.

Material and Methods

Plasmid Construction

Human MET was purchased from Origene (Rockville, MD). Murine Met and TPR-MET were generously provided by Dr. George F. Vande Woude (Van Andel Research Institute, Grand Rapids, MI). All three genes were cloned into MSCV-IRES-GFP (MIG) using the Gateway Cloning system (Invitrogen Corporation, Carlsbad, CA). All point mutations were created using the Quikchange kit (Agilent Technologies, Cedar Creek, TX) according to the manufacturer's instructions.

Cell culture

Ba/F3 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Invitrogen) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), L-glutamine (Invitrogen), penicillin/streptomycin (Invitrogen), fungizone (Invitrogen), and WEHI-conditioned media. Ba/F3 cells were infected with retrovirus expressing human or murine MET WT, R970C (R968C in murine version), T992I (T990I in murine version), or TPR-MET. Stable cell lines were sorted for GFP expression on a FACSAria flow cytometer (BD Biosciences, San Jose, CA). Cells were counted daily using Guava ViaCount reagent and Guava Personal Cell Analysis flow cytometer (Guava Technologies, Hayward, CA).

WEHI-independence Assays

Parental Ba/F3 cells or those expressing human or murine MET WT, R970C (R968C in murine version), T992I (T990I in murine version), or TPR-MET were washed three times and resuspended in RPMI media with 10% FBS, penicillin/streptomycin, and fungizone and counted daily.

Immunoblotting

Human MET WT, R970C, T992I, or TPR-MET were transfected into 293 T17 cells using Fugene6 transfection reagent (Roche, Indianapolis, IN). After 48 hours, 293 T17 cells were lysed in 1 × lysis buffer (Cell Signaling, Danvers, MA), and diluted in sample buffer (75 mM Tris pH 6.8, 3% SDS, 15% glycerol, 8% β-mercaptoethanol, 0.1 % bromophenol blue). Whole cell lysates were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) and subjected to immunoblot analysis with antibodies

specific for phospho-tyrosine (4G10, Millipore), total or phospho-MET (Cell Signaling), or β-actin (Millipore).

Genotypic Analysis

All patient material was de-identified and obtained with informed consent with approval from the institutional review board of Oregon Health & Science University. *MET* juxtamembrane and activation loop domains were sequenced in CLL, AML, CMML, and normal individuals as described(19). For colorectal, endometrial, thyroid, and melanoma patient samples, mass spectrometric genotyping of MET R970 and T992 was performed with the Sequenom MassARRAY system and the OncoCarta assay panel (Sequenom, San Deigo, CA) as described(20).

Statistical Analysis

The incidence of MET^{R970C} and MET^{T992I} in each cohort was estimated by the proportion of individuals with mutations (i.e., the number of individuals with mutations divided by the total number of individuals in a cohort) and is expressed as a percentage. The 95% confidence intervals were calculated by using the exact binomial distribution. For Ba/F3 transformation experiments and densitometry of immunoblots, a Student's t-test was performed for each MET variant compared with MET^{WT} and samples with p value less than 0.05 were deemed significant.

Results and Discussion

To determine the frequency of the sequence variants MET^{R970C} and MET^{T992I} in patients with a variety of malignancies, we screened DNA from 96 CLL, 191 AML, 32 CMML, 109 colorectal cancer, 73 endometrial cancer, 168 thyroid cancer, and 115 melanoma patients as well as 96 normal individuals. Screening of these cohorts revealed that all groups contained low frequency MET^{R970C} or MET^{T992I} sequence variants, and most cohorts contained both variants. Matched buccal cell samples were available for 3 cases exhibiting each variant (T992I – 2 melanoma and 1 endometrial cancer; R970C – 2 thyroid and 1 endometrial cancer) and all 6 cases showed presence of the variant allele in the germline material. Interestingly, we also identified one normal individual with MET^{R970C} and one normal individual with MET^{T992I} (Table 1). These alleles have not been detected previously in normal samples. Thus, our findings as well as those by others suggest these sequence variants occur at similar frequencies among many different types of malignancy as well as in individuals without cancer (Table 1)(11–18,21). In addition, 95% confidence intervals of the incidence of each sequence variant are overlapping for each diagnosis compared with all other diagnoses and compared with the individuals without cancer (Table 1). This indicates a lack of evidence for statistically significant differences in the incidence of either sequence variant in individuals with or without cancer. Since we (and others) have also found that these variants are generally germline, we functionally characterized these variants for transforming capacity.

We created Ba/F3 cells stably expressing human or murine MET^{WT}, human MET^{R970C}, human MET^{T992I}, murine Met^{R968C}, murine Met^{T990I}, or TPR-MET and withdrew IL-3 from culture media. Ba/F3 cells that have been transformed by an oncogene such as TPR-MET are capable of proliferating without IL-3 as shown in Figure 1A; however, we did not observe transformation by any of the full-length MET transgenes, including the sequence variants (Figure 1A). Importantly, Ba/F3 cells are not a universally effective system for evaluation of tyrosine kinase oncogenecity, depending on the particular kinase and the physical location of the mutation in the protein. However, previous reports of the MET^{R970C} and MET^{T992I} variants suggested that these variants were capable of transforming Ba/F3

cells, hence this system was employed to directly test this prior claim(14). In addition to the Ba/F3 system, we next assessed the phosphorylation status of wild-type MET compared with each variant—a technique that was also previously employed to demonstrate increased phosphorylation of these sequence variants(14). We expressed human MET^{WT}, MET^{R970C}, MET^{T992I}, or TPR-MET in 293 T17 cells and immunoblotted with antibodies specific for global phospho-tyrosine (4G10), phospho-MET, total MET, or β-actin. While cells expressing TPR-MET exhibited markedly increased levels of global phosphotyrosine as well as phospho-MET, we did not observe any differences in phosphorylation levels with the MET R970C or T992I variants, again arguing against a direct transforming or dysregulated role for MET^{R970C} or MET^{T992I} (Figure 1B, C).

Several forms of MET have been implicated in oncogenesis including fusion with TPR and point mutation at residues D1228, Y1230, and M1250(6,7,9,10). The capacity for MET^{R970C} and MET^{T992I} to contribute to oncogenesis has been a topic of debate. First identified by Schmidt et al.(15), MET^{T992I} was thought to represent a rare polymorphism due to lack of disease segregation and failure to induce focus formation or phosphorylation in NIH3T3 cells. Lee et al. also observed no functional consequence of these variants using similar assays but did observe slightly faster tumor growth in nude mice(12). Consequences of these variants were identified in assays for cytoskeletal function(13,14,21). Of note, neither variant was previously observed in samples from healthy individuals(13,14). We find no difference in transformative capacity or phosphorylation status of either variant compared with wild-type MET and suggest these alleles are not transforming. This conclusion is also based on the presence of these variants in a wide variety of malignancies as well as individuals without cancer. We cannot rule out the possibility that these alleles may play a role in disease pathogenesis in some other manner such as promoting changes in migration or differentiation. In this way, these alleles may predispose an individual towards cancer when combined with an oncogene that drives cellular proliferation. However, we find no evidence that these alleles directly promote transformation. Given the recent emergence of MET inhibitors as clinical cancer therapeutics(22), it will be important to carefully evaluate these alleles for their role in oncogenesis.

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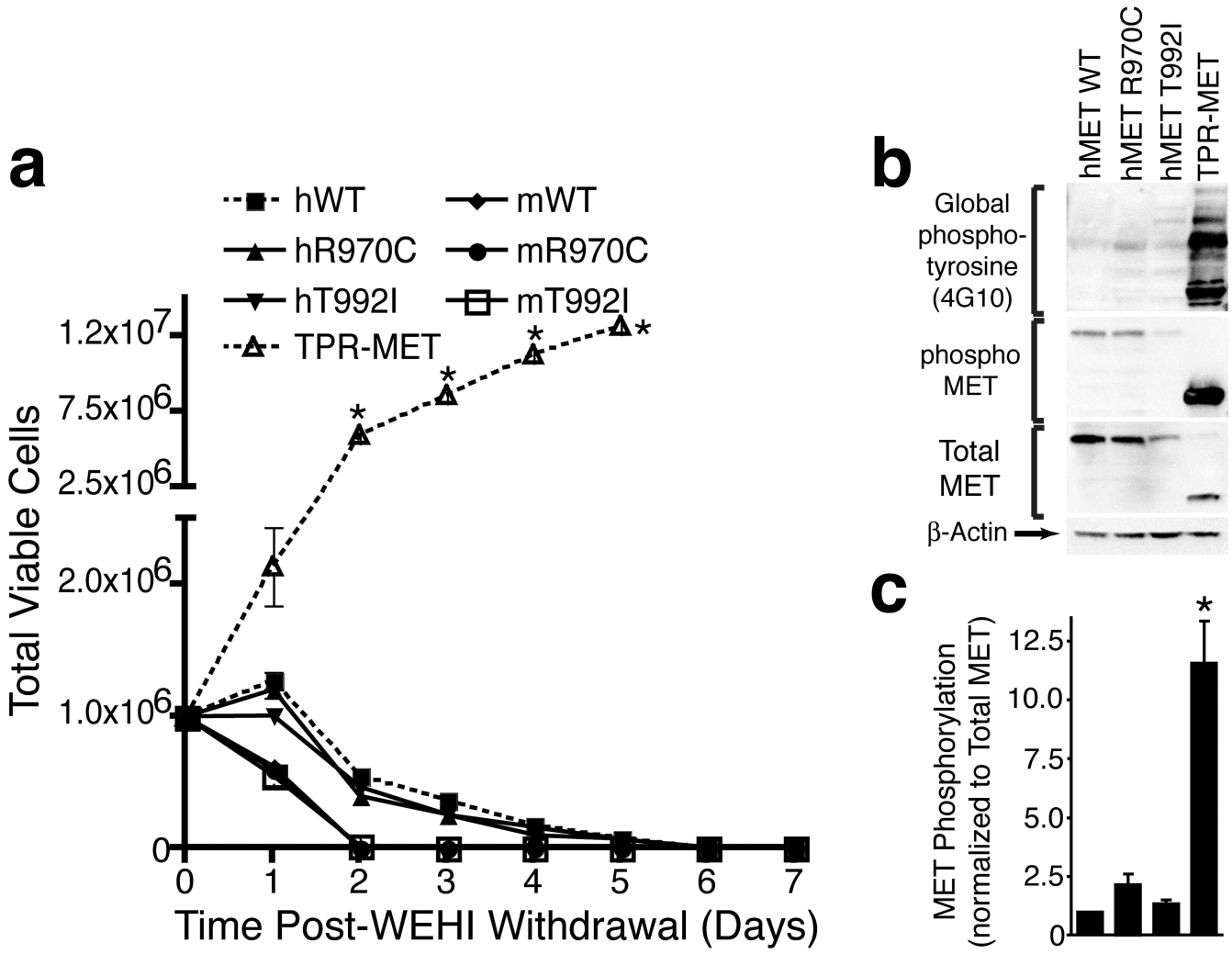

Figure 1. Characterization of MET Sequence Variants in Ba/F3 Cells

(A) Ba/F3 cells overexpressing human or murine MET^{WT}, human MET^{R970C}, human MET^{T992I}, murine Met^{R968C}, murine Met^{T990I}, or TPR-MET were plated in media without IL-3. Cells were counted daily for one week. Values represent mean ± s.e.m. (n=3). * indicates $p < 0.002$ with t-test in comparison to human MET^{WT}.

(B) Whole cell lysates from 293 T17 cells overexpressing human MET^{WT}, MET^{R970C}, MET^{T992I}, or TPR-MET were subjected to immunoblot analysis with antibodies specific for phospho-tyrosine (4G10), phoshpo-MET, total MET, or β-actin.

(C) Densitometric analysis of immunoblots shown in panel (B). Phospho-MET is normalized to total MET and values are expressed as fold of WT. Values represent mean ± s.e.m. (n=3). * indicates $p < 0.05$ with t-test in comparison to human MET^{WT}.

Cohort (n)	Incidence of R970C	95% Exact CI	Reference
Normal (96)	1 (1%)	0–6%	Reported Here
CLL (96)	0	0–4%	Reported Here
AML (191)	0	0–2%	Reported Here
CMML (32)	2 (6%)	0–21%	Reported Here
Colorectal Cancer (109)	1 (1%)	0–5%	Reported Here
Endometrial Cancer (73)	1 (1%)	0–7%	Reported Here
Thyroid Cancer (168)	2 (1%)	0–4%	Reported Here
Melanoma (115)	1 (1%)	0–5%	Reported Here
Total (880)	**8 (0.9%)**	**0–2%**	
Papillary renal carcinoma (129)	0	0–3%	Schmidt et al. 1999
Other solid tumors (201)	0	0–2%	Schmidt et al. 1999
Breast Cancer (30)	0	0–12%	Lee et al. 2000
CLL (95)	0	0–4%	Brown et al. 2008
CLL (8)	1 (13%)	0–53%	Tjin et al. 2006
Follicular Lymphoma (15)	1 (7%)	0–32%	Tjin et al. 2006
Burkitt's Lymphoma (12)	1 (8%)	0–38%	Tjin et al. 2006
Diffuse Large B-cell Lymphoma (39)	1 (3%)	0–13%	Tjin et al. 2006
Small Cell Lung Cancer (32)	0	0–11%	Ma et al. 2003
Non-small cell lung carcinoma (20)	0	0–17%	Teng et al. 2006
Lung carcinoma (361)	0	0–1%	Teng et al. 2006
Fillicular Carcinoma (21)	0	0–16%	Wasinius et al. 2005
Papillary Carcinoma (53)	0	0–7%	Wasinius et al. 2005
Medullary Carcinoma (13)	0	0–25%	Wasinius et al. 2005
Mesothelioma (43)	0	0–8%	Jagadeeswaran et al. 2006
Gastric Carcinoma (85)	0	0–4%	Lee et al. 2000
Total (1157)	**4 (0.35%)**	**0–1%**	
Cohort (n)	**Incidence of T992I**	**95% Exact CI**	**Reference**
Normal (96)	1 (1%)	0–6%	Reported Here
CLL (96)	2 (2%)	0–7%	Reported Here
AML (191)	2 (1%)	0–4%	Reported Here
CMML (32)	0	0–11%	Reported Here
Colorectal Cancer (109)	0	0–3%	Reported Here
Endometrial Cancer (73)	2 (3%)	0–10%	Reported Here
Thyroid Cancer (168)	0	0–2%	Reported Here
Melanoma (115)	2 (2%)	0–6%	Reported Here
Total (880)	**9 (1.0%)**	**0–2%**	
Papillary renal carcinoma (129)	1 (1%)	0–4%	Schmidt et al. 1999

Cohort (n)	Incidence of R970C	95% Exact CI	Reference
Other solid tumors (201)	0	0–2%	Schmidt et al. 1999
Breast Cancer (30)	1 (3%)	0–17%	Lee et al. 2000
CLL (95)	1 (1%)	0–6%	Brown et al. 2008
Small Cell Lung Cancer (32)	1 (3%)	0–16%	Ma et al. 2003
Non-small cell lung carcinoma (20)	1 (5%)	0–25%	Teng et al. 2006
Lung carcinoma (361)	5 (1%)	0–3%	Teng et al. 2006
Follicular Carcinoma (21)	2 (10%)	1–30%	Wasinius et al. 2005
Papillary Carcinoma (53)	3 (6%)	1–16%	Wasinius et al. 2005
Medullary Carcinoma (13)	1 (8%)	0–36%	Wasinius et al. 2005
Mesothelioma (43)	0	0–8%	Jagadeeswaran et al. 2006
Gastric Carcinoma (85)	0	0–5%	Lee et al. 2000
Total (1083)	**16 (1.5%)**	**0–2%**	