Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele

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Tyrosine kinase-activating mutations in Met have been observed in hereditary papillary renal carcinomas as well as in other cancers. These mutations have been examined in several *in vitro* systems, where they cause constitutive Met activation, focus formation, and cell motility, and are tumorigenic in xenografts. To study the influence of these mutations on tumorigenesis *in vivo*, we generated mice with targeted mutations in the murine *met* locus. The following five mouse lines with mutant Met were created: WT, D1226N, Y1228C, M1248T, and M1248T/L1193V. We observed that mice harboring D1226N, Y1228C, and M1248T/L1193V mutations developed a high frequency of sarcomas and some lymphomas, whereas the M1248T mice developed carcinomas and lymphomas. Of considerable interest, we observed trisomy of chromosome 6 and duplication of the mutant *met* allele in a majority of the tumors, similar to what has been reported in patients with hereditary renal papillary carcinomas. These results demonstrate that activating Met mutations and *met* amplification play key roles in promoting tumorigenesis *in vivo*. Moreover, our findings show that different mutations in the Met kinase domain can influence the types of cancers that develop.

Met is the tyrosine kinase receptor for hepatocyte growth factor/scatter factor (HGF/SF). Met is expressed predominantly in epithelial cells, whereas HGF/SF is expressed in a paracrine manner by mesenchymal cells (1, 2). Signaling through the Met receptor is crucial for embryonic development as well as for mediating cell growth, motility, invasion, and angiogenesis (3–6). Met and HGF/SF are well known oncogenes, and inappropriate Met and/or HGF/SF expression is associated with multiple human solid tumors and poor clinical prognosis (7) (www.vai.org/vari/metandcancer). Several distinct germ-line and somatic activating mutations in Met have been observed in hereditary papillary renal carcinoma (HPRC) (8, 9) as well as in other carcinomas (10–12). HPRC accounts for ≈10% of renal cancers and is characterized by multifocal, bilateral papillary tumors (13). Although Met is a dominant oncogene, patients with germ-line missense mutations in the kinase domain have incomplete penetrance and develop HPRC late in life (8). The long tumor latency may be due to the requirement of duplication of the mutant allele, which is frequently observed in patients with HPRC (14, 15). These events suggest that Met mutations require at least a “second hit” for tumors to progress.

Although the oncogenic potential of these Met mutations has been demonstrated through *in vitro* studies (16, 17), their causal role for *in vivo* tumorigenesis in mouse models has not been demonstrated. To directly address the physiological role of activating Met mutations, we generated mice with targeted mutations in the murine *met* locus, representative of both somatic and germ-line activating mutations present in HPRC. We found that the different activating Met mutations predispose mice to diverse tumor types, including sarcomas, lymphomas, and carcinomas, some of which are

metastatic. Moreover, similar to HPRC, we also discovered trisomy of chromosome 6 and nonrandom duplication of the mutant *met* allele in the majority of tumors. Our results lead us to conclude that *met* amplification and genetic instability are necessary for tumor progression within the presence of activating Met mutations.

Materials and Methods

Generation of *met* Mutant Mice. A cDNA clone containing the coding region from the *Sph*I site in exons 14 (3,051 bp) through 20 was inserted into the pSXL DUE vector containing a poly(A) sequence. The bacterial *neomycin* (*neo*) gene was inserted 3' of the poly(A) tail, and the herpes simplex virus *thymidine kinase* (*tk*) gene was ligated downstream of the 3' recombination region to allow for counterselection against random integration of the construct. Mutations were introduced into individual constructs by PCR [M1248T (ATG → ACG), Y1228C (TAC → TGC), D1226N (GAC → AAC), and M1248T/L1193V (ATG → ACG/CTT → GTT)] and verified by sequencing. The *Spe*I linearized construct was electroporated into embryonic stem cells and injected into blastocysts by using methods described in refs. 18 and 19. Germ-line transmission was confirmed by Southern hybridization on tail DNA and PCR genotyping (for details, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). Experiments using mice were approved by the Van Andel Research Institute Institutional Animal Care and Use Committee.

Immunoprecipitation and Western Blot Analyses. Approximately 100 mg of liver tissue from adult male mice was homogenized in RIPA buffer [20 mM Tris (pH 7.5)/150 mM NaCl/0.1% SDS/1% Nonidet P-40/50 mM sodium fluoride/1 mM sodium orthovanadate/1 mM EDTA/0.5% deoxycholate/Complete Proteinase Inhibitor Mixture Tablets (Roche Diagnostics)]. Protein concentrations were quantified by using the DC Protein Assay (Bio-Rad). For immunoprecipitation, 1 mg of protein was incubated overnight with anti-Met antibody (B-2, Santa Cruz Biotechnology) in 1 ml of RIPA buffer at 4°C. Immune complexes were collected with protein G-Sepharose beads (Amersham Pharmacia) and washed three times in RIPA buffer. Immune complexes were eluted in 30 μl of Laemmli sample buffer (pH 6.8) (Sigma) and boiled for 10 min before being loaded on to a gel. Immunoprecipitated lysates were separated on a 0.8% Tris-glycine gel (Invitrogen), transferred onto a poly(vinylidene difluoride) membrane (Invitrogen), and examined by Western analysis using an anti-Met antibody (B-2, Santa Cruz Biotechnology). Western blot analyses were performed as described in ref. 20.

Abbreviations: HGF/SF, hepatocyte growth factor/scatter factor; HPRC, hereditary papillary renal carcinoma; mu, murine *met* construct.

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Table 1. Histopathological analysis of *met* mutant mice

Tumor/lesion	M1248T ^{mu/+}	Y1228C ^{mu/+}	M1248T/L1193V ^{mu/+}	D1226N ^{mu/+}	WT ^{mu/+}
Sarcomas					
Hemangiosarcoma	—	10	4*	13	—
Fibrosarcoma	—	1	1*	2*	—
Myxoma	—	1 [†]	—	—	—
Leiomyosarcoma	—	—	1	—	—
Spindle-like	—	—	1	—	—
Lymphomas					
Histiocytic sarcoma	5	5	1	—	1
Lymphoblastic	—	—	1	—	2
Lymphocytic	1	1	—	—	—
Follicular center	1	—	—	—	—
Carcinomas					
Transitional cell	1	—	—	—	—
Squamous cell	1	—	—	—	—
Lung adenocarcinoma	1	—	—	—	1
Lung adenoma	1	—	—	—	1
Basosquamous adenocarcinoma	1	—	—	—	—
Hepatoma	1	—	1	—	—
Nonremarkable	1	0	3	3	4

Some mice contained more than one tumor. Some mice also had pathological phenotypes that were not reported in this study; therefore, these mice were not included in the “nonremarkable pathology” group. Lung adenomas and hepatomas may have been observed due to the old age of the mice.

*Tumors in which metastatic lesions were observed.

[†]The myxoma was contiguous with a hemangiosarcoma (shown in Fig. 3D).

phorylated Met was observed at equal, but extremely low, levels in the liver extracts of each of the lines (data not shown).

Male and female heterozygous mice were intercrossed to determine the viability of homozygous mutant animals. Both homozygous WT^{mu/mu} and D1226N^{mu/mu} mice were obtained, but only heterozygous offspring were produced from the other *met* mutant lines. This result was not unexpected given that the D1226N mutation had weak activity in *in vitro* studies (16, 17). Approximately 130 embryos at various developmental stages were examined from each line. For reasons not yet apparent, viable homozygous embryos were not observed in the Y1228C and M1248T lines or the M1248T/L1193V line after embryonic day 6.5 or 13.5, respectively.

Survival Analysis of *met* Mutant Mice. To determine the effect of activating Met mutations on the lifespan of the *met* mutant mice, pairwise Kaplan–Meier analysis was performed on each line (Fig. 2). Median survival times (months) for each line were as follows: WT^{+/+}, 22.1 ± 4.3; WT^{mu/+}, 20.5 ± 4.7; M1248T/L1193V^{mu/+}, 19.0 ± 5.3; Y1228C^{mu/+}, 15.9 ± 6.4; D1226N-1^{mu/mu}, 12.5 ± 6.0; D1226N-2^{mu/mu}, 10.6 ± 6.7; and M1248T^{mu/+}, 18.3 ± 5.8. There was no significant difference in survival between WT^{+/+} and WT^{mu/+} mice ($P = 0.3$). The M1248T^{mu/+} and M1248T/L1193V^{mu/+} mice also had no significant difference in survival compared with WT^{mu/+} mice ($P = 0.9$ and $P = 0.4$, respectively). This result was unexpected, given that M1248T and M1248T/L1193V are the most active mutations in focus formation and tumorigenic assays in nude

mice (16, 17). Although the M1248T^{mu/+} and M1248T/L1193V^{mu/+} mice live a normal lifespan, neoplasias generally were discovered in the older mice upon necropsy. By contrast, the Y1228C^{mu/+} mice had a significantly shorter lifespan than WT^{mu/+} mice ($P = 0.03$). The D1226N^{mu/mu} mice had the shortest lifespan of all of the mutated lines. Only eight D1226N^{mu/+} mice were aged and had a lifespan of 20 ± 6.6 months. Because of the long lifespan of these mice, D1226N^{mu/mu} mice were used in the following studies. The *met* mutant lines are not congenic, and, therefore, the influence of genetic background on the tumor profiles cannot be excluded. However, it is important to note that two D1226N^{mu/mu} lines originating from separate founders had no statistical difference in their lifespan ($P = 0.07$) or tumor profile, suggesting that our results are not due to background effects.

***met* Mutant Mice Develop Unique Tumor Phenotypes.** We performed histological analysis on ≈18 mice from each line (Tables 1 and 2).

Fig. 3. *met* mutant mice develop unique tumor profiles. (A) Hemangiosarcoma observed s.c. in a D1226N^{mu/mu} mouse. (B) Transitional cell carcinoma of the ureter observed in a M1248T^{mu/+} mouse. (C) Squamous cell carcinoma of the anus observed in a M1248T^{mu/+} mouse. (D) Adjacent myxoma (M) and hemangiosarcoma (H) observed in a Y1228C^{mu/+} mouse. (E and F) Primary fibrosarcoma from a M1248T/L1193V^{mu/+} mouse (E) and the metastatic lesion (M) in the lung (L) (F). (Hematoxylin/eosin images, ×200 magnification.)

Table 2. Tumor incidence in *met* mutant mice

Mutant	Total mice	Survival range, months	Tumor incidence, %
M1248T ^{mu/+}	17	4–29	58.8
Y1228C ^{mu/+}	19	1–23	89.5
M1248T/L1193V ^{mu/+}	18	3–27	50.0
D1226N ^{mu/mu}	18	2–19	83.8
WT ^{mu/+}	9	10–28	44.4

Some mice contained more than one tumor. Only malignant tumors were included in the tumor incidence calculation (i.e., lung adenomas were not included).

Fig. 4. FISH of *met* mutant tumors. (A) Relative copy number of *met* was measured by using dual-color interphase FISH on tumor touch preps. Photomicrographs with RP23–416H6 *met* probe (green), RP24–462C10 probe (red), and DAPI counterstaining are shown as follows: diploid nuclei containing two RP23–416H6 and two RP24–462C10 signals (Top Left); trisomy of chromosome (Chr.) 6 (Top Right); duplication of only the *met* locus (Middle Left); loss of the *met* locus (Middle Right); and loss of one copy of Chr. 6 (Bottom Left). (Bottom Right) A photomicrograph of interphase FISH with RP23–416H6 probe (Chr. 6, green), RP23–151I21 probe (Chr. 8, red), and DAPI counterstaining; trisomy of Chr. 6 and disomy of Chr. 8 are present. (B) The number of mutant *met* alleles was determined by using quantitative PCR. M1248T tumors 6 and 7, M1248T/L1193V tumor 2, and Y1228C tumors 1 and 2 all had significant levels of Chr. 6 trisomy based on FISH analysis (Table 3). The mutant *met* allele was significantly increased in comparison with the mu/+ control. Based on FISH, D1226N tumor 5 had no Chr. 6 trisomy, and no increase in mutant *met* was observed by quantitative PCR when compared with the mu/mu control. *P* values for each comparison are as follows: M1248T tumor 6, $P = 2.4 \times 10^{-5}$; M1248T tumor 7, $P = 0.001$; M1248T/L1193V tumor 2, $P = 0.002$; Y1228C tumor 1, $P = 0.0001$; Y1228C tumor 2, $P = 4.0 \times 10^{-6}$; and D1226N tumor 5, $P = 0.7$.

to be a factor for tumor development (14, 15). To determine whether the *met* locus was duplicated in tumor samples from *met* mutant mice, we performed dual-color interphase FISH on touch preparations of tumors and normal tissues from each of the *met* lines by using a bacterial artificial chromosome clone specific for the *met* locus in band A2 of chromosome 6 (RP23–416H6) as well as a control probe for band D3 in chromosome 6 (RP24–462C10). The control probe allowed us to distinguish between duplication of chromosome 6 and localized amplification of the *met* locus. Strikingly, cells with trisomy and tetrasomy of chromosome 6 were observed in 97% and 30% of the *met* mutant tumors examined ($n = 30$), respectively (Fig. 4A and Table 3). By contrast, normal tissues examined from each *met* mutant line had polyploidy in <2.1% of their nuclei (Table 4). Moreover, when the same tumors were examined with markers for chromosomes 8 and 16, no changes in the copy number of chromosome 16 were observed, and only two

Fig. 5. Model of activating mutations within the Met kinase domain. The M1248T mutation is located within the P+1 loop (teal), whereas the D1226N and Y1228C mutations lie within the activation loop (purple). The weaker L1193V mutation (light blue) lies near catalytic residues (green). The model is based on the crystal structure of the insulin receptor (29) as derived from Birchmeier *et al.* (7). [Reproduced with permission from Nature Reviews (www.nature.com/reviews) Molecular Cell Biology (7) (Copyright 2004, Macmillan Magazines Ltd.).]

tumors exhibited more than two copies of chromosome 8 (Fig. 4A). Cells with amplification of only the *met* locus, but not the chromosome 6D3 region, were noted in half of the tumors examined, albeit at a lower frequency than the trisomic and tetrasomic cells, and were most common in D1226N^{mu/mu} tumors. Interestingly, the loss of one copy of chromosome 6 and/or loss of only the *met* locus also was observed in several tumors that also contained chromosome 6 polyploidy. We suspect that the WT *met* allele is lost in the nuclei where one *met* locus is observed. Collectively, these results demonstrate that tumor formation in *met* mutant mice is associated with nonrandom polyploidization of chromosome 6 and the *met* locus.

The Mutant *met* Allele Is Duplicated in Mutant *met* Tumors. To determine whether the mutant *met* allele was being amplified in the murine tumors, we performed quantitative PCR with a probe specific to the mutant *met* allele. Five tumors with significant chromosome 6 trisomy and one tumor without trisomy were examined (Fig. 4B). Each of the tumors with trisomy had significant increases in the copy number of the mutant *met* allele when compared with the nontumor heterozygous control. Because the tumor extracts examined contained a heterogeneous mixture of normal and tumor cells, the copy numbers for the mutant allele did not equal absolute values. Note that a control tumor lacking trisomy 6 had no increase in the mutant allele when compared to the nontumor mu/mu control. These results demonstrate that duplication of the mutant *met* allele is required for tumorigenesis.

Discussion

Activating mutations in the tyrosine kinase domain of Met are associated with HPRC. An essential role for these mutations in the etiology of HPRC is supported by the observation that ectopic expression of the same Met mutations in cultured cell lines causes constitutive Met activation, focus formation, cell motility, and tumorigenesis (16, 17). Here, we show that these mutations, under the control of the endogenous *met* promoter, are responsible for spontaneous tumorigenesis *in vivo*. These results provide convincing evidence that activating Met mutations play a key role in promoting *in vivo* tumorigenesis.

Interestingly, the *met* mutant mice we generated in this study showed remarkable differences in tumor profiles. Why the