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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, while 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 7 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.

Key words: Met, tyrosine kinase, somatic mutation, invasive growth, metastases.

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 radio-metabolic 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 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 aminoacid 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-Beltram 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) which 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 unfavourable micro environmental conditions -*e.g.* 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.

Materials 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 1. All patients received a thorough sequential multistep diagnostic work-up 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-PET. The immunohistochemical profile was defined by a panel of at least ten markers, according to accepted procedures outlined by Horlings et al. (Horlings et al., 2008) and further refined by Rossi et al. (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. (Horlings et al., 2008) and by Rossi et al. (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 co-expression 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 re-evaluated 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 1216 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 not-transformed 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 Burhard et al. (Burhard et al., 2006), and then analyzed on ALFexpress II Sequencer (GE Healthcare Europe). FISH analysis was carried out on 4 µm 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, USA).

MET mutant expressing constructs.

MET mutations p.Cys385Tyr (g.1154G>A) and p.Val1312Ile (g.3932G>A) were generated using QuickChange Site-Directed Mutagenesis XL kit (Stratagene, LaJolla) 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 P156RRLsinPPThCMVMCSpre lentiviral vector.

Lentiviral cell infection and Western blotting.

COS-7 and T47D (from ATCC) 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 co-transfection 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, USA). Antibodies against the MET phosphor-epitopes [PY1349] and [PY1234/1235] were from BioSource International; anti actin from Santa Cruz. Conjugated secondary antibodies were from Amersham (Arlington Heights, VA).

Biological assays.

Cell proliferation assays, either in absence or in presence of HGF (50 ng/mL), were performed using ATP-lite Luminescent Assay System (PerkinElmer Life And Analytical Sciences, Inc., MA, USA). The experiments were repeated three times each one performing triplicates. *Anchorage-independent growth* was assayed plating $5X10^3$ 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, St.Louis, MO), then colonies were visualized using 0.02% iodonitrotetrazolium chloride (0.02% in PBS, Sigma-Aldrich, St.Louis, MO) and counted at a DMIL microscope (Leica) equipped with a digital camera (DFC320; Leica). For the *wound healing assay*, confluent cell layers were starved for 24 hours in 2% FCS. The assay was performed as previously described (Michieli et al., 2004) and quantified by time-lapse microscopy (one image every twenty minutes, for thirty-six hours), plotting the distance between the scratch margins calculated as mean of ten different measurements expressed as percentage of the initial distance. When required, medium was supplemented with *HGF*. For *invasion assays*, $5X10^4$ cells were seeded in Transwell™ permeable supports (Cole-Parmer Vernon Hills, Illinois 60061) with the upper side of the porous polycarbonate membrane coated with 9,8 µg/mL Matrigel™ (BD Biosciences, Franklin Lakes, NJ USA). Assays were carried out in presence of 2% FCS, with or without 20 ng/mL *HGF*, for 24 hours. 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 quantified using Image J software.

Results

Patients 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 immunohistochemical (IHC) profile it was possible to identify the putative site of origin in 24 patients, while the remaining 23 cases were classified as 'truly' *cancers of unknown primary origin* (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 behaviour. 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 not-transformed areas adjacent to the neoplastic lesions. Mutations were found only in tumor specimens, while 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%). Since 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 (7 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 microsatellite instability (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 which is similar to *Met* (63% homology; Ronsin, 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 harbouring *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 harbouring *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*.

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

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. 3, panel A). 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 two-hundred folds in basal conditions and two-hundred and fifty folds upon *HGF* stimulation in p.Val1312Ile. The *SEMA mutant* behaved, in wound healing assay, as the wild type receptor, whereas significantly augmented upon *HGF* stimulation (Fig. 3, panel B). As invasive behaviour 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 harbouring either mutations (p.Val1312Ile and p.Cys385Tyr), both in basal conditions and upon *HGF* stimulation. *TK mutant* displayed a hyper-invasive behaviour, 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. 3, panel C). The clonogenic properties of *Met* mutants were assessed by anchorage independent growth assay (Fig. 3, panel D). *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 hyper-metastatic phenotype, they at least provide evidence that the *Met* receptors mutated in CUPs were functional.

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 harbouring *MET* mutations (Lorenzato et al., 2002), On the other hand, in the vast majority of tumors, *MET* activation is due to over-expression : the event exacerbates malignancy by usurping the (otherwise physiological) anti-apoptotic and pro-invasive properties of the proto-oncogene, as an 'expedience' to gain fitness under adverse micro-environmental conditions (for a review see Comoglio et al., 2008).

We now demonstrate that *MET* mutations occur with remarkable high frequency (30%) in metastatic cancers of unknown primary origin (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, while 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. While it has been extensively demonstrated that mutations activating the kinase are tumorigenic (Michieli et al., 1999; Graavel et al., 2004), the oncogenic activity of the SEMA mutations was not

fully predicted. This unexpected behaviour suggests that the non-catalytic 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* signalling, 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: (i) a potential functional marker, with diagnostic and prognostic implications; (ii) a rationale for clinical trials with MET inhibitors.

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Figure Legends

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.

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 minutes). 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).

Figure 3. The invasive growth phenotype of *TK* (p.Val1312Ile) and *SEMA* (p.Cys385Tyr) *MET* mutants.

Results are means +/- 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 twenty minutes, for thirty-six hours), plotting the distance between the scratch margins calculated as mean of ten 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 µm/sec *vs* 0,087 µm/sec) and upon ligand stimulation (0,250 µm/sec *vs* 0,100 µm/sec). Pictures represent WT *Met* and *TK mutant* at time 0 and after 15 hours.

C) Invasion Assay. Each bar represents the relative ratio (per cent) of migrated cells in respect to wild type T47D, used as a control (CRTL). [(Met_X/CTLR)* 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 two weeks. Each bar represents the relative ratio (per cent) of number of colonies in respect to wild type T47D, used as a control (CRTL). [(Met_X/CTLR)* 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.

Sample ID	06	10	11	20	24	29	30
Gender	M	M	F	M	M	M	M
Age (yrs)	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	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN	UNKNOWN
Histology	Undifferentiated carcinoma	Undifferentiated carcinoma	Undifferentiated carcinoma	Undifferentiated carcinoma	Undifferentiated carcinoma	Undifferentiated carcinoma	Undifferentiated carcinoma
MET[1] 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
KRAS[2] exon2	wt	wt	wt	wt	n.t	wt	wt
PIK3CA[3] exon 9-20	wt	wt	wt	wt	n.t	wt	wt
EGFR[4] exon18	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
RON[5] SEMA exons 1-5	wt	wt	wt	wt	n.t	wt	wt
RON exon 6-13	wt	wt	wt	wt	n.t	wt	wt
RON exons14-20	wt	wt	wt	wt	n.t	wt	wt

[1] : (NM_000245.2)
[2]: (NM_004985.3)
[3] : (NM_006218.2)
[4] : (NM_005228.3)
[5] : (NM_002447.2)

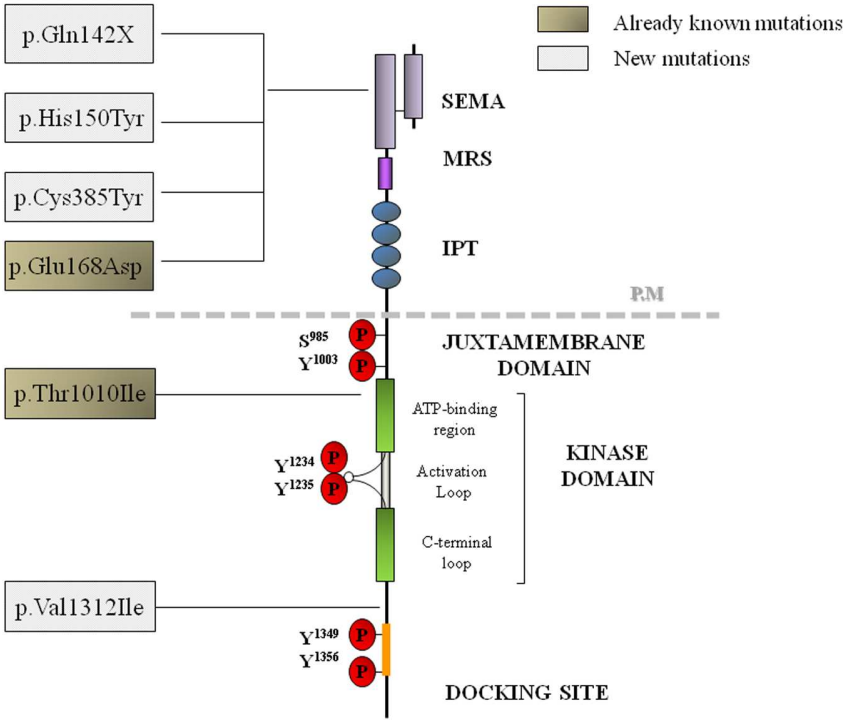


Figure 1. MET mutations in CUPs.
Met receptor structure displaying mutations found in CUPs.
§ PM: plasma membrane.
*P: sites of phosphorylation.
ǂ MRS: MET Related Sequence.
• IPT: protein-protein interaction site

203x152mm (200 x 200 DPI)

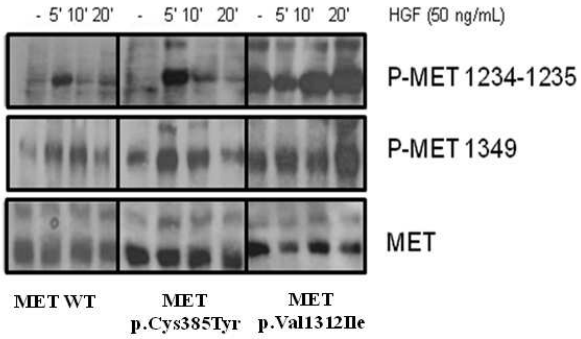
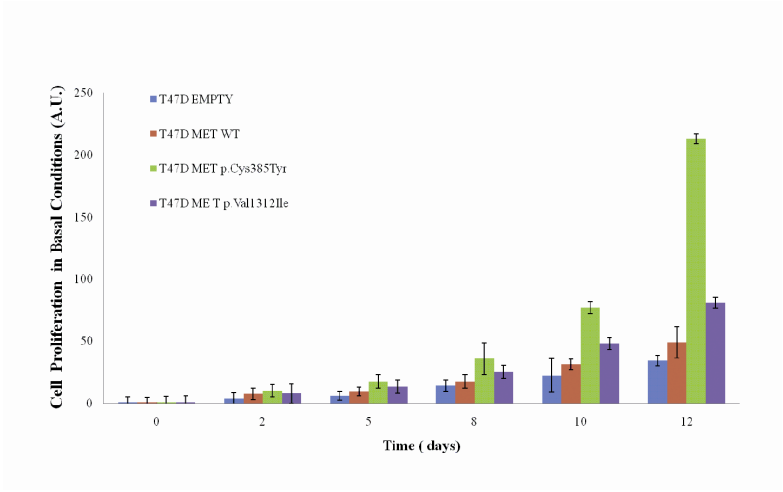
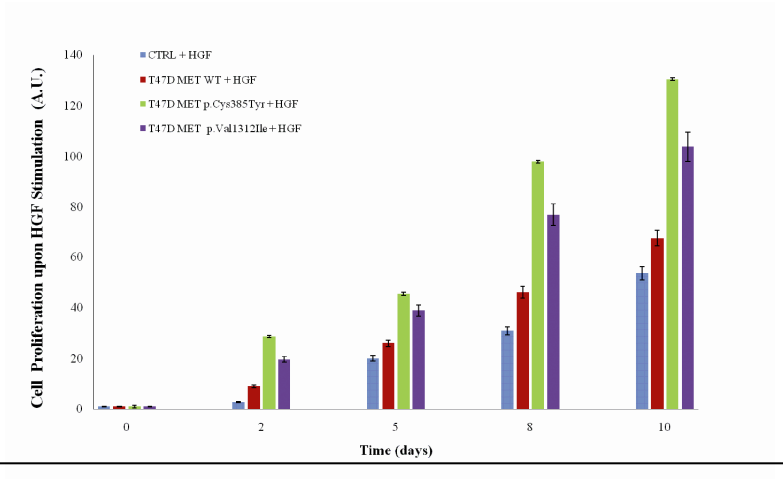


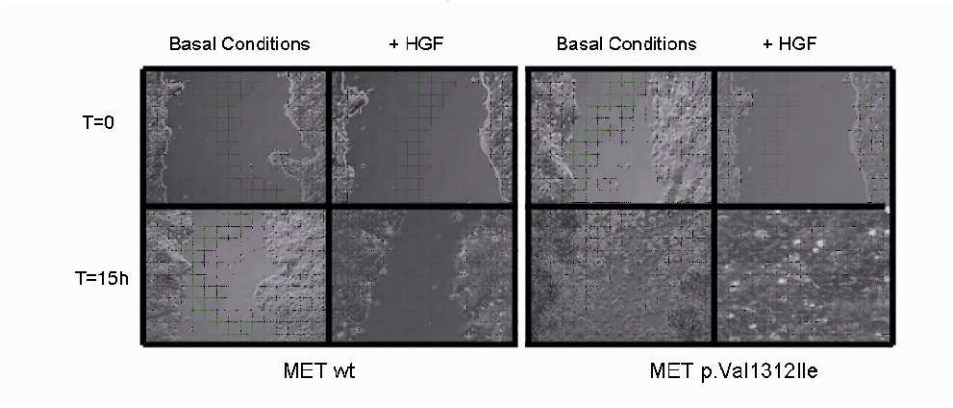
Figure 2. MET mutations phosphorylation status.
Receptors phosphorylation was assessed in basal conditions and upon HGF stimulation in a time-course experiment (5, 10 and 20 minutes). The panel shows T47D extracts expressing wild type Met (MET WT), Met harboring the SEMA (p.Cys385Tyr) or the TK (p.Val1312Ile)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

254x190mm (96 x 96 DPI)

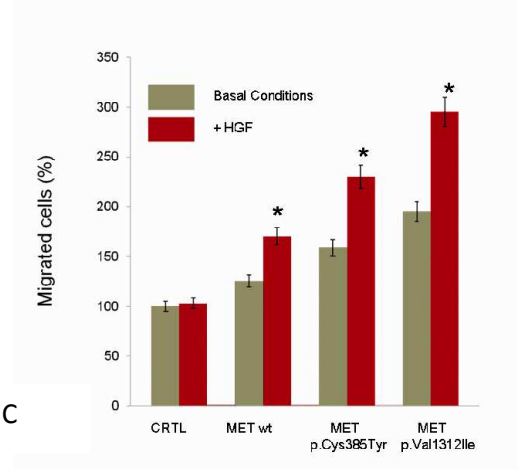

Cell Proliferation in Basal Conditions (A.U.)
T47D EMPTY
T47D MET WT
T47D MET p.Cys385Tyr
T47D MET p.Val1312Ile
Time (days)


Cell Proliferation upon HGF Stimulation (A.U.)
CTRL + HGF
T47D MET WT + HGF
T47D MET p.Cys385Tyr + HGF
T47D MET p.Val1312Ile + HGF
Time (days)

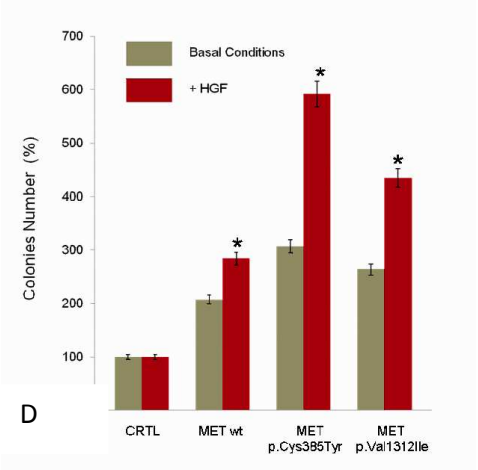
A


Basal Conditions
+ HGF
Basal Conditions
+ HGF
T=0
T=15h
MET wt
MET p.Val1312Ile

B


Migrated cells (%)
Basal Conditions
+ HGF
CRTL
MET wt
MET p.Cys385Tyr
MET p.Val1312Ile

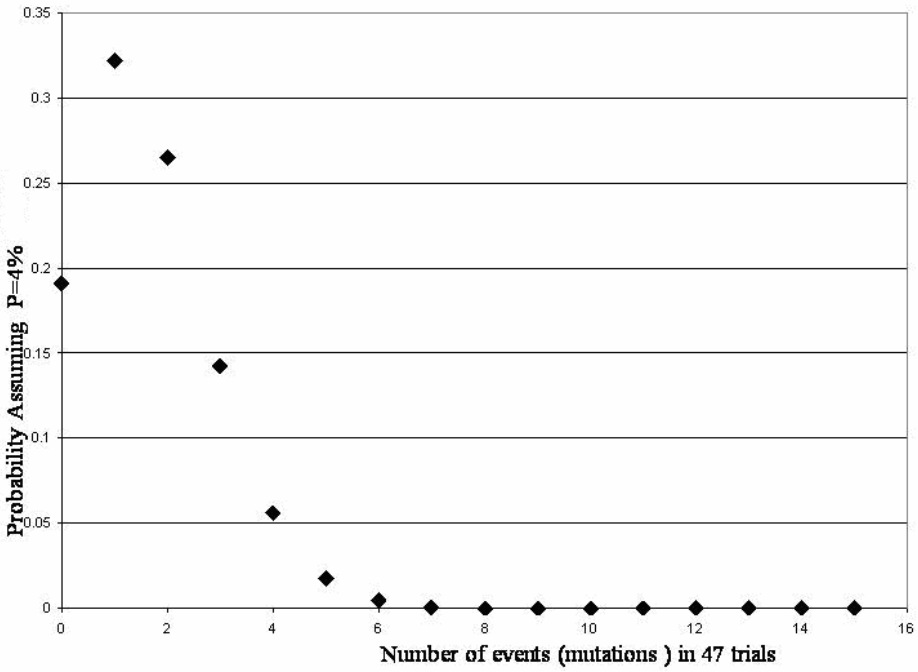
C


Colonies Number (%)
Basal Conditions
+ HGF
CRTL
MET wt
MET p.Cys385Tyr
MET p.Val1312Ile

D

Supporting Material.

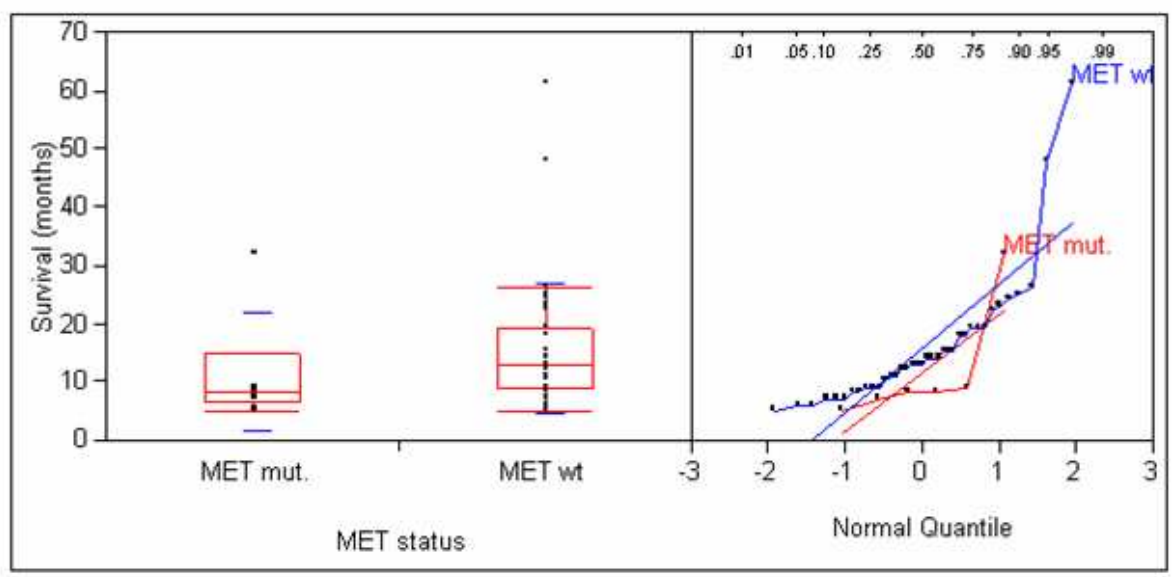
Supporting figure 1. Binomial distribution of expected number of events (mutations) according to MET mutational frequency reported for unselected primary cancers (4%, COSMIC Database). Expected probability of the occurrence of seven events (mutations) in forty-seven trials (analyzed population) would be of 0.00091. The observed MET mutational frequency in the early metastatic cancer cohort is actually extremely higher, and is further increased in the CUPs onset.



15	2.95E-11
14	3.74E-10
13	4.3E-09
12	4.46E-08
11	4.15E-07
10	3.44E-06
9	2.53E-05
8	0.000163
7	0.00091
6	0.004338
5	0.0173
4	0.056161
3	0.142535
2	0.265285
1	0.322008
0	0.191271

Supporting figure 2. Analysis of patients survivals by MET status

Survivals (expressed in months) of MET wild type vs MET mutated patients have been analyzed and represented as continous variables. For each group box plots summarize the distribution of points at each factor level. The ends of the box are the 25th and 75th quantiles. The difference between the quartiles is the interquartile range. The line across the middle of the box identifies the median sample value. Corresponding quantiles plot and table are also represented.



Quantiles

Level	Minimum	10%	25%	Median	75%	90%	Maximum
MET mut.	5	5	6,5	8	14,75	32	32
MET wt	5	6,8	9	13	19	25,2	61

Supporting table 1. A summary on patients clinical data.

‡ wt denotes MET wild type status.

° n.a denotes data not available, patient withdrawn at follow up

Early Metatstatic Carcinomas

ID	Gender	Age (yrs)	Metastatic site (DNA source)	Number of Metastatic Sites	Primary Origin Site	Histology	Survival (months)	MET Status Normal Tissue	MET Status Tumor
Primary origin subsequently solved by immunohistochemistry									
7	M	82	Brain	2	Lung	Small cell carcinoma	12	wt	wt
8	M	68	Brain	2	Lung	Adenocarcinoma	9	wt	wt
13	M	52	Brain	3	Lung	Adenocarcinoma	11	wt	wt
19	F	63	Brain	2	Lung	Adenocarcinoma	9	wt	wt
40	M	43	Liver	2	Lung	Adenocarcinoma	25	wt	wt
2	M	75	Bone (vertebrae)	2	Liver	Carcinoma	48	wt	wt
43	M	59	Liver	3	Biliary tract	Adenocarcinoma	6	wt	wt
33	F	49	Liver	2	Pancreas	Neuroendocrine carcinoma	14	wt	wt
47	M	64	Liver	2	Pancreas	Adenocarcinoma	5	wt	wt
1	F	70	Bone (vertebrae)	2	Breast	Muciparous carcinoma	14	wt	wt
15	F	48	Brain	2	Breast	Adenoarcinoma	61	wt	wt
17	F	58	Brain	3	Melanoma	Melanoma	7	wt	wt
5	F	63	Brain	2	Lung	Neuroendocrine carcinoma	14	wt	wt
9	M	64	Brain	2	Lung	Adenomcarcinoma	24	wt	wt
16	F	78	Brain	2	Lung	Small cell carcinoma	12	wt	wt
18	F	64	Bone (vertebrae)	2	Lung	Adenocarcinoma	13	wt	wt
21	M	67	Brain	2	Lung	Adenocarcinoma	19	wt	wt
22	M	57	Liver	3	Lung	Adenocarcinoma	19	wt	wt
30	M	77	Liver	2	Lung	Adenocarcinoma	7	wt	wt
23	F	45	Liver	2	Breast	Neuroendocrine carcinoma	15	wt	wt
27	M	66	Liver	3	Stomach	Adenocarcinoma	8	wt	wt
32	M	47	Liver	2	Stomach	Adenocarcinoma	9	wt	wt
34	F	52	Liver	2	Biliary tract	Adenocarcinoma	19	wt	wt
44	F	61	Liver	2	Biliary tract	Adenocarcinoma	11	wt	wt
Total n°		24							
Gender M/F		11/13							
Mean age(yrs)		60							
Unknown Primary Origin (CUP)									
3	M	55	Brain	2	UNDETERMINED	Undifferentiated carcinoma	22	wt	wt
4	M	64	Bone (vertebrae)	2	UNDETERMINED	Undifferentiated carcinoma	18	wt	wt
6	M	67	Brain	3	UNDETERMINED	Undifferentiated carcinoma	8	wt	p.His150Tyr
11	F	45	Brain	4	UNDETERMINED	Undifferentiated carcinoma	7	wt	p.Cys385Tyr
12	M	63	Brain	3	UNDETERMINED	Undifferentiated carcinoma	15	wt	wt
14	M	57	Brain	4	UNDETERMINED	Adenocarcinoma	23	wt	wt
20	M	58	Brain	3	UNDETERMINED	Undifferentiated carcinoma	5	wt	p.Glu168Asp
10	M	50	Brain	2	UNDETERMINED	Undifferentiated carcinoma	9	wt	p.Gln142X
25	F	43	Liver	2	UNDETERMINED	Adenocarcinoma	26	wt	wt
26	F	65	Liver	2	UNDETERMINED	Undifferentiated carcinoma	15	wt	wt
28	M	47	Liver	3	UNDETERMINED	Undifferentiated carcinoma	13	wt	wt
29	M	65	Liver	2	UNDETERMINED	Adenocarcinoma	8	wt	p.Thr1010Ile
31	M	57	Liver	4	UNDETERMINED	Undifferentiated carcinoma	n.a	wt	wt
35	M	67	Liver	2	UNDETERMINED	Undifferentiated carcinoma	8	wt	wt

Supporting table 2. Exons and primers used for mutational analysis for each gene in study. Multiple primer pairs were designed for exons larger than 350 bases as indicated.

Gene				
MET	Exon	Forward primer	Reverse primer	Sequencing primer
	1	GATGCCCGGCTGAGTCAC	GAACAGCAGTCAGGTCTCTTAGG	GTCTCTTAGGGGAACAAAGAAAG
	2-1	GTTGGGAAGCTTTATTTCTGATAG	TCCGACTGTATGTCAGCAGTATG	AGCAGTATGATTGTGGGGAAAG
	2-2	GGTGTTTGGAAAGATAACATCAAC	GGAACAGAACCTGATTATTCTTGTG	GAAAGATAACATCAACATGGCTC
	2-3	GTTCAGAGATTCTTACCCCATTAAG	AAGCACAAAAGAAGCCCTGG	CCATTAAGTATGTCCATGCCTTTG
	3	CTCCAGGCTCTGAAAATACACAC	AAACGAAAACAAATTAATTGCAC	CACACTGAAAGGTTTCTTACCAG
	4	ATTGATTTACAATGAGGGGAACT	GAGGAGGGGTAAATTTAAGATGA	AGCCCTGCTAATCTGTTATTACC
	5	ACCGTTATGACAGGATTTGCAC	CCAAGAGAGAGATGGTGCTTTAG	AAAAACAAACCAAACCAAACC
	6	ATTGTGGGAAAATGAAAGAATTT	GATGAGCTTCTTGAGCAATTTTA	TACATTTTGTTTGTTCGTTTTCC
	7	TCTTGAAACTCTGCTTGCTATTC	AGGGGAGATAAAAACAAAACAAA	AAACAACCTAACCAGAAAATTCC
	8	CGGGACAACACAATACAGTACAT	CATCTTGATTTTTCTTCTCCACA	ACACACACACACAAAACAATCTG
	9	CACTTAGGAACCATTGAGTTATATCC	ATTCTACAGCAAAATCATCCTTG	TCCTTTTGATTTGTGGATATAATTC
	10	AACTTCCATTTGATGTTGACTGT	TGCAAGGAAATTAACTAGCAAAC	CATCTTTGCCCTCTAACCATGTG
	11	TGTATCGTGTTTCCAGAAATGTG	TGCAGTCCACGGTTATTCTTATC	ATTGTTGTACTTGGCCATTGTAT
	12	GGCCTGTGTTTGCAGTATATTT	AAACCCACACATAAATGAGCTTT	TATATTCCTTTGCCATTGTTAGC
	13	CCAAAGTGCTACAACCTGTGT	TATAAGACAGCACACAAGAATCG	CGACGACAATCTTAAACTGTAATG
	14	TTAAGATTGTCGTCGATTCTTGT	AGCCATGTAATTTTGTGTCAAAT	TCACAACCCACTGAGGTATATGT
	15	TAAAAGCTCTTCCTGTTTCAGTC	CACTTGTTATCACTGCTCTGTCA	TTGCTTTCACCATTGTCTAAGTT
	16	TGAAGCTCATAAAGGGTTTGATA	AAAACAAATTTTCAGGATTAGGC	CCATAATTTCAGTGGTAGCTGAT
	17	ACAAGATGCTAACTGTGTGGTTT	TTAAATGTGCATCTTTGGCTACT	TTACCATTTCATTGCTCTTCCTA
	18	CTTGAGCCATTAAGACCAAACTA	TTTGCATAAGAAGAGAAAACAGC	GCTTAACTAGCATTGAACAGTGG
	19	TCCTTCAGAAGTTATGGATTTCA	TATGAAGAAAACTGGAATTGGTG	TTCAAATACTGAAGCCACTTGTT
	20	AGCCAAGTTTAGTTACCAAGACC	CCAGCATTTTAGCATTACTTCAT	CCAAAAAGAAAGACATGCTGTAA
	21	GACCCCTTGTAAGTAGTCTTTCTG	AATAGTGCAATTTTGGCAAGAG	CTTTTAAAGGTCAGGCAGTGAA
RON	1-1	ATCCTCTAGGGTCCCAGCTC	CAGGCTCTGGACAGACTTCA	ACTTCAGGTCAGGCCCAAG
	1-2	CTACGAGGGCGACAGAAATG	TATGGTGGGCTGAGAAGAGG	GAGGGCGACAGAAATGAGAG
	1-3	TGCTTCCTGCATGACCTAGA	CTTGGGCAGCACTGACAAC	GCACTGACAACGCCACAA
	1-4	AGCCCACGCTCAGTGTCTAT	GCTCAGCTCAGTGGCAAGTT	AGCACAGGGTAGGGCTGTC
	1-5	TGGTCCTCGACTGCAGATTT	ACTGGGCGACTGGAAGAAGT	TCAATTAGTGTGTCCAGCAGGT
	1-6	GGTCTTTGTGACTGGCAAGG	CCTCAGTGATGGAAGGGAAG	CCCCAACTCTGTCGTCTGTG
	2	CACAAGGCTGAACCCTGACT	CTAGGGGACTGTGGGGATG	CTTCCCTTCCCTGACCTTTC
	3	GTCCTCATCCCCACAGTCC	ATGTCAATGCCTCCCTGGAT	TCTCCATCCCCATTTTGCT
	4	GCCCTACCACCCTAGCCTAC	CTGCCAGGGAAGCCATAC	CCCTAGCCTACTGTGTACCG
	5	AGGTGGGGCTGAGAGAGC	AGGGGAGGGACCAGATTGTA	CACATGAGGACTCAGGCTGT
	6	GAGGCATGGGTGGAGAAAT	GCTTAGGCAGGTCCTCCAC	AAATGCCATTCTCTGGCTCA
	7	GCATGCATCTAGGCCTGTGT	GCACAGACAGGGCAAGGTAG	CCTGTGTAATTCCTGGCTGA
	8	TGGCCATCACAAAGAGGGTA	CTTTGGGTGTTCTCCAAAGC	TGAGCCCAATCTCTTGTTCC
	9	ATGAGAGAGCCAGCTTTGGA	CTGCAGCTCAGGGAACTCAT	GAGCCAGCTTTGGAGAACAC
	10	GGAGGAAGGCTGGATGAGTT	GTAGGCTGGCCCCTACTTTC	GGGGTTCCTGGCTAATCACT
	11	ACCCAGTGCCAACCTAGTTC	CCCAGGATATGACATTCACCA	CATTCACCATCTACGCAGACC
	12	GGACCTCCCTGGGAAACAC	GGGTAGGGGCTGATTAAAGG	CTACAGGCTGGGCCTGAGTT
	13	GGATCTAAGTCCTCCCCAGAG	GGAGAGGGAATGAGGAGCTT	CAAGCCACCAAGGATTCTCT
	14	CCACACCCCTGCCTATTCT	TTTCACTGTGTTAGCCAGGAC	GATAGTGGAGGCATGAGAAGC
	15	TCTGAGGTCTGGTTGCCAAT	CTCTGCCTTCCCATCTTCTG	GCCAATGAGAATGTGTGGTG
	16	CCTGAGCAGGAGACCTTCAT	ACCCAGAGCCAGATGAACAC	CTGCCCTGTGCCTGACTT
	17	CAGTGTTCATCTGGCTCTGG	AGCACCGCACACCCTCAT	TGCTTCACCCCAGCTACTCT
	18	TGGGTTCAGGGCCTGGTA	AAGCCATGTGGACTGTAGGG	AGGGCCAGTCCTAAGTGTGA
	19	ATTGGGGGTGAGTTGAGTCC	GCTCTGTCTCCTGTCTCTTCCA	AGTTCCCTCCCCACTACCAC
	20-1	CAGAATCCTTGGGTGGAAAT	TGGACGCACATTCATCTCAT	CTTGGGTGGAAATTGCCTTA
	20-2	GGGAGGTGGAGCAGATAGTG	CTCAAGGCAGCTAAGCAGGT	CACTGCTTGGGGACCATTAT
PIK3CA	9	GGGAAAAATATGACAAAGAAAGC	CTGAGATCAGCCAAATTCAGTT	TAGCTAGAGACAATGAATTAAGGGAAA
	20	CTCAATGATGCTTGGCTCTG	TGGAATCCAGAGTGAGCTTTC	TTGATGACATTGCATACATTCG
EGFR	18	TGATCTGTCCCTCACAGCAG	TCAGGAAAATGCTGGCTGAC	TTCAGGGCATGAACTACTTGG
	19	GCTGAGGTGACCCTTGTCTC	ACAGCTTGCAAGGACTCTGG	TGGAGCCTCTTACACCCAGT
	20	CCCAGTGTCCCTCACCTTC	CCACACAGCAAAGCAGAAAC	GCTGGTAACATCCACCCAGA
	21	CCCTGTGCTAGGTCTTTTGC	AAAGGAATGTGTGTGTGCTG	CATTCATGCGTCTTCACCTG
KRAS	2	GGTGGAGTATTTGATAGTGTATTAACC	AGAATGGTCCTGCACCAGTAA	TCATTATTTTTATTATAAGGCCTGCTG