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MET mutations in cancers of unknown primary origin (CUPs)†.

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Abstract

Cancer of unknown primary origin (CUP) defines metastatic disease of unknown origin, accounting for 3–5% of all cancers. Growing evidence demonstrates that inappropriate execution of a genetic program named “invasive growth,” driven by the MET oncogene, is implicated in the metastatic process. MET activation in cancers is mainly consequent to overexpression, whereas mutations are rarely found. We reasoned that the occurrence of MET somatic mutations might

sustain premature occult dissemination of cancer cells, such as that observed in CUPs. We sequenced MET in genomic DNA obtained from 47 early metastatic cancers. By extensive immunohistochemical analysis a primary site was afterward postulated in 24 patients, whereas 23 cases remained of unknown primary (CUPs). MET somatic mutations were found in seven cases, all belonging to the CUP cohort. Mutational incidence (30%) was thus significantly higher than the expected one (4%), in the absence of high mutational background. Several nucleotide changes were novel and clustered either in the kinase domain or in the extracellular semaphorin domain. Mutated receptors were functional and sustained the transformed phenotype, suggesting that MET activating mutations are genetic markers associated with the CUP syndrome. *Hum Mutat* 31:1–7, 2010. © 2010 Wiley-Liss, Inc..

Keywords

MET;tyrosine kinase;somatic mutation;invasive growth;metastases

1. Introduction

It is widely accepted that systemic neoplastic spread is a late event, resulting from accumulation of genetic alterations occurred in local progression [Fearon and Vogelstein, 1990]. However, in some instances, distant dissemination arises at a very early stage, so that metastases reach clinical relevance before primary lesion [Hüsemann et al., 2008; Weinberg, 2008]. In a number of cases, integration of immunohistochemistry [Park et al., 2007] and sophisticated imaging and radiometabolic techniques [Regelink et al., 2002; Sève et al., 2007] helps in identifying the primary tumor site [Pentheroudakis et al., 2007]; nevertheless, sometimes metastases remain really “orphan.” The latter defines a highly malignant syndrome known as cancer of unknown primary origin (CUP), which may display undifferentiated phenotype. These tragic cases may be described as not only of unknown origin, with respect to organ site, but also of unknown pathogenesis. Representing a still unsolved clinical problem lacking effective therapeutic regimens, CUPs describe a specific entity that occurs with an incidence that cannot be ignored: 3–5% of all human cancers [Briasoulis et al., 2009].

On the other hand, an increasing body of evidence suggests that the invasive malignant phenotype requires the aberrant execution—in time and space—of a physiological genetic program named “invasive growth,” resulting from the integration of different biological activities, including cell–cell dissociation (“scatter”), migration, invasion, and cellular proliferation. The program is driven by specific factors, among which is Scatter Factor, also known as Hepatocyte Growth Factor (HGF), and semaphorins [Trusolino and Comoglio, 2002]. The receptor for Scatter Factor/HGF is encoded by the MET oncogene (NM_000245.2) [Boccaccio and Comoglio, 2006], located on chromosome 7q31, made of 21 exons codifying for a trans-membrane tyrosine kinase protein. The extracellular region contains a 500 amino acid SEMA domain, distinctive of the semaphorin superfamily [Comoglio and Boccaccio, 2001; Tamagnone et al., 1999], involved in receptor dimerization and activation [Gherardi et al., 2003; Kong-Beltran et al., 2004]; a cysteine-rich domain (known as MET-Related Sequence [MRS]); and a protein–protein interaction site made of four immunoglobulin-like structures (IPT domain). The intracellular portion of the receptor is made of a juxtamembrane region; a catalytic site containing two tyrosines (Y1234 and Y1235) that controls the enzymatic activity, and a C-terminal regulatory tail with two tyrosines (Y1349 and Y1356) that, upon phosphorylation, create a docking site for intracellular signal transducers. The latter is responsible for the concomitant activation of multiple intracellular signaling pathways, triggering a cascade of biological responses, ultimately leading to the invasive phenotype [Ponzetto et al., 1994].

In human cancers MET activation confers a selective advantage for tumor progression [Engelman et al., 2007]. It generally occurs as a late event, mainly consequent to receptor overexpression driven by unfavorable microenvironmental conditions, for example, hypoxia [Pennacchietti et al., 2003]; in some instances overexpression is due to gene amplification [Comoglio et al., 2008]. Somatic point mutations are rarely found, accounting for no more than 3–4% of unselected primary cancers (COSMIC database: <http://www.sanger.ac.uk>). We hypothesized that MET

activation by somatic mutations at early stages of cancer onset might sustain aberrant execution of the invasive growth program and result in precocious metastatic phenotype, such as that identifying CUPs.

2. Material and methods

CUP Patients Selection

We analyzed 47 specimens from a cohort of cancer patients in which the first manifestation detected were two or more metastases. Formalin-fixed paraffin-embedded (FFPE) samples derived from the two different institutions: Department of Pathology at University of Verona and Department of Pathology at University of Torino. The majority of collected samples were obtained from biopsies; brain metastases derived from surgical specimens. Clinical data are listed in Supp. Table S1. All patients received a thorough sequential multistep diagnostic workup following the European Society of Medical Oncology (ESMO) guidelines [Briasoulis et al., 2009]. Diagnosis of CUPs was reached in those patients with no detectable site of primary origin, after exhaustive anamnesis and diagnostic evaluation including complete physical examination, complete laboratory tests, whole-body CT scan, and FDG-positron emission tomography (PET). The immunohistochemical profile was defined by a panel of at least 10 markers, according to accepted procedures outlined by Horlings et al. [2008] and further refined by Rossi et al. [2009].

Immunohistochemistry (IHC)

As mentioned above, given that the accuracy of IHC is a crucial step in CUP diagnosis, immunophenotype of each case was carefully validated according to accepted standards as reported by Horlings et al. [2008] and by Rossi et al. [2009]. Panels of up to 10 antibodies were used on FFPE tumor sections, according to the manufacturer's recommendations. The gastric origin of the metastatic lesions was recognized by the coexpression of various immunohistochemical markers, such as cytokeratins 7 and 20 and CDX2. Metastatic lesions from the biliary-pancreatic region proved to be positive for cytokeratin 19. Immunohistochemical reactions were evaluated independently by two pathologists; controversial cases were reevaluated jointly until a consensus was reached.

Genetic Analysis

PCR primers designed to amplify the entire coding sequence of MET (NM_000245.2) and RON (NM_002447.2), KRAS (NM_004985.3) exon 2, EGFR (NM_005228.3) exons 18–19–20–21 and PIK3CA (NM_006218.2) exons 9–20 are listed in Supp. Table S2. A total of 1,216 PCR products were generated from independent aliquots of DNA and subjected to direct sequencing. The somatic origin of each mutation was confirmed by sequencing, together with neoplastic DNA, normal DNA obtained by laser capture microdissection of nontransformed areas adjacent to the neoplastic lesions, whereas, in the case of small samples, normal DNA was extracted from different histological specimens of the same patient (archived at Pathological Banks), as previously described [Moroni et al., 2005]. Mutations were detected only in transformed tissues. Microsatellite instability (MSI) was assessed by using the panel of repeats as previously described [Suraweera et al., 2002]. These five mononucleotide markers (BAT25, BAT26, NR21, NR24, and NR27) were amplified according to the modifications proposed by Buhard et al. [2006], and then analyzed on ALFexpress II Sequencer (GE Healthcare Europe). FISH analysis was carried out on 4- μ m FFPE, as already described [Casorzo et al., 2005], using the following commercially available DNA probes: CEP 7 locus D7Z1-SpectrumGreen™ and locus-specific LSID7S522-SpectrumOrange™ probe for band 7q31.1 and LSI-EGFR-SpectrumOrange™ probe for band 7p12 (Vysis/Abbott Molecular, IL).

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

MET Mutant Expressing Constructs

MET mutations p.Cys385Tyr (c.1154G>A) and p.Val1312Ile (c.3932G>A) were generated using QuickChange Site-Directed Mutagenesis XL kit (Stratagene, LaJolla, CA) according to the manufacturer's instructions and confirmed by direct sequencing. The entire sequence of all engineered cDNAs was checked and no other unexpected mutations were found. cDNAs, either wild-type or mutated, were then cloned into P156RRLsinPPT_hCMVMCSpre lentiviral vector.

Lentiviral Cell Infection and Western Blotting

COS-7 and T47D (from ATCC, Rockville, MD) were cultured in either in DMEM or RPMI-1640 media (Invitrogen, Carlsbad, CA), supplemented with 10% FCS (Sigma-Aldrich, St. Louis, MO). Viral particles were produced by transient cotransfection of 293T cells with 10 µg PRRL2-WT-MET; PRRL2-p.Cys385Tyr-MET, PRRL2-p.Val1312Ile-MET in combination with 3 µg of envelop plasmid (pMD2-VSV-G), 5 µg of core packaging plasmid (pMDLg/pRRE), and 2.5 µg of pRSV-REV as previously described [Vigna and Naldini, 2000]. Expression of wild-type Met, p.Cys385Tyr-Met, and p.Val1312Ile-Met was tested by Western blot analysis. Antibodies used are listed below. Anti MET: DQ-13 and DL-21 [Prat et al., 1991]; C-12 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies against the MET phosphor-epitopes (PY1349) and (PY1234/1235) were from BioSource International (Carmarillo, CA); antiactin from Santa Cruz (Santa Cruz, CA). Conjugated secondary antibodies were from Amersham (Arlington Heights, VA).

Biological Assays

Cell proliferation assays, either in the absence or in the presence of HGF (50 ng/ml), were performed using ATP-lite Luminescent Assay System (Perkin-Elmer Lifeand Analytical Sciences, Inc., Reading, MA). The experiments were repeated three times, each one performing triplicates. Anchorage-independent growth was assayed plating 5×10³ cells in 0.5% agarose. HGF was supplied in the overly medium (40 ng/ml). Cell viability was assessed by Alamar blue staining (Resazurin sodium salt, Sigma-Aldrich), then colonies were visualized using 0.02% iodinitrotetrazolium chloride (0.02% in phosphate-buffered saline [PBS], Sigma-Aldrich), and counted at a DMIL microscope (Leica, Banockburn, IL) equipped with a digital camera (DFC320; Leica). For the wound-healing assay, confluent cell layers were starved for 24 hr in 2% fetal calf serum (FCS). The assay was performed as previously described [Michieli et al., 2004] and quantified by time-lapse microscopy (one image every 20 min, for 36 hr), plotting the distance between the scratch margins calculated as mean of 10 different measurements expressed as percentage of the initial distance. When required, medium was supplemented with HGF. For invasion assays, 5×10⁴ cells were seeded in Transwell™ permeable supports (Cole-Parmer, Vernon Hills, IL) with the upper side of the porous polycarbonate membrane coated with 9.8 µg/ml Matrigel™ (BD Biosciences, Franklin Lakes, NJ). Assays were carried out in presence of 2% FCS, with or without 20 ng/ml HGF, for 24 hr. Migrated cells were stained with crystal violet, then detected using a microscope (DMIL; Leica) equipped with a digital camera (DFC320; Leica), and photographed with GIMP 2.2.8 software (The GIMP-GNU Image Manipulation Program) and vnquantified using Image J software.

3. Results

Patient Selection

Forty-seven cancer patients in which the first manifestation detected were two or more metastases were selected as described in the method section. Clinical data are listed in Supp. Table S1. The diagnosis of "early metastatic cancer"

was reached in those patients with no detectable site of primary origin after exhaustive anamnesis and diagnostic evaluation, including physical examination, complete laboratory tests, whole-body CT scan, and FDG-PET. Based on the IHC profile it was possible to identify the putative site of origin in 24 patients, while the remaining 23 cases were classified as “truly” CUPs. The putative primary suggested by IHC was more often lung (50%), followed by liver–biliary tract or pancreas (25%): these tissue-of-origin profiles are coherent with published data obtained at autopsy [Pentheroudakis et al., 2007; Varadhachary et al., 2008]. The two cohorts displayed the typical early metastatic cancer clinical characteristics, even though CUPs featured a more aggressive behavior. Predominant histopathology was adenocarcinoma in the cohort with solved primary (70.8%) and undifferentiated carcinoma in CUPs (78.2%).

MET Mutations in CUPs

In the overall population of early metastatic cancers MET (MIM# 164860) was found mutated in 7 out of the 47 (~15%) samples analyzed. All mutations found in this study have been validated by processing independent aliquots (extracted from the same paraffin block at different times) of tumor DNA in multiple PCR amplification and sequencing rounds. The somatic origin of the mutations was confirmed by processing, together with tumor DNA, normal matched DNA obtained by laser capture micro dissection of nontransformed areas adjacent to the neoplastic lesions. Mutations were found only in tumor specimens, whereas normal matched DNA showed wild-type MET. Mutations were confirmed by sequencing tumor genomic DNA both in forward and reverse directions. Surprisingly all mutations occurred in undifferentiated carcinomas with no identifiable tissue of origin (Supp. Table S1). In this “truly” CUP cohort the overall occurrence of MET mutations is extremely high (30%). Because mutations occur as digital events (mutation absence/presence), the binomial distribution represents the statistical function that correctly describes this phenomenon [Taylor, 1982]. By its simple application to data on MET mutations reported by COSMIC database (<http://www.sanger.ac.uk>) the results become relevant in respect to the unselected cancer population in which MET is mutated in 4% of cases. The incidence is noteworthy even if compared to that of upper aero-digestive track tumors, which actually display the highest registered mutational rates (14%, data from COSMIC Database). In both cases the probability of the event (mutation) occurrence in a population of 47 samples, would be strictly near to zero; indeed, the value obtained in fully CUPs (seven cases) hugely exceeds the expected one (Supp. Fig. S1).

Four somatic mutations (p.Gln142X; p.His150Tyr; p.Glu168Asp, and p.Cys385Tyr) clustered in exon 2, which codifies for the SEMA domain, one mutation (p.Thr1010Ile) was found in exon 14, which codifies for the juxtamembrane domain and one (p.Val1312Ile) affected a residue located in exon 20, encoding for the tyrosine kinase domain, nearby the active site (Fig. 1). Two out of six mutations found in this study (p.Glu168Asp and p.Thr1010Ile) have already been described [Di Renzo et al., 2000; Ma et al., 2008; Seiwert et al., 2009]; the other four are novel changes. As reported above, these results were validated through detection of the same mutation by processing independent aliquots of tumor DNA in multiple PCR amplification and sequencing rounds; moreover, no mutations were found in matched DNA extracted from adjacent normal tissue. In order to rule out the possibility of high mutational background due to repair deficiency or environmental exposure, we performed standard MSI assays. We analyzed six out of seven MET mutated patients (lacking material from the seventh) and none of them showed MSI (Table 1). To further support this result we checked in the same cases the mutational profile of “hot spot” regions of three oncogenes frequently mutated in cancers: KRAS (MIM# 190070) exon 2, EGFR (MIM# 131550) exons 18–19–20–21, and PIK3CA (MIM# 171834) exons 9–20 (Table 1); no mutations were found. As further control, we also checked the full-length coding sequence of RON (MIM# 600186). The RON gene, located on chromosome 3p21.3, has a similar size to MET, being made by 20 exons. Ron belongs to the Scatter Factor receptor family encoding a receptor that is similar to Met (63% homology) [Ronsin et al., 1993], featuring an extracellular portion containing a SEMA domain, a transmembrane region, and an intracellular tyrosine kinase domain. In four out of the seven MET mutated patients the cancer genomic DNA available was enough to sequence the whole RON gene; in other two cases the analysis was limited to the exons encoding for the SEMA and the

tyrosine kinase domains. In none of the CUP patients harboring MET mutations, RON was found to be mutated. We also analyzed MET gene copy number (by fluorescent in situ hybridization [FISH]) in the MET mutated patients and MET amplification was never detected (some samples carrying an increased MET copy number in consequence to a low grade of chromosome 7 polysomy; Table 1). To further investigate if other genes—frequently mutated in cancers—could be the driving force in CUP patients harboring MET wild type, we performed the mutational analysis of the tree “hot spot” oncogenes: KRAS exon 2; EGFR exons 18–19–20–21 and PIK3CA exons 9–20. No mutations were detected. On the contrary, four mutated samples were detected in the non-CUP group. Two cases (ID samples 13 and 19) carried EGFR somatic changes, p.Leu858Arg and c.2184_2283del, respectively. The other two mutated cases (ID samples 21 and 32) featured the KRAS Gly12Asp activating mutation. The above data strengthen the selective association between the CUP phenotype and MET, but not KRAS, EGFR, or PIK3CA.

Figure 1. MET mutations in CUPs. Met receptor structure displaying mutations found in CUPs. §PM: plasma membrane. *P: sites of phosphorylation. ‡MRS: MET-related sequence. •IPT: protein–protein interaction site.

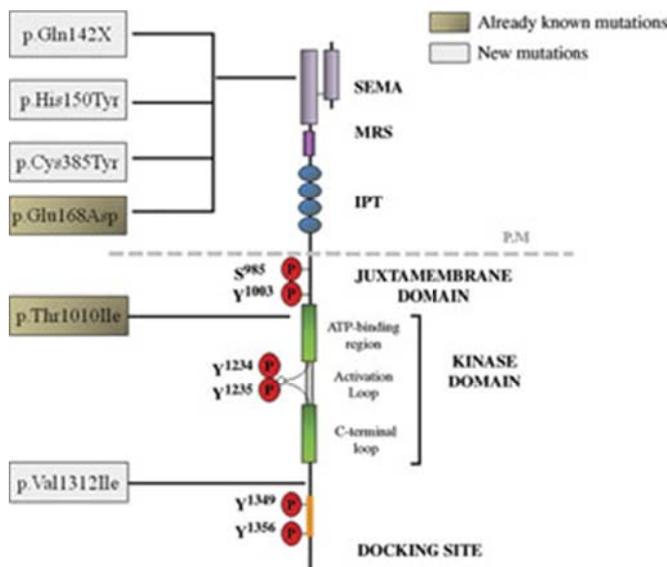


Table 1. Details on the Seven MET Mutated Patients

Sample ID	6	10	11	20	24	29	30
Gender	M	M	F	M	M	M	M
Age (years)	67	50	45	58	67	56	55
Metastatic DNA source (site)	Brain (Cerebellum)	Brain (Frontal cortex)	Brain (Frontal cortex)	Brain (Tentorium)	Liver	Liver	Liver
Site of primary origin	UNKNOWN						
Histology	Undifferentiated carcinoma						
MET mutation	p.His150Tyr	p.Gln142X	p.Cys385Tyr	p.Glu168Asp	p.Glu168Asp	p.Val1312Ile	p.Thr1010Ile
MET/CEP7 (FISH)	3.7/3.2	2.7/2.5	4.7/4.8	3.5/3.6	n.t.	2.9/2.8	2.172.0
MSI	Stable	Stable	Stable	Stable	n.t.	Stable	Stable
KRASbexon2	wt	wt	wt	wt	n.t.	wt	wt
PIK3CAexon 9–20	wt	wt	wt	wt	n.t.	wt	wt

Sample ID	6	10	11	20	24	29	30
EGFRd exon1 8	wt	wt	wt	wt	n.t	wt	wt
EGFR exon 19	wt	wt	wt	wt	n.t	wt	wt
EGFR exon 20	wt	wt	wt	wt	n.t	wt	wt
EGFR exon 21	wt	wt	wt	wt	n.t	wt	wt
RONeSEMA exons 1–5	wt	wt	wt	wt	n.t	wt	wt
RON exons 6–13	wt	wt	wt	wt	n.t	wt	wt
RON exons 14–20	wt	wt	wt	wt	n.t	wt	wt

MSI = microsatellite instability; n.t. = not tested; the MET/CEP7 denotes the ratio between the mean number of MET copies and the mean number of centromere 7 observed for each samples by FISH analysis.

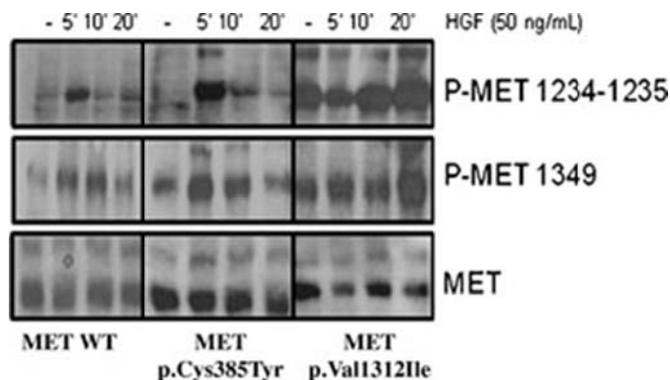
- a a(NM_000245.2).
- b b(NM_004985.3).
- c c(NM_006218.2).
- d d(NM_005228.3).
- e e(NM_002447.2).

Within the limits of the small cohort analyzed in this study, the median survival of patients harboring MET-mutations was of 8 months, whereas that of MET wild-type patients was 13 months (Supp. Fig. S2). Taken together, these data suggest a potential role of MET mutational profile in CUPs as a negative prognostic marker.

Functional Characterization of MET Mutations

We focused on the novel nucleotide changes identified, and among them the p.Cys385Tyr and the p.Val1312Ile mutations, localized in the SEMA or the tyrosine kinase domain, respectively. Their oncogenic potential was investigated by reproducing the mutations in cDNAs and expressing the mutated Met proteins in two different mammalian cell lines: T47D and COS-7. All experiments were performed in both cell lines, obtaining comparable results. Notably, T47D do not express endogenous Met. Lentiviral expression of wild-type receptor was used as a control. We investigated receptors phosphorylation and downstream signaling. We demonstrated that p.Val1312Ile (TK mutant) was constitutively phosphorylated, both on the catalytic domain (Y1234 and Y1235) and the docking-site tail (Y1349) (Fig. 2). Results were confirmed by in vitro kinase activity assay (data not shown). The two major downstream effectors of MET signaling, AKT and ERK, were coherently persistently activated (data not shown). On the other hand, as reasonably expected, the p.Cys385Tyr receptor (SEMA mutant) was not basally phosphorylated (Fig. 2). However, as described below, both mutations were able to activate the invasive growth response.

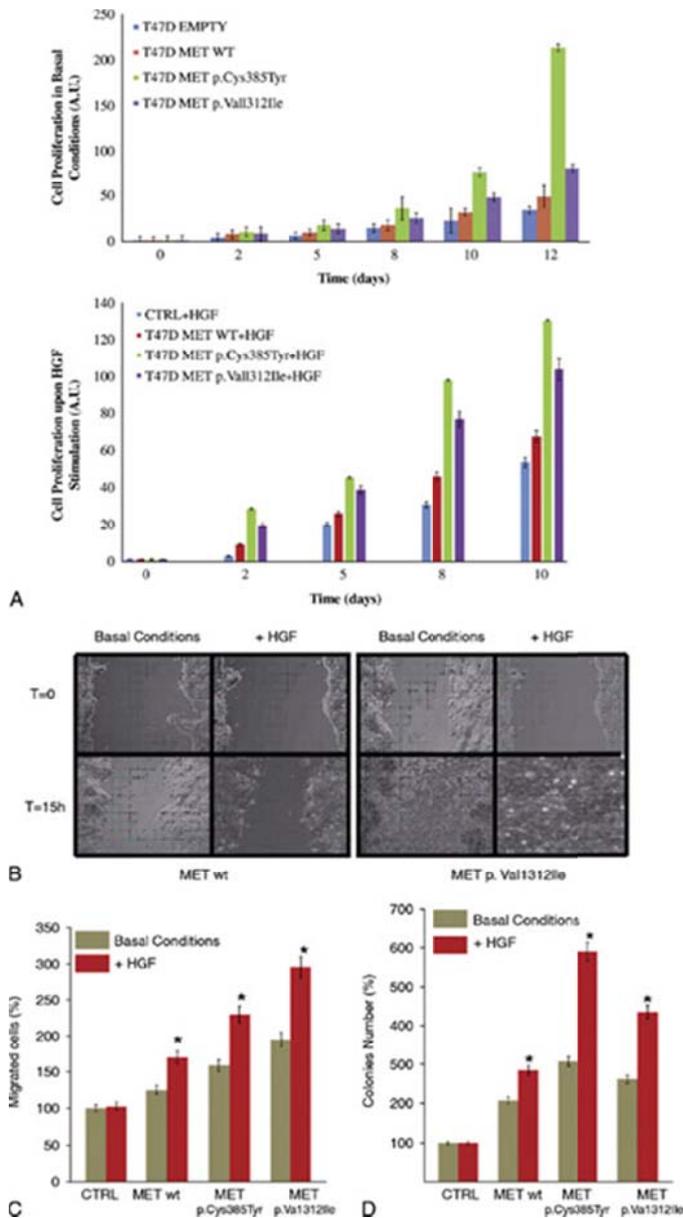
Figure 2. MET mutations phosphorylation status. Receptors phosphorylation was assessed in basal conditions and upon HGF stimulation in a time-course experiment (5, 10, and 20 min). The panel shows T47D extracts expressing wild-type Met (MET WT), Met harboring the SEMA (p.Cys385Tyr), or the TK (p.Val1312Ile) mutations. Blots were decorated with antibodies against pospho-Met proteins (Y1234–1235 and Y1249), and total Met protein (DL21).



We analyzed the key features of the invasive growth phenotype: proliferation, motility, invasion, and anchorage-independent growth (Fig. 3). The TK mutant (p.Val1312Ile) showed an increase in proliferation rate, as well as, unexpectedly, the SEMA mutant (p.Cys385Tyr). Cell proliferative rates were also tested upon HGF stimulation and were further increased in both mutants (Fig. 3A). Cell motility was assessed by a cell monolayer wound-healing assay, both in basal conditions and upon ligand stimulation. We observed that TK mutant featured an enhanced motile phenotype when compared to wild-type Met. "Healing speed" increased about 200-fold in basal conditions and 250-fold upon HGF stimulation in p.Val1312Ile. The SEMA mutant behaved, in the wound-healing assay, as the wild-type receptor, whereas significantly augmented upon HGF stimulation (Fig. 3B). As invasive behavior of cancer cells is reflected, *in vitro*, by their ability to migrate through matrigel-coated Boyden chambers [Terranova et al., 1986] we performed a matrigel invasion assay and showed that the invasive capacity was significantly increased by transduction of cDNAs harboring either mutations (p.Val1312Ile and p.Cys385Tyr), both in basal conditions and upon HGF stimulation. TK mutant displayed a hyperinvasive behavior, which was further enhanced in the presence of a ligand gradient. SEMA mutant's phenotype appeared to be less aggressive but still significantly higher ($P < 0.005$) compared to wild-type Met (Fig. 3C). The clonogenic properties of Met mutants were assessed by anchorage-independent growth assay (Fig. 3D). TK mutant showed a significant increase ($P < 0.001$) in number of colonies formed. Interestingly, clonogenic growth was enhanced at high degree in cells expressing the SEMA mutant. Anchorage-independent growth of both mutants was significantly augmented by HGF supplementation. Although these *in vitro* data do not provide incontrovertible proof supporting the key role of the described mutations in generating the hypermetastatic phenotype, they at least provide evidence that the Met receptors mutated in CUPs were functional.

Figure 3. The invasive growth phenotype of TK (p.Val1312Ile) and SEMA (p.Cys385Tyr) MET mutants. Results are means \pm SD. Comparisons were made using a two-Tailed Student's *t*-test. P-Values less than 0.05 were considered to be statistically significant. A: Cell proliferation of T47D expressing wild-type Met, SEMA mutant, or TK mutant in basal condition and upon HGF supplementation. Untransduced T47D were used as a control (CTRL). B: Wound-healing Assay. Migration into the wound was quantified by time-lapse microscopy (one image every 20 min, for 36 hr), plotting the distance between the scratch margins calculated as mean of 10 different measurements expressed as percentage of the initial distance. In TK mutant motility was double compared with wild-type Met both in basal conditions (0.176 $\mu\text{m}/\text{sec}$ vs. 0.087 $\mu\text{m}/\text{sec}$) and upon ligand stimulation (0.250 $\mu\text{m}/\text{sec}$ vs. 0.100 $\mu\text{m}/\text{sec}$). Pictures represent WT Met and TK mutant at time 0 and after 15 hr. C: Invasion assay. Each bar represents the relative ratio (percent) of migrated cells in respect to wild-type T47D, used as a control (CTRL). $[(\text{MetX}/\text{CTRL}) * 100]$, where X = WT, SEMA mutant, TK mutant]. Experiments were conducted in basal conditions and upon HGF stimulation. Error bars indicate standard deviations. *Denotes statistically significant differences. D: Anchorage-independent Growth (Soft Agar) Assay. Colonies were scored after 2 weeks. Each bar represents the relative ratio (percent) of number of colonies in respect to wild-type T47D, used as a control (CTRL). $[(\text{MetX}/\text{CTRL}) * 100]$, where X = WT, SEMA mutant, TK mutant]. Experiments were conducted in

basal conditions and upon HGF stimulation. Error bars indicate standard deviations. *Denotes statistically significant differences.



4. Discussion

MET activation by point mutations is a (relatively) rare event, occurring in 3% of all cancer types. Mutations occur preferentially in hereditary and sporadic papillary renal cancers [Schmidt et al., 1997], gastric and childhood hepatocellular carcinomas [Lee et al., 2000; Park et al., 1999], thoracic neoplasms (lung cancers and malignant pleural mesotheliomas) [Cipriani et al., 2009], as well as melanomas [Puri et al., 2007]. Activation of MET by mutations occurs as a late event, and it has been associated with the acquisition of metastatic potential. For instance, it has been demonstrated that, during progression toward metastasis, head and neck carcinomas select cells harboring MET mutations [Lorenzato et al., 2002]. On the other hand, in the vast majority of tumors, MET activation is due to overexpression: the event exacerbates malignancy by usurping the (otherwise physiological) antiapoptotic and pro-invasive properties of the proto-oncogene, as an "expedience" to gain fitness under adverse microenvironmental conditions (for a review, see [Comoglio et al., 2008]).

We now demonstrate that MET mutations occur with remarkable high frequency (30%) in metastatic CUPs. The relatively high incidence of activating MET mutations found in CUPs suggests the involvement of the oncogene in this still obscure pathology. Within the limits of the cohort analyzed in this study, MET mutational profile in CUPs clearly classifies MET mutated patients as those displaying the most aggressive disease. It should be noted, however, that the CUP group is enriched for tumors lacking differentiation markers, whereas the non-CUP group is enriched for specific tumor types, and thus the two groups are biased for differentiation grade and organ of origin. Therefore, MET mutation may as well reflect either differentiation grade and/or organ of origin. In this respect a preferential expression of MET in cancer stem cells has been postulated [Boccaccio and Comoglio, 2006]. However, this report provides experimental evidence that the mutations found in CUPs are functional, as their transduction in reporter cells in vitro confers a clear invasive growth phenotype.

The mutations detected in CUPs affect both the tyrosine kinase (catalytic) domain (p.Val1312Ile) as well as the extracellular SEMA domain (p.Gln142X; p.His150Tyr; p.Glu168Asp, and p.Cys385Tyr). Interestingly, the three mutations affecting the SEMA domain of the receptor have been found in hematogenous metastases to the brain, a sanctuary site that can be reached only by most aggressive clones, capable to cross the blood–brain barrier. Although it has been extensively demonstrated that mutations activating the kinase are tumorigenic [Graveel et al., 2004; Michieli et al., 1999], the oncogenic activity of the SEMA mutations was not fully predicted. This unexpected behavior suggests that the noncatalytic domain of the MET receptor might be somehow involved in tumor progression. Although the mechanistic explanation of such a phenotype goes behind the scope of this report, we can speculate a yet unknown role in the control of the invasive growth program of the SEMA domain, a protein–protein interaction motif. Mutations localized within the SEMA domain might interfere either with the ligand binding or with the three-dimensional structure of the receptors.

Several therapeutic strategies have been developed to block MET signaling, each focusing on one of the sequential steps that regulates MET activation [Comoglio et al., 2008]. To date, dozens of small inhibitors and a few monoclonal antibodies have been actively pursued to antagonize MET activation. Based on experiences from EGFR inhibition, the occurrence of genetic alterations of MET activity might induce direct oncogene addiction and therefore predict therapeutic responsiveness. In addition to canonical mutations occurring within the kinase region, the described novel mutations affecting the SEMA domain may help identifying the responsive patient subset for personalized anti-MET therapy.

Overall, the above described mutations have two clinical implications for the CUPs—still an orphan disease—providing: (1) a potential functional marker, with diagnostic and prognostic implications; (2) a rationale for clinical trials with MET inhibitors.

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References

- Boccaccio C, Comoglio PM. 2006. Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer* 6:637–645.
- Briasoulis E, Pavlidis N, Felip E. 2009. Cancers of unknown primary site: ESMO clinical recommendations for diagnosis, treatment and follow up. *Ann Oncol* 20:iv154–iv155.

- Buhard O, Cattaneo F, Wong YF, Yim SF, Friedman E, Flejou JF, Duval A, Hamelin R. 2006. Multipopulation analysis of polymorphisms in five mononucleotide repeats used to determine the microsatellite instability status of human tumors. *J Clin Oncol* 24:241–251.
- Casorzo L, Luzzi C, Nardacchione A, Picciotto F, Pisacane A, Risio M. 2005. Fluorescence in situ hybridization (FISH) evaluation of chromosomes 6, 7, 9 and 10 throughout human melanocytic tumorigenesis. *Melanoma Res* 15:155–160.
- Cipriani NA, Abidoye OO, Vokes E, Salgia R. 2009. MET as a target for treatment of chest tumors. *Lung Cancer* 63:169–179.
- Comoglio PM, Boccaccio C. 2001. Scatter factors and invasive growth. *Semin Cancer Biol* 11:153–165.
- Comoglio PM, Giordano S, Trusolino L. 2008. Drug development of MET inhibitors: targeting oncogene addiction and expedience. *Nat Rev Drug Discov* 7:504–516.
- Di Renzo MF, Olivero M, Martone T, Maffe A, Maggiora P, Stefani AD, Valente G, Giordano S, Cortesina G, Comoglio PM. 2000. Somatic mutations of the MET oncogene are selected during metastatic spread of human HNSC carcinomas. *Oncogene* 19:1547–1555.
- Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Jänne PA. 2007. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 316:1039–1043.
- Fearon ER, Vogelstein B. 1990. A genetic model for colorectal tumorigenesis. *Cell* 61:759–797.
- Gherardi E, Youles ME, Miguel RN, Blundell TL, Iamele L, Gough J, Bandyopadhyay A, Hartmann G, Butler PJ. 2003. Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. *Proc Natl Acad Sci USA* 100:12039–12044.
- Graveel C, Su Y, Koeman J, Wang LM, Tessarollo L, Fiscella M, Birchmeier C, Swiatek P, Bronson R, Vande Woude G. 2004. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. *Proc Natl Acad Sci USA* 101:17198–17203.
- Horlings HM, van Laar RK, Kerst JM, Helgason HH, Wesseling J, van der Hoeven JJ, Warmoes MO, Floore A, Witteveen A, Lahti-Domenici J, Glas AM, Van't Veer LJ, de Jong D. 2008. Gene expression profiling to identify the histogenic origin of metastatic adenocarcinomas of unknown primary. *J Clin Oncol* 26:4435–4441.
- Hüsemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmüller G, Klein CA. 2008. Systemic spread is an early step in breast cancer. *Cancer Cell* 13:56–58.
- Kong-Beltran M, Stamos J, Wickramasinghe D. 2004. The Sema domain of Met is necessary for receptor dimerization and activation. *Cancer Cell* 6:75–84.
- Lee JH, Han SU, Cho H, Jennings B, Gerrard B, Dean M, Schmidt L, Zbar B, Vande Woude GF. 2000. A novel germ line juxtamembrane Met mutation in human gastric cancer. *Oncogene* 19:4947–4953.
- Lorenzato A, Olivero M, Patanè S, Rosso E, Oliaro A, Comoglio PM, Di Renzo MF. 2002. Novel somatic mutations of the MET oncogene in human carcinoma metastases activating cell motility and invasion. *Cancer Res* 62:7025–7030.
- Ma PC, Tretiakova MS, MacKinnon AC, Ramnath N, Johnson C, Dietrich S, Seiwert T, Christensen JG, Jagadeeswaran R, Krausz T, Vokes EE, Husain AN, Salgia R. 2008. Expression and mutational analysis of MET in human solid cancers. *Genes Chromosomes Cancer* 47:1025–1037.

- Michieli P, Basilico C, Pennacchietti S, Maffè A, Tamagnone L, Giordano S, Bardelli A, Comoglio PM. 1999. Mutant Met-mediated transformation is ligand-dependent and can be inhibited by HGF antagonists. *Oncogene* 18:5221–5231.
- Michieli P, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L, Comoglio PM. 2004. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell* 6:61–73.
- Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, Gambacorta M, Siena S, Bardelli A. 2005. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 6:279–286.
- Park SY, Kim BH, Kim JH, Lee S, Kang GH. 2007. Panels of immunohistochemical markers help determine primary sites of metastatic adenocarcinoma. *Arch Pathol Lab Med* 131:1561–1567.
- Park WS, Dong SM, Kim SY, Na EY, Shin MS, Pi JH, Kim BJ, Bae JH, Hong YK, Lee KS, Lee SH, Yoo NJ, Jang JJ, Pack S, Zhuang Z, Schmidt L, Zbar B, Lee JY. 1999. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res* 59:307–310.
- Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM. 2003. Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. *Cancer Cell* 3:347–361.
- Pentheroudakis G, Golfinopoulos V, Pavlidis N. 2007. Switching benchmarks in cancer of unknown primary: from autopsy to microarray. *Eur J Cancer* 43:2026–2036.
- Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, Graziani A, Panayotou G, Comoglio PM. 1994. A multifunctional docking site mediates signalling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* 77:261–271.
- Prat M, Crepaldi T, Gandino L, Giordano S, Longati P, Comoglio P. 1991. C-terminal truncated forms of Met, the hepatocyte growth factor receptor. *Mol Cell Biol* 11:5954–5962.
- Puri N, Ahmed S, Janamanchi V, Tretiakova M, Zumba O, Krausz T, Jagadeeswaran R, Salgia R. 2007. c-MET is a potentially new therapeutic target for treatment of human melanoma. *Clin Cancer Res* 13:2246–2253.
- Regelink G, Brouwer J, de Bree R, Pruim J, van der Laan BF, Vaalburg W, Hoekstra OS, Comans EF, Vissink A, Leemans CR, Roodenburg JL. 2002. Detection of unknown primary tumours and distant metastases in patients with cervical metastases: value of FDG-PET versus conventional modalities. *Eur J Nucl Med Mol Imaging* 29:1024–1030.
- Ronsin C, Muscatelli F, Mattei MG, Breathnach R. 1993. A novel putative receptor protein tyrosine kinase of the met family. *Oncogene* 8:1195–1202.
- Rossi G, Nannini N, Costantini M. 2009. Morphology and more specific immunohistochemical stains are fundamental prerequisites in detection of unknown primary cancer. *J Clin Oncol* 27:649–650.
- Schmidt L, Duh FM, Chen F, Kishida T, Glenn G, Choyke P, Scherer SW, Zhuang Z, Lubensky I, Dean M, Allikmets R, Chidambaram A, Bergerheim UR, Feltis JT, Casadevall C, Zamarron A, Bernues M, Richard S, Lips CJ, Walther MM, Tsui LC, Geil L, Orcutt ML, Stackhouse T, Lipan J, Slife L, Brauch H, Decker J, Niehans G, Hughson MD, Moch H, Storkel S, Lerman MI, Linehan WM, Zbar B. 1997. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. *Nat Genet* 16:68–73.
- Seiwert TY, Jagadeeswaran R, Faoro L, Janamanchi V, Nallasura V, El Dinali M, Yala S, Kanteti R, Cohen EE, Lingen MW, Martin L, Krishnaswamy S, Klein-Szanto A, Christensen JG, Vokes EE, Salgia R. 2009. The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. *Cancer Res* 69:3021–3031.

- Sève P, Billotey C, Broussolle C, Dumontet C, Mackey JR. 2007. The role of 2-deoxy-2-[F-18]fluoro-D glucose positron emission tomography in disseminated carcinoma of unknown primary site. *Cancer* 109:292–299.
- Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, Seruca R, Iacopetta B, Hamelin R. 2002. Evaluation of tumor microsatellite instability using five quasimonomorphic mononucleotide repeats and pentaplex PCR. *Gastroenterology* 123:1804–1811.
- Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM. 1999. Plexins are a large family of receptors for transmembrane, secreted and GPI-anchored semaphorins in vertebrate. *Cell* 99:71–80.
- Terranova VP, Hujanen ES, Loeb DM, Martin GR, Thornburg L, Glushko V. 1986. Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells. *Proc Natl Acad Sci USA* 83:465–469.
- Trusolino L, Comoglio PM. 2002. Scatter-factors and semaphoring receptors: cell signaling for invasive growth. *Nat Rev Cancer* 4:289–300.
- Varadhachary GR, Talantov D, Raber MN, Meng C, Hess KR, Jatkoe T, Lenzi R, Spigel DR, Wang Y, Greco FA, Abbruzzese JL, Hainsworth JD. 2008. Molecular profiling of carcinoma of unknown primary and correlation with clinical evaluation. *J Clin Oncol* 26:4442–4448.
- Vigna E, Naldini L. 2000. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidate for gene therapy. *J Gene Med* 2:308–316.
- Weinberg RA. 2008. Leaving home early: reexamination of the canonical models of tumor progression. *Cancer Cell* 14:283–284.