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Ciclo XXIV

TITOLO TESI

RESPONSE TO TREATMENT WITH CHEMICAL AND BIOLOGICAL INHIBITORS OF c-MET MUTATED FORMS

Settore/i scientifico disciplinari di afferenza

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Presentata da: Dr. MARIA MADDALENA ANGIONI

Coordinatore Dottorato Prof. GAETANO DI CHIARA

Tutor/Relatore Prof. AMEDEO COLUMBANO
Prof. SILVIA GIORDANO

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ABSTRACT

c-MET is a receptor tyrosine kinase that, after binding with its ligand, hepatocyte growth factor (HGF), activates many signaling pathways, driving proliferation, motility, migration and invasion. Although c-MET is important in the control of tissue homeostasis under normal physiological conditions, it has also been found to be aberrantly activated in human cancers via mutation, amplification or protein overexpression. Activating point mutations were identified in the kinase domain of MET, either in the germline of patients affected by hereditary papillary renal carcinoma (HPRC) or in spontaneously occurring tumors; in particular, nine missense mutations (defined MET^{PRC} mutations), leading to constitutive activation of MET protein, have been identified in HPRC families. Given the importance of MET as a target for cancer therapies, clinical trials aimed at inhibiting it through the use of tyrosine kinase inhibitors (TKIs) have recently been started.

The aim of project was: (i) to evaluate if MET^{PRC} mutants are sensitive to PHA-665752 (a small kinase inhibitor of MET), (ii) if some mutants are insensitive to the inhibitor, to investigate the mechanisms responsible for resistance, (iii) to check if the resistant mutants are still sensitive to other chemicals inhibitors or monoclonal antibodies against MET, (iv) to identify activating point mutations in human surgically resected lung cancers.

We have found that some MET^{PRC} mutants cannot be inhibited by PHA-665752. Treatment with this TKI does not alter either receptor phosphorylation or MET mutants-induced biological activities (migration, invasion, anchorage-independent growth). We showed that these mutants are insensitive also to JNJ-38877605, a

multitargeted tyrosine kinase inhibitor.. When we performed the mutational analysis on lung cancer samples, in one tumor we found the presence of one of the identified“resistant” mutations.

To determine whether the mutants resistant to PHA-665752 could be inhibited with other strategies, we treated the mutant-expressing cells with the monoclonal antibody DN30, directed against the extracellular portion of the receptor. Our results showed that DN30 was indeed able to inhibit all the MET^{PRC} mutants.

In conclusion, we have identified some MET^{PRC} mutants which do not respond to the ATP competitive kinase inhibitors. Since the identified MET^{PRC} mutations are located in the kinase domain and alter its conformation; it is likely that the competitive inhibitors are unable to interact with the ATP binding site in the context of the mutated receptors; this would render these mutants "resistant" to the action of tyrosine kinase inhibitors. However, these mutated forms still remain responsive to treatment antibodies directed against the MET extracellular portion: This observation is important since the use of monoclonal antibodies represent a therapeutic alternative for patients with tumors carrying MET mutants resistant to TKIs.

INTRODUCTION

The MET (HGF receptor) tyrosine kinase.

The MET gene, discovered as an oncogene more than two decades ago (Trusolino,L. and Comoglio,P.M., 2002; Cooper,C.S. et al. 1984), encodes for a tyrosine kinase receptor that binds to, and is activated by, the growth and motility factor HGF (also called Scatter Factor 1). The MET receptor is a disulphide-linked heterodimer composed of an extracellular 50-kDa α chain and transmembrane 145-kDa β chain. The extracellular moiety contains a conserved "Sema" domain of 500 amino acids, known to be a protein-protein interaction domain, and a cystein-rich motif of 80 amino acids, called MET related sequence (MRS). The intracellular portion of the receptor can be divided into three functional domains: i) a juxtamembrane domain, playing an inhibitory function through a serine residue (S985) phosphorylated by protein kinase C or Ca2+/calmodulin-dependent kinases (Gandino,L. et al. 1994) and a tyrosine residue (Y1003) that, upon phosphorylation, binds to the E3 ubiquitin ligase Cbl which promotes receptor ubiquitinylation, endocytosis and degradation; ii) the tyrosine kinase catalytic domain, that contains the major phosphorylation site represented by the tyrosine residues 1234 and 1235, whose activity is induced in an autocatalytic fashion by receptor trans-phosphorylation (Naldini,L. et al. 1991); and iii) a carboxi-terminal tail with a unique docking site responsible for the recruitment of a wide spectrum of downstream signaling molecules; among them are the phosphatidylinositol 3-kinase (PI3K), the GRB2/SOS complex, the non-receptor tyrosine kinase Src, the transcription factor STAT3 and the adaptors Shc and Gab-1, that provide additional docking sites for many signaling molecules.

All the structural features described so far define a receptor family (the "Scatter Factor Receptor Family") comprising, besides MET, also Ron (Macrophage Stimulating Protein Receptor) and its chicken orthologue Sea.

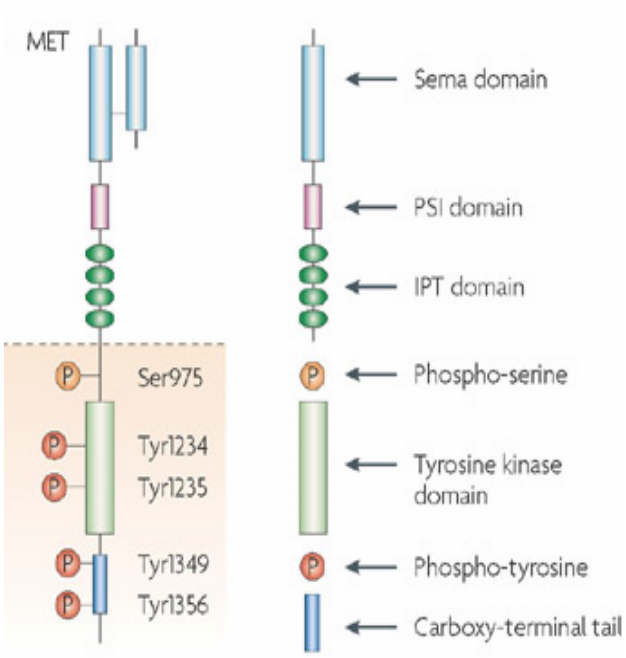


Fig 1.1

MET is a single-pass, disulphide-linked α/β heterodimer that is formed by proteolytic processing of a common precursor in the post-Golgi compartment. The extracellular portion of MET is composed of three domain types. The N-terminal 500 residues fold into a large Sema domain, which encompasses the whole α-subunit and part of the β-subunit, and shares sequence homology with domains found in the semaphorin and plexin families. The Sema domain is followed by a PSI domain – also found in plexins, semaphorins and integrins,– that spans about 50 residues and contains four disulphide bonds. The PSI domain is connected to the transmembrane helix via four IPT domains, which are related to immunoglobulin-like domains and are named after their presence in plexins and transcription factors. The intracellular portion includes the tyrosine kinase catalytic site flanked by distinctive juxtamembrane and carboxy-terminal sequences. Phosphorylation of Tyr1234 and Tyr1235 within the catalytic site positively modulates the enzymatic activity, whereas phosphorylation of Ser975 in the juxtamembrane segment downregulates the kinase activity. The carboxy-terminal tail includes two critical tyrosines (Tyr1349 and Tyr1356) that, once phosphorylated, act as a promiscuous docking site for the recruitment of several transducers and adaptors.

[**Adapted from the following article:** Comoglio, PM., Giordano, S. Trusolino, L. (2008) Drug development of MET inhibitors: targeting oncogene addiction and expedience. Nat Rev Drug Discov. 7:504-16.]

The MET ligand: Hepatocyte Growth Factor (HGF).

Hepatocyte growth factor is the ligand of the MET tyrosine kinase. This growth factor was independently discovered by two groups who identified it through different experimental approaches: the first one characterized it as a potent motility factor and thus named it as "scatter factor"; the second one identified the factor for its ability to promote growth of hepatocytes, hence the name of hepatocyte growth factor (Naldini,L. et al., 1991; Uehara, Y. et al., 1995). These two factors, later, turned out to be the same molecule (Schmidt, C. et al., 1995; Huh, C. G. et al., 2004), which is usually known as HGF.

HGF is secreted by mesenchymal cells and acts mainly on cells of epithelial origin. It is secreted as a single inactive polypeptide and it is cleaved by serine proteases into a 69-kDa alpha-chain and a 34-kDa beta-chain. A disulfide bond between the alpha and beta chains is present in the active, heterodimeric molecule. The protein belongs to the plasminogen subfamily of S1 peptidases but it has no detectable protease activity.

MET and the Invasive Growth program.

MET activation evokes pleiotropic biological responses, both *in vitro* and *in vivo*, often referred to as "*invasive growth*". This is a complex genetic program, specifically induced by the Scatter Factor Receptors MET and Ron. It consists in a series of obligate rate-limiting steps physiologically occurring during embryogenesis and tissue repairing. In the first step of this process, cells acquire the ability to dissociate from their neighbors, by breaking intercellular adherent junctions ("*scattering*") and then leave their original environment and reach the circulation ("*directional migration*" and "*invasion*"). Cell survival in the bloodstream is facilitated by MET-induced protection from apoptosis and ability to transiently grow in an anchorage-independent

manner. Finally, cells extravasate, face the new environment, proliferate and eventually undergo terminal differentiation (Giordano, S. et al., 1993).

In vivo, MET is expressed in epithelial cells of many organs (Sonnenberg,E. et al., 1993) ; under physiologic conditions MET contributes to the establishment of normal tissue patterning and to the onset and persistence of normal organ architecture. In fact, during embryogenesis, the *invasive growth* process is an essential step that ensures the correct structural tissue organization; in adulthood , when the architectural tissue organization is already well established, *MET* activity becomes dispensable but it is still required when tissues are damaged and cells have to reacquire the ability to dissociate, migrate and repair the regenerating tissues (Uehara, Y. et al., 1995; Schmidt, C. et al., 1995; Huh, C. G. et al., 2004).

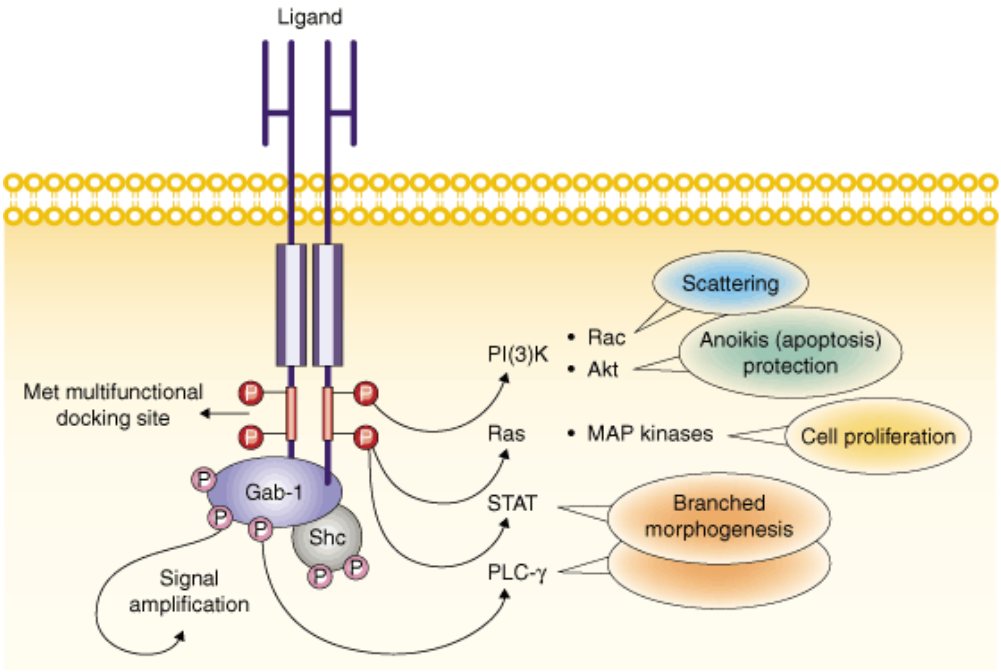


Fig 1.2

After ligand-induced dimerization, the tyrosine kinase domain (purple) phosphorylates two key tyrosines included in a specific four-residue sequence (red) in the receptor cytoplasmic tail. These generate a docking site with specificity for the indicated signal transducers. The specificity of the biological response (invasive growth) results from the receptor's individual signaling profile originated by combining the nature of the pathways with their intensity, duration and synchrony. Moreover, the receptor phosphorylates signal amplifiers at multiple sites, lowering the threshold for the response.

[**From the following article:** Comoglio, PM. (2001) Pathway specificity for Met signalling. Nat Cell Biol. 3:E161-2.]

MET and Cancer.

In transformed tissues, deregulation of the invasive growth program is responsible for cancer progression and metastasis. Constitutive MET activation forces neoplastic cells to disaggregate from the tumor mass, erode basement membranes, infiltrate stromal matrices, and eventually colonize new territories to form metastases, somehow recapitulating the physiological *invasive growth* program (Trusolino,L. and Comoglio,P.M., 2002).

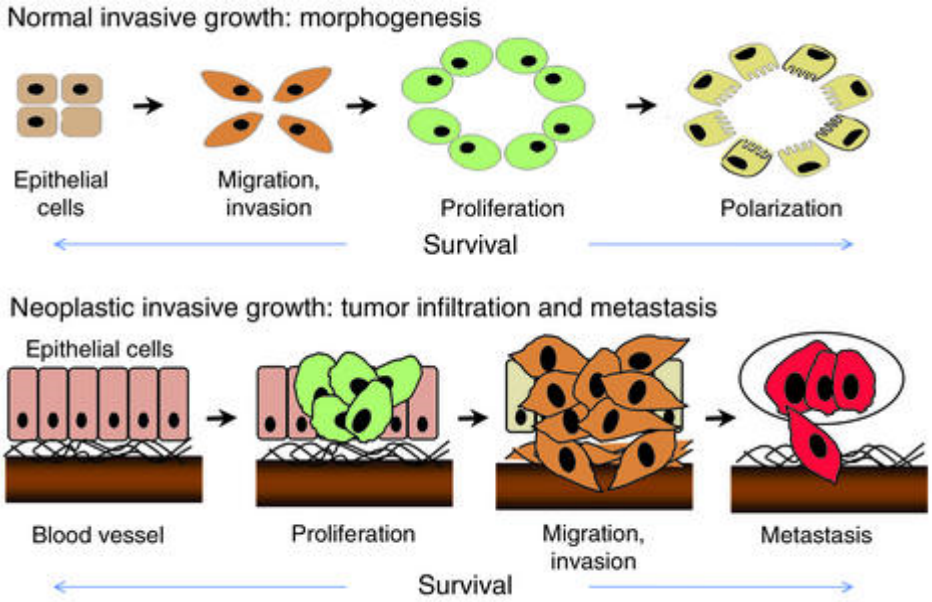


Fig 1.3

The invasive growth program under physiological and pathological conditions. In both settings, invasive growth results from analogous biological processes - cell-cell dissociation and migration, cell proliferation and survival - but the endpoints are different. Normal cells exploit invasive growth to colonize new territories and build polarized three-dimensional structures, thus forming the parenchymal architecture of several organs. Cancer cells implement this program aberrantly to infiltrate the adjacent surroundings and form metastases.

[From the following article: Comoglio, P.M. and Trusolino, L. (2002) Series Introduction: Invasive growth: from development to metastasis. J Clin Invest. 109: 857–862.]

Indeed, data produced by many laboratories provide compelling evidence that HGF-MET signaling plays an important role in the development and malignant progression of tumors. First, cell lines that ectopically overexpress MET or HGF become tumorigenic and metastatic in nude mice, while MET down-regulation decreases their tumorigenic potential; second, MET or HGF transgenic mice (Wang,R. et al., 2001; Takayama,H. et al., 1997) develop metastatic tumors; third, aberrant MET expression (usually overexpression) has been found in many kinds of solid tumors and correlates

with poor prognosis (Birchmeier,C. et al., 2003). Furthermore, MET signaling is involved in the regulation of tumor angiogenesis, either directly, through the pro-angiogenic activity of HGF that induces the formation of new vessels and the sprouting of the pre-existing ones, or indirectly, through the regulated secretion of angiogenic factors, such as VEGFA (Gille, J., et al. 1998), interleukin-8 (IL-8) and trombospondin-1 (Rosen, E.M. et al., 1993; Zhang, Y.W. et. al, 2003). Moreover, not only endothelial cells, but also macrophages (Galimi, F. et al., 2001) and other leucocytes (Skibinski, G., 2003) express MET and it has been shown that activation of this receptor in these cells can contribute to tumor growth and metastasis formation. Several experimental evidences have indeed demonstrated that therapeutic MET targeting also impairs the function of inflammatory cells, interfering with the pro-tumorigenic role of the tumor microenvironment (Zhang, Y. W. et.al, 2003).

Nowadays, several mutations were founded in the MET gene (Fig 1.4).

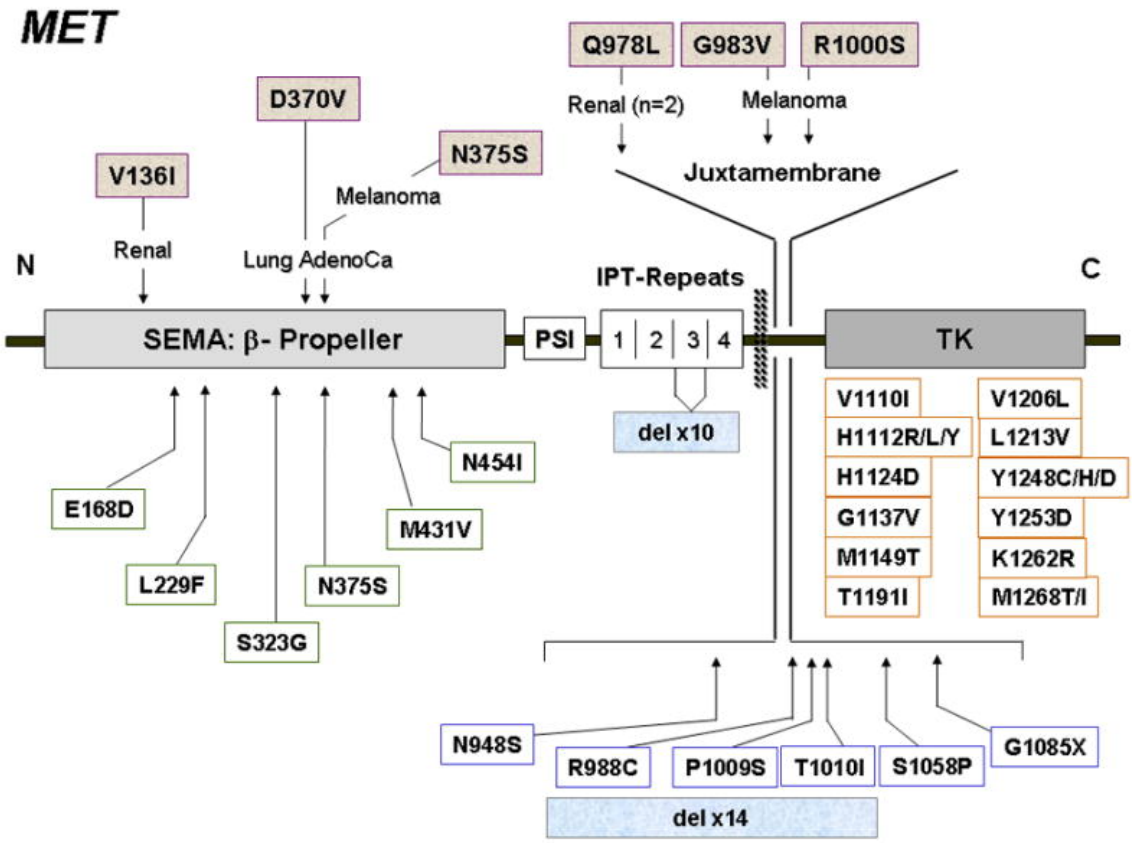


Fig 1.4

Mutations of MET in human solid tumors. MET receptor is shown in the schematic diagram highlighting different functional domains of the receptor: extracellular semaphorin (Sema) domain, PSI domain, the four IPT-repeats, transmembrane (TM) domain, juxtamembrane (JM) domain and cytoplasmic tyrosine kinase (TK) domain. The MET mutations identified in different human solid cancers are represented in the top. Summary of various mutations of MET previously reported in human solid cancers, including renal cell carcinomas (both sporadic and hereditary), gastric carcinoma, hepatocellular carcinoma, glioma, squamous cell carcinoma of the head and neck, SCLC, NSCLC, mesothelioma and melanoma, are shown in the bottom for comparison.

[**From:** Ma PC, et al. (2008) Expression and mutational analysis of MET in human solid cancers. Genes Chromosomes Cancer. 47:1025-37.]

The main proof that MET is directly involved in tumorigenesis came from the identification of germ-line activating mutations in patients with hereditary papillary renal carcinoma (HPRC) (Schmidt, L. et al., 1997).

Schmidt et al. sequencing the MET gene from affected members of HPRC families and from tumor samples of patients with sporadic papillary carcinoma, identified nine different mutations (referred to as MET^{PRC} mutations) that result in amino acid substitutions in the kinase domain of the receptor. Three of these mutations (D1228N, D1228H, and M1250T) are located in codons homologous to those mutated in the tyrosine kinase receptors *Kit* and *Ret*. Mutated *Kit* alleles are found in patients with mastocytosis and acute myeloid leukemia of M2 subtype (Piao, X., Bernstein, A., 1996; Beghini, A. et al., 1998) and missense mutations in *Ret* are associated with multiple endocrine neoplasia type 2B (MEN2B) (Hofstra, R. M. et al., 1994). This suggests that alteration of these residues is a critical event in deregulating tyrosine kinase receptors.

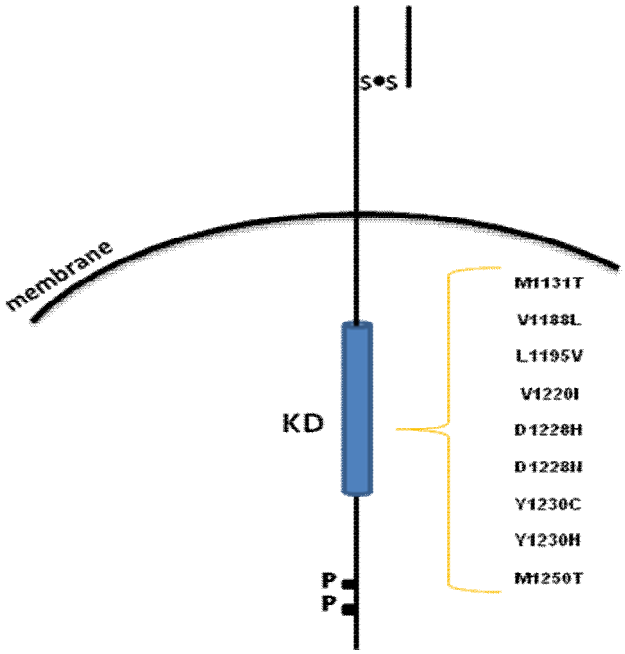


Fig 1.5

Schematic picture of the MET receptor structure. In blue is represented the MET intracellular tyrosine kinase domain, and the two tyrosines in the C-terminal region -that are required for interactions with a number of effector proteins- were indicated (P). The orange brace bracket recapitulate the list of MET^{PRC} mutations of the kinase domain (KD) found in HPRC. KD: kinase domain; P: phosphorylation site; S▪S: disulfide bond.

Mechanisms of MET activation in cancer.

MET activation in human tumors can be induced through different mechanisms:

1) *MET overexpression.*

Overexpression is the most frequent alteration in human tumors. Overexpressed receptors undergo spontaneous dimerization and subsequent activation, even in the absence of ligand. Increased MET expression can be due to: i) MET gene amplification, most common in colorectal tumors (Birchmeier et al., 2003), in 21% of lung cancers become resistant to EGFR inhibitors (Bean et al., 2007), gastric cancers, where 10–20% of all primary tumors and up to 40% of the scirrhous histological subtype have increased MET gene copy numbers (Sakakura et al., 1999; Kuniyasu et al., 1992).

Recently, it has been shown that gastric and lung cancer cell lines harboring amplification of the MET locus are addicted to the constitutive activity of this receptor for their growth (Corso et al., 2008; Lutterbach et al., 2007; Smolen et al., 2006). (ii) Enhanced MET transcription, induced by other oncogenes, such as Ras, Ret and Ets or transcription factors such as MACC1 (Stein,U. et al., 2009). (iii) Hypoxia-activated transcription, leading to higher amounts of receptor that hypersensitize the cells to HGF and promote tumor invasion (Pennacchietti,S. et al. 2003), (iv) loss of negative regulators, like microRNAs (miRNAs), is It is known that miRNAs play a role in human cancers where they can act either as oncogenes, down-regulating tumor suppressor genes, or as onco-suppressors, targeting molecules critically involved in promotion of tumor growth. Regarding the interplay between MET and miRNAs, Migliore C. et al. have identified three miRNAs (miR-34b, miR-34c, and miR-199a*) that negatively regulate MET expression (Migliore et al., 2008).

2) *MET structural alterations*.

Sequencing the MET gene from affected members of HPRC families led to the identification of missense point mutations in the receptor kinase domain that have been shown to be responsible for this hereditary form of cancer. The same and other point mutations in the intracellular portion of the receptor have been identified also in sporadic occurring tumors, like childhood hepatocellular carcinomas, sporadic papillary renal carcinomas, gastric carcinomas (Birchmeier, C. et al., 2003) and head and neck squamous cell carcinomas. In this last case, it has been shown that these mutations are selected during the metastatic spread and confer invasive properties to expressing cells (Nilkovitch-Miagkova, A. and Zbar, B., 2002). *In vitro* studies have proven what has been observed in human cancers (Soman, N.R. et al., 1991).

MET activation can originate also from abnormal post-translational processing, as shown in cell lines with a defect in furine activity. In fact, lack of cleavage of the single chain MET precursor and failure to originate the two-chains mature heterodimer result in a constitutively active molecule (Nilkovitch-Miagkova,A. and Zbar,B., 2002).

Increased MET activation can also derive from impaired receptor down-regulation. Mutations that prevent binding of the Cbl ubiquitin ligase, responsible for MET ubiquitinylation and endocytosis, lead to increased amount of receptors expressed at the cell surface and to enhanced signal transduction (Trusolino,L. and Comoglio,P.M., 2002). Finally, naturally truncated and active MET receptors have been detected in malignant human muscoloskeletal tumors (Nilkovitch-Miagkova,A. and Zbar,B., 2002).

3) HGF-dependent paracrine/autocrine activation.

MET can be activated by its ligand, HGF, either in a paracrine or an autocrine manner. Autocrine activation occurs when tumor cells aberrantly express both HGF and its receptor, as shown in osteosarcomas and rhabdomyosarcomas, gliomas and carcinomas of thyroid, breast, and lung (Birchmeier,C. et al., 2003).

4)HGF-independent mechanisms.

MET activation can happen in a HGF-independent manner through its transactivation via other membrane receptors, including adhesive receptors, like CD44 and integrins (Trusolino, L., et al. 2001; Van, de Wetering et al. 1999), and signal transducing receptors, such as Ron, EGF receptor family members, FAS and B Plexins (Kruger, R. P., et al., 2005; Giordano, S. et al., 2002). It is interesting to note that all these receptors are individually believed to be involved in cancer progression.

On these bases, MET is considered an important target in anti-cancer therapy with a possible anti-metastatic potential.

Targeting MET.

In recent times, molecules targeting MET reached the access to clinical trials. Most of them are small molecules tyrosine kinase inhibitors, while few are biological antagonists and monoclonal antibodies targeting either the ligand or the receptor (Fig 1.6).

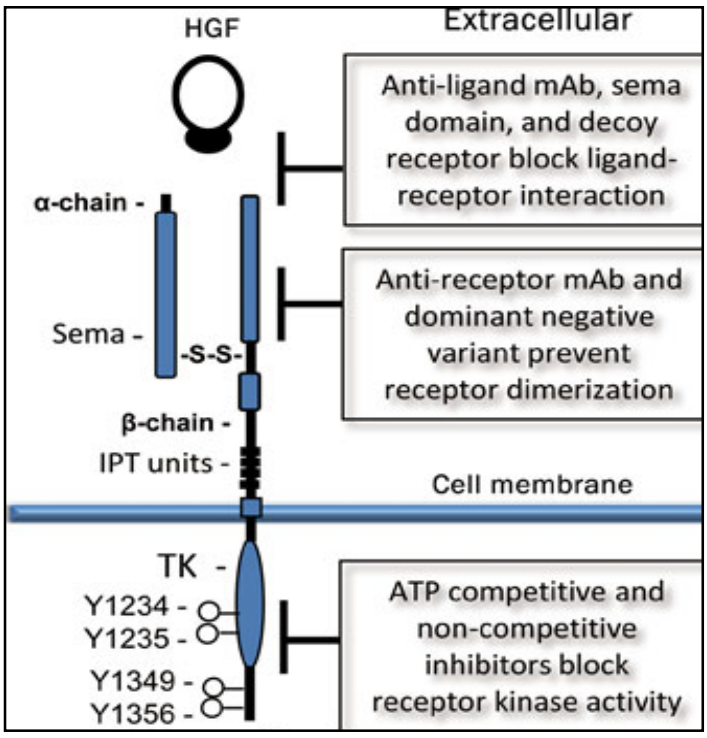


Fig 1.6

Schematic representation of the structures of human c-MET and its potential signaling inhibition using various candidate therapeutic agents.

[**Adapted from:** Wang MH, et al. (2010) Potential therapeutics specific to c-MET/RON receptor tyrosine kinases for molecular targeting in cancer therapy. Acta Pharmacol Sin. 31:1181-8.]

1) MET/HGF competitors.

The first attempts to interfere with cancer progression by targeting the HGF/MET system came in the late 1990s and aimed at interfering with HGF binding to MET, through the use of antagonistic compounds ('competitors').

One of the most promising competitors is NK4, a variant of HGF comprising only the four-kringles of the α chain; NK4 binds to MET without inducing receptor activation and thus behaves as a full antagonist (Date, K., et al., 1997). Its major limit relies in its inability to interfere with the development of tumors in which MET is activated in a HGF-independent manner.

A chimeric factor containing selected domains of HGF and MSP and able to signal through MET/Ron heterodimers was proven to be able to dissociate the trophic properties of HGF, such as proliferation and protection against apoptosis, from its pro-invasive ability. This opened the possibility of exploiting some 'favorable' effects of HGF to reduce, for example, chemotherapy-related cytotoxicity (Michieli, P. et al., 2002).

Recently, it has been shown that a mutated and uncleavable form of pro-HGF (HGF is first produced as a single, inactive precursor that is then cleaved in the two-chains mature form) obtained through a single amino acid substitution that prevents the cleavage of the single chain precursor in the mature form, can both displace HGF from MET and competitively inhibit the proteolytic activation of the endogenous pro-HGF (Mazzone, M. et al., 2004).

Another molecule with therapeutic potential is a soluble form of the MET extracellular portion (the so-called decoy-MET), a recombinant protein corresponding to the entire extracellular domain of MET. This molecule acts both on MET and HGF, as it blocks receptor dimerization and sequesters the circulating HGF (Michieli, P. et al., 2004).

2) Monoclonal antibodies.

Monoclonal antibodies are currently used to target other RTKs in cancer and are providing good therapeutic results. The best known examples, already approved by FDA, are Cetuximab (against EGFR) in head and neck and colorectal cancer, Trastuzumab (against HER-2) in locally advanced and metastatic breast cancer and Bevacizumab (against VEGF) in metastatic colon cancer and non-small-lung cancer (Barni, S., et al., 2007; Rocha-Lima, C.M. et al. 2007). It is important to underline that

monoclonal antibodies can interfere with RTKs' activity either targeting the receptor (Cetuximab or Trastuzumab) or the ligand (Bevacizumab).

Compared to the use of TKIs, one advantage of the monoclonal antibodies is the high target specificity. In addition, some antibodies may elicit immune responses such as antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity, which offer different tumor-killing mechanisms in addition to direct inhibition of the target.

Like the c-MET TKIs, the antibodies have also been evaluated preclinically and demonstrated significant antitumor activity in HGF/c-MET-dependent tumor models, with good tolerability.

Concerning the MET–HGF system, promising results have been obtained both with anti-HGF (one of them has just entered the first phase II clinical trial), and anti-MET antibodies.

Recently, a panel of fully human monoclonal antibodies that bind to and neutralize human HGF has been developed (Burgess, T. et al., 2006). Kim and colleagues identified another monoclonal antibody, L2G7, active not only *in vitro*, but also able to interfere with tumor growth and to induce tumor regression in mouse models (Kim, K. J. et al. 2006).

The potential use of mAbs targeting MET in human cancer therapy induced the production of a growing number of these molecules. As bivalent antibodies exhibited both agonistic and antagonistic activity towards the receptor, allowing a partial activation of MET downstream pathways (Christensen, J. G., et al. 2005), one monovalent antibody (Fab), named 5D5, was engineered to inhibit HGF-dependent MET activation (Martens, T. et al., 2006).

Finally, our group identified a mAb, named DN30, that efficiently down-regulates MET receptor. This specific mAb exploits its effect in inhibiting HGFR signaling by a dual mechanism: on one hand it reduces the number of receptor molecules on the cell surface; on the other hand it promotes the release of a decoy HGFR (Petrelli, A. et al., 2006) which is endowed with a dominant negative activity. Another important observation is that the inhibitory mechanism activated by this mAb does not require HGFR tyrosine kinase activity. This feature represents a relevant advantage in the perspective of a therapeutic approach, because, in clinical practice, it is frequent to combine different drugs to improve the effect on the target molecule. In the case of HGFR, it would thus be possible to combine kinase inhibitors with the mAb, allowing the contemporary action on both HGFR activation and receptor levels; this is likely to enhance the therapeutic efficacy of target therapy in HGFR-overexpressing tumors, with the aim of interfering with both tumor growth and the acquisition of an invasive–metastatic phenotype.

3) Small molecules.

From a pharmacological point of view, the most promising tools for cancer therapy are believed to be the competitors for the ATP binding site of the receptor, the so-called 'small kinase inhibitors' or 'small molecules'. The reason for the increasing interest in the development of these compounds is their good efficacy in clinics (for example Gleevec, targeting c-kit and BCR-ABL, Iressa and Tarceva, targeting EGFR and Sorafenib, targeting several RTKs) and to their ability to inhibit receptor activation due not only to ligand binding but also to over-expression or interaction with co-receptors. This last issue is of particular interest dealing with MET, as activation due to receptor over-expression is quite frequent in human tumors

(Desiderio, M. A., 2007). Moreover, as already discussed, several data strongly suggest that MET cross-talk with other membrane receptors may lead to its activation in the absence of the ligand For all these reasons, it is important to develop compounds able to efficiently switch off MET signal. Despite the potentiality of small molecules in cancer therapy, it must be taken into consideration that if, on one hand, these molecules are very effective and promising, on the other hand, they may create problems for side-effects because ATP analogues are likely not absolutely specific for a given tyrosine kinase, and thus, toxicity is a big concern.

Initial attempts to identify MET-ATP binding site competitors brought to the identification and characterization of K252a, a Staurosporine analogue, behaving as a broad spectrum kinase inhibitor (Morotti, A.,et al., 2002).

Searching for more selective compounds, two new small-molecule inhibitors have been developed: SU11274 and PHA-665752. At nanomolar concentrations, they both strongly inhibit HGF-induced activation of MET in cultured cells and tumorigenicity in mouse models (Berthou, S. et al., 2004; Ma, P. C., et al., 2005).

Recently, Zou and colleagues identified a new small-molecule inhibitor, named PF-2341066: it is an orally available ATP-competitive compound selective for MET. *In vivo*, this compound showed a good tolerability and a dose-dependent anti-tumor activity (Zou, H. Y. et al., 2007).

Agents that showed encouraging clinical benefit as well as acceptable safety profiles in early-stage trials have progressed rapidly in the clinic. These include ARQ197, XL184 and PF-2341066, all of which are currently being evaluated in Phase III trials **(Table 1**).

A novel MET/RON inhibitor (Compound I, from Amgen) has been recently identified and characterized. This molecule specifically inhibits both the receptors belonging to

the same RTK family and prevents the activation of the *invasive growth* program in response to the interaction with the cognate ligands. Compound I showed specific anti-tumor activity in animal models known to be dependent on either MET or Ron activation (Zhang Y, et al., 2007).

Finally, an indirect approach to interfere with MET activity has been achieved using geldanamycin (GA), an anti-tumor drug that binds to and inhibits HSP90 chaperone activity by preventing proper folding and functioning of certain oncoproteins and, amongst them, MET (Xie, Q. et al., 2005).

4) Clinical trials.

Recently, some molecules targeting MET reached the access to clinical trials: they all share low levels of toxicity but further investigations are required to optimise the clinical settings (**Table 1**).

Table 1. c-MET pathway inhibitors in development.

Agent	Developer	Comments
Selective TKIs		
ARQ197	ArQule/Daiichi Sankyo/Kyowa Hakko Kirin	c-MET enzyme K_i: ≈355 nM; cellular IC_{50}: 300 – 1000 nM Phase III (NSCLC), Phase II (CRC, gastric, HCC, germ cell and MiT tumors) Clinical activity: PR and SD reported in lung, neuroendocrine, prostate, testicular, gastric, colorectal, ovarian, pancreatic and renal cancers
JNJ38877605	Johnson & Johnson	c-MET enzyme IC_{50}: ~4 nM; cellular IC_{50}: ~50 nM Phase I (advanced solid tumors): completed
INCB28060	Novartis/Incyte	c-MET enzyme IC_{50}: ≈0.13 nM; cellular IC_{50}: ≈1 nM Phase I (advanced tumors)
EMD1214063	EMD Serono	c-MET enzyme IC_{50}: ~1 nM; cellular IC_{50}: 1 – 6 nM
EMD1204831		c-MET enzyme IC_{50}: ~12 nM; cellular IC_{50}: ~15 nM Phase I (advanced solid tumors)
AMG337	Amgen	c-MET enzyme and cellular IC_{50}: unknown Phase I (advanced solid tumors)
Non-selective TKIs		
PF-2341066 (crizotinib)	Pfizer	c-MET enzyme IC_{50}: ~4 nM; cellular IC_{50}: 11 – 13 nM Also active against ALK: ~2-fold less potent Phase III (ALK-altered NSCLC), Phase I–II (lung,

Table 1. c-MET pathway inhibitors in development.

Agent	Developer	Comments
		ALCL and other tumors) Clinical activity: PR and SD reported in NSCLC and inflammatory myofibroblastic sarcoma patients with ALK rearrangement
XL184 (cabozantinib)	Exelixis	c-MET enzyme IC_{50}: ~1.8 nM; cellular IC_{50}: ~8 nM Also active against VEGFR, RET, KIT, FLT3 and TIE2 Phase III (medullary thyroid cancer), Phase II (glioblastoma/astrocytoma, NSCLC and RDT design in nine tumor types: breast, gastric, NSCLC, ovarian, pancreatic, prostate, SCLC, liver and melanoma) Clinical activity: PR and SD reported in various tumors including thyroid, carcinoid, neuroendocrine, parotid, appendiceal, liver, colorectal, renal, melanoma, mesothelioma, renal, liver, lung, glioblastoma and cutaneous T-cell lymphoma
GSK1363089/ XL880 (foretinib)	GlaxoSmithKline	c-MET enzyme IC_{50}: ~0.4 nM; cellular IC_{50}: ~23 nM Also active against VEGFR, AXL, PDGFR, KIT, FLT3 and TIE2 Phase II (breast, NSCLC, papillary renal, gastric and head and neck), Phase I (liver) Clinical activity: PR and SD reported in thyroid, renal, colorectal, carcinoid, melanoma, nasopharyngeal, urethral, ovarian, mesothelioma and gastric cancers
MGCD265	Methylgene	c-MET enzyme IC_{50}: ~24 nM; cellular IC_{50}: ~40 nM Also active against RON, VEGFR1/2/3 and TIE2 Phase II (NSCLC), Phase I (advanced tumors)
E7050	Eisai	c-MET enzyme IC_{50}: ~14 nM; cellular IC_{50}: 6 – 37 nM Also active against VEGFR2 Phase II (HCC), Phase I (advanced solid tumors)
AMG208	Amgen	c-MET enzyme IC_{50}: ~4 nM; cellular IC_{50}: 10 – 100 nM Also active against RON Phase I (various tumors)
MP470	SuperGen	c-MET enzyme and cellular IC_{50}: unknown Also active against KIT, PDGFR, FLT3, RET and RAD51 Phase I (various tumors): completed Clinical activity: PR and SD reported in lung cancer
BMS-777607	Bristol-Myers Squibb	c-MET enzyme IC_{50}: ~3.9 nM; cellular IC_{50}: 20 – 160 nM Also active against Ron, AXL, TYRO3 and MER Phase I–II (advanced solid tumors): completed
MK-2461	Merck	c-MET enzyme IC_{50}: ~2.5 nM; cellular IC_{50}: 26 – 900 nM Also active against RON, FLT1, 3 and 4, and FGFR1, 2 and 3 Phase I–II (various tumors): completed
Therapeutic Abs		
MetMAb (PRO143966)	Genentech/Roche	Humanized anti-human c-MET monovalent antibody Phase II (NSCLC and triple negative breast cancer) Clinical activity: CR and SD reported in lung, gastric and melanoma cancers

Table 1. c-MET pathway inhibitors in development.

Agent	Developer	Comments
AMG102/(rilotumumab)	Amgen	Humanized anti-human HGF IgG2 Phase II (SCLC, NSCLC, CRC, prostate, glioma, RCC, gastric or esophagogastric junction adenocarcinoma, mesothelioma and gynecologic tumors) Clinical activity: PR and SD reported in glioblastoma and other tumors
AV-299	Aveo	Humanized anti-human HGF antibody Phase II (lung), Phase I (advanced solid tumors, lymphomas and MM)

Mechanisms of resistance to tyrosine kinase inhibitors.

In the field of acquired resistance to kinase inhibitors, three major mechanisms of resistance have begun to emerge: (i) genetic alterations of the target, such as gene amplifications that leads to receptor overexpression and thus render the amount of available drug not sufficient to block the target; (ii) mutations in the target kinase that abrogate the inhibitory action of the drug [e.g., T790M in epidermal growth factor receptor (EGFR) and T315I in ABL]; (iii) activation of signaling pathways that bypass the continued requirement for the original target; (iv) constitutive activation of downstream transducers.

Among the most common mechanisms of resistance, genetic modifications include - but are not limited- to: point mutations, deletion and amplification of genomic areas.

As previously reported, unequivocal evidence that implicates MET in human cancer is provided by the activating mutations that have been discovered in both sporadic and inherited forms of human renal papillary carcinomas (Schmidt, L. et al., 1997). Activating mutations have also been described in sporadic tumors such as childhood hepatocellular carcinomas (Park, W. S. et al., 1999), sporadic papillary renal carcinomas (Schmidt, L. et al., 1997), gastric carcinomas (Lee, J. H. et al., 2000), lung carcinomas (Kong-Beltran, M. et al., 2006) and head and neck squamous cell

carcinomas (Di Renzo, M. F. et al., 2000). The table 2 recapitulate that such mutations, which alter sequences within the kinase domain, have also been found in a large types of cancer and metastatic lesions.

Category	Cancer type	HGF/SF expression	Met expression	Poor prognosis	Mutation of Met	*In vitro* studies	Animal model
Carcinomas	Bladder	Y	Y	Y	N	Y	Y
	Breast	Y (A)	Y	Y	N	Y	Y
	Cervical	N	Y (60%)	Y	N	N	N
	Cholangiocarcinoma	N	N	N	N	N	Y
	Colorectal	Y	Y	N	N	Y	Y
	Oesophageal	N	Y	N	N	N	N
	Gastric	Y	Y (46%)	Y	Y	Y	Y
	Head and neck	Y	Y	Y	Y	Y	N
	Kidney	Y	Y	N	Y	N	N
	Liver	Y	Y	Y	Y	Y	Y
	Lung	Y (A)	Y	Y	N	Y	Y
	Nasopharyngeal	Y	Y	Y	N	Y	N
	Ovarian	N	Y	N	Y	Y	Y
	Pancreas/Gall bladder	Y	Y	N	N	Y	Y
	Prostate	Y	Y	N	N	Y	Y
	Thyroid	Y (A)	Y	Y	N	Y	N
Musculoskeletal sarcomas		Y	Y	N	N	Y	N
	Osteosarcoma	Y	Y	N	N	Y	Y
	Synovial sarcoma	Y	Y	N	N	N	N
	Rhabdomyosarcoma	N	Y	N	N	Y	Y
Soft tissue sarcomas	MFH/Fibrosarcoma	Y	Y	N	N	N	Y
	Leiomyosarcoma	Y	Y	N	N	Y	Y
	Kaposi's sarcoma	Y	Y	N	N	N	Y
Haematopoietic malignancies	Multiple myeloma	Y (~85%)	Y	Y	N	Y	Y
	Lymphomas	Y	Y	N	N	Y	Y
	Adult T-cell leukaemia	N	Y (62%)	N	N	Y	N
	Acute myelogenous leukaemia	Y	N	N	N	Y	N
	Chronic myeloid leukaemia	Y	N	N	N	N	N
Other neoplasms	Glioblastomas/ Astrocytomas	Y (~80%)	Y (100%)	Y	Y	Y	Y
	Melanoma	Y	Y	N	N	Y	Y
	Mesothelioma	Y (~35–80%)	Y (~75–100%)	N	N	Y	N
	Wilms' tumor	Y	Y	N	N	N	N

*A version of this table that includes the full list of references is available in Online table 1 and at www.vai.org/HgfSf-METandcancer. For each cancer type, headings correspond to studies showing: HGF/SF expression in tumour biopsies; Met expression in tumour biopsies; expression of HGF/SF or Met correlating with poor prognosis; sporadic or germline-activating mutations in Met; tumour cells *in vitro* expressing Met or HGF/SF, some with correlation to *in vitro* neoplastic-like activities; and animal models supporting the role of Met and HGF/SF in cancer, including human tumour xenografts in immune-compromised mice, mice with HGF/SF or Met transgenes, or other animal models displaying dependence on HGF/SF or Met in cancer development. A, autocrine; MFH, malignant fibrous histiocytoma; %, percentage of tumours examined that are positive; N, no report; Y, citations available in Online table 1 and at www.vai.org/HgfSf-METandcancer for each entry. For example, citations for Met expression in bladder cancer can be found online (as indicated by Y), but there are no reports (N) of Met mutations in this cancer.

Tab 2
Hepatocyte growth factor/scatter factor, MET and cancer references.

[**From the following article:** Birchmeier, C. et al., (2003) Met, metastasis, motility and more. *Nature Reviews Molecular Cell Biology* 4, 915-925]

THE SCIENTIFIC PROBLEM

AND THE AIM OF THE WORK

Targeted cancer therapies are based on the use of drugs that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. By focusing on molecular and cellular changes that are specific to cancer, targeted cancer therapies may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells. The development of targeted therapies, therefore, requires the identification of good targets: in other words, targets that are known to play a key role in cancer cell growth and survival. In cancers driven by a dominant oncogene, targeted therapies have led to remarkable improvements in response and survival, whereas in others the outcome has been more modest.

Once a target has been identified, a therapy must be developed; most targeted therapies are either small-molecule drugs or monoclonal antibodies. Small-molecule drugs are typically able to diffuse into cells and can act on targets that are found inside the cell. Most monoclonal antibodies cannot penetrate the cell plasma membrane and are directed against targets that are outside the cell or on the cell surface.

Many targeted cancer therapies have been approved for the treatment of specific types of cancer, others are being studied in clinical trials, and many more are in preclinical testing.

Unfortunately, many patient's tumor types are refractory to targeted therapies (intrinsic resistance). Moreover, even if an initial response to targeted therapies is obtained, the vast majority of tumors subsequently become refractory (i.e., acquired resistance) and patients eventually progress. In the majority of cases this is caused by expansion of clones containing mutated forms of the target, which confer insensitivity to the drug.

In addition, multiple factors including pharmacokinetics issues, such as suboptimal drug delivery, further contribute to resistance formation. Loss of target dependence due to the activation of parallel signaling pathways has also been reported as cause for acquired drug insensitivity.

Taking together, recent basic and clinical research is trying to improve the efficacy of targeted therapies by developing new generations of rationally designed targeted agents, and translating this information to the clinic to select patients for appropriate therapy.

However, one key aspect to improve the potential of targeted therapies is, first of all, a better understanding the intrinsic or acquired resistance mechanisms that limit their efficacy.

In this scenario, the aim of my PhD project was to evaluate the activity of some available anti-MET therapies (small molecules and monoclonal antibody) targeting the MET receptor harboring mutations in the kinase domain and, if some mutants were insensitive to the inhibitor, to investigate the mechanisms responsible for resistance. Then, I aimed to evaluate if the mutants resistant to small kinase inhibitors are still sensitive to other chemicals inhibitors or monoclonal antibodies against MET. Finally, since the anti-MET therapies are ongoing in NSCLC patients, I also screened surgically resected lung cancers to identify activating point mutations

RESULTS

Evaluation of sensitivity of MET ^{PRC} mutants to the MET TKI inhibitor PHA-665752

As previously shown, sequencing the MET gene from affected members of HPRC families and from tumor samples of patients with sporadic papillary carcinoma allowed the identification of nine different MET mutations (referred to as MET^{PRC} mutations) that result in amino acid substitutions in the protein. All MET^{PRC} mutations belong to the "gain of function" type and lead to constitutive activation of the protein (see Tab 3.1). This results in constitutive tyrosine phosphorylation of the receptor that can be experimentally used as a read-out of its activation.

MISSENSE MUTATION (AMINOACID SUBSTITUTION)	REFERENCE NUMBER OF THE MUTANT
M1131T	MET ^{PRC}1
V1188L	MET ^{PRC} 2
L1195V	MET ^{PRC} 3
Y1230C	MET ^{PRC} 4
Y1230H	MET ^{PRC} 5
V1220I	MET ^{PRC} 6
D1228H	MET ^{PRC} 7
D1228N	MET ^{PRC} 8
M1250T	MET ^{PRC} 9

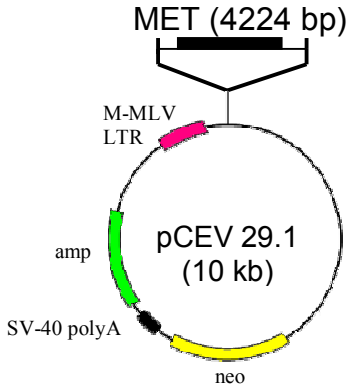
Tab 3.1

Schematic summary of all MET^{PRC} mutants: aminoacid substitution and corresponding reference number of the mutant (chosen to make the reading easier).

To evaluate if MET^{PRC} mutants are sensitive or resistant to the MET small kinase inhibitor PHA-665752, we first transiently transfected MET wt and all the MET^{PRC} mutants in the COS-7 cell line**,** derived from the kidney of African green monkey.

This cell line is a fibroblast-like cell line established from CV-1 simian cells which were transformed by an origin-defective mutant of SV40 encoding for wild-type T-antigen. This cell line is suitable for high efficient transient transfection (Gluzman, Y., 1981). All the mutants cloned in the plasmidic mammalian expression vector pCEV 29.1 were available in the laboratory (Giordano, S., et al. 2000).

A



B

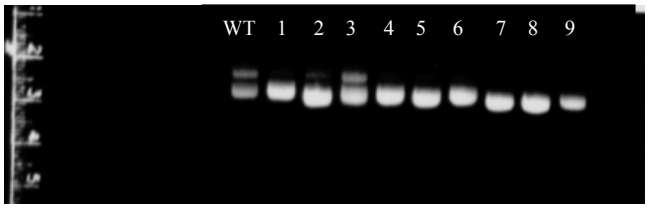


Fig 3.1

(A) The plasmidic construct pCEV29.1. Wild-type and mutant MET cDNAs were cloned into the pCEV29.1 expression vector (Giordano, S., et al. 2000) and the quality of the plasmidic DNA was validated by agarose gel electrophoresis (B).

Transfections were performed by the DEAE-dextran technique. Briefly, DEAE-dextran transfection is one of the oldest chemical, nonviral methods developed to transfer RNA or DNA to cultured mammalian cells. The standard transfection protocol involves pretreatment of cells with chloroquine, followed by exposure of the cells to a DEAE-dextran and DNA solution. Sixteen hours after transfection, cells were treated with the small molecule tyrosine kinase inhibitor PHA-665752 [250] nM. Twenty four hours later, cells were lysed with boiling Laemmli buffer (Laemmli, UK., 1970), proteins were quantified by Pierce BCA (bicinchoninic acid) Protein Assays and analyzed by western blot (WB). As shown in Fig 3.2, we observed that, while phoshorylation of MET wt and of some MET^{PRC} mutants (i.e. M1130T, V1188L, V1220I, M1250T) was inhibited in presence of PHA-665752, other mutants (namely L1195V, Y1230C, Y1230H, D1228H, D1228N) were still phosphorylated.

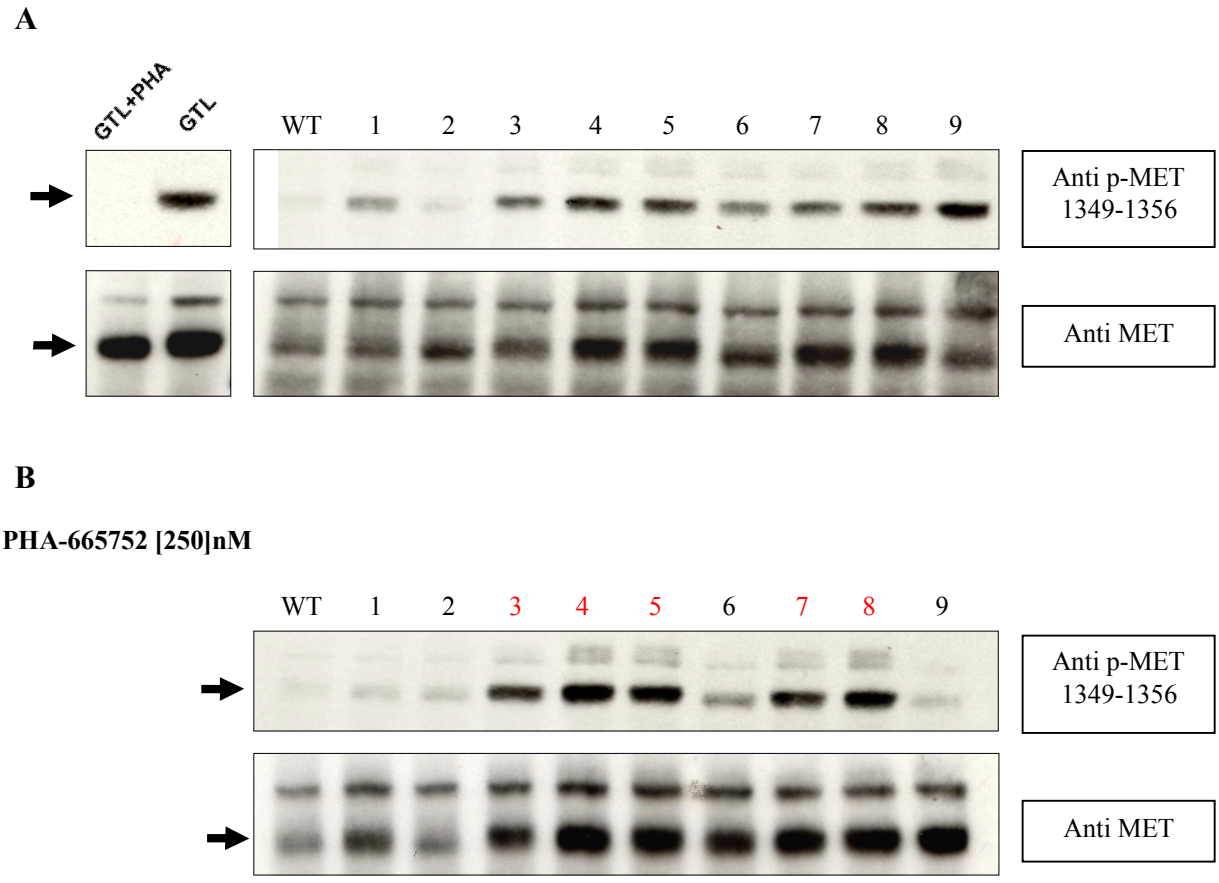


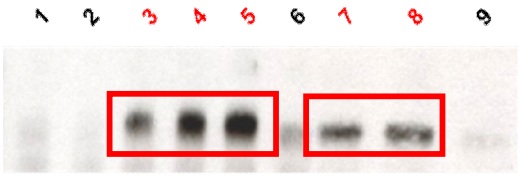
Fig 3.2

Equal amounts of pCEV 29.1 containing the cDNA of MET wt or of the different MET ^{PRC} mutants were transfected in COS-7 cells with DEAE-dextran procedure. After transfection, cells were untreated (A) or treated (B) with the ATP-competitive tyrosine kinase inhibitor PHA-665752 [250] nM. After 24 hours of treatment, cells were washed with phosphate-buffer saline (PBS) and lysed with Laemmli buffer. Proteins were quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL) and analysed by Western Blots. As shown, blots probed with anti-phospho MET antibodies (directed against the phosphorylated tyrosines 1349/1356) showed that some MET^{PRC} mutants (the red ones namely L1195V, Y1230C, Y1230H, D1228H, D1228N, operatively defined as mutants 3, 4, 5, 7 and 8 respectively) remained phosphorylated, and thus active, also in presence of the inhibitor. GTL16 cells, derived from a gastric carcinoma, over-expressing a constitutively phosphorylated receptor, were used as positive control for the TKI and antibody functionality.

In order to assess whether the lack of receptor inhibition was a dose-dependent effect or a real inability to respond to the drug treatment, we repeated the same experiments using a dose of PHA-665752 ten times higher than the IC50. As shown in Fig 3.3, tyrosine phosphorylation of the MET mutants 4, 5, 7 and 8 was not inhibited even at these high doses. MET mutant 3 was only partially inhibited, suggesting the existence of a different mechanism of drug resistance.

A

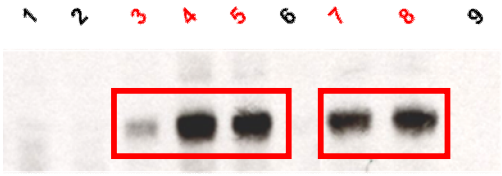
PHA-665752 [250] nM



Anti p-MET 1349-1356

B

PHA-665752 [500] nM



Anti p-MET 1349-1356

Fig 3.3

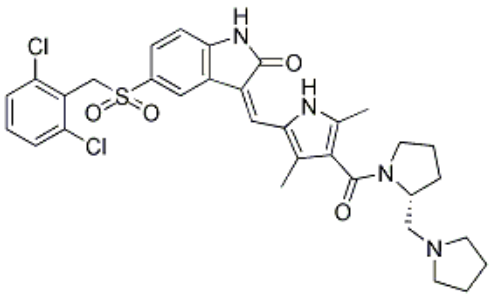
COS-7 cells transiently transfected with equal amounts of pCEV 29.1 containing the cDNA of MET wt or the different MET^{PRC} mutants were treated with the PHA-665752 TKI at two different concentrations: [250] nM (A) and [500] nM (B). After 24 hrs of drug treatment, cells where lysed in boiling Laemmli buffer. WB analysis revealed that MET phosphorylation of the resistant mutants (the red ones numbers 3, 4, 5, 7, 8 respectively) was persistent also at higher doses of TKI.

PHA-665752 was identified as a small ATP-competitive molecule, inhibitor of the catalytic activity of c-MET kinase (Ki of 4 nM, IC50 of 9 nM). PHA-665752 also exhibited >50-fold selectivity for c-MET compared with a panel of diverse tyrosine

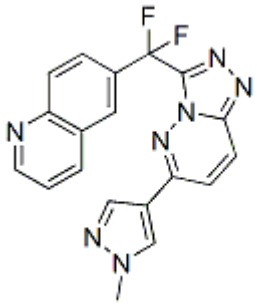
and serine-threonine kinases. In cellular studies, PHA-665752 potently inhibited HGF-stimulated and constitutive c-MET phosphorylation, as well as HGF and c-MET-driven phenotypes such as cell growth (proliferation and survival), cell motility, invasion, and/or morphology of a variety of tumor cells (Christensen, JG., 2003).

To evaluate if the inability to respond to PHA-665752 was shared also by other ATP-competitive MET inhibitors, we treated COS-7 cells expressing the different mutants with another small molecule tyrosine kinase inhibitor, the JNJ-38877605.

JNJ-38877605 is an orally bioavailable, highly specific MET inhibitor (selective over other 229 kinases tested). This agent inhibits c-MET with IC50 at 4 nmol/L and has a different chemical structure than PHA-665752 (see Fig 3.4).



PHA-665752



JNJ-38877605

Fig 3.4

Chemical structure of the small molecules TKIs PHA-665752 and JNJ-38877605.

As shown in Fig 3.5 all the mutants that were responsive to PHA-665752, were also responsive to JNJ-38877605, and mutants resistant to PHA-665752 remained active also in the presence of JNJ-38877605.

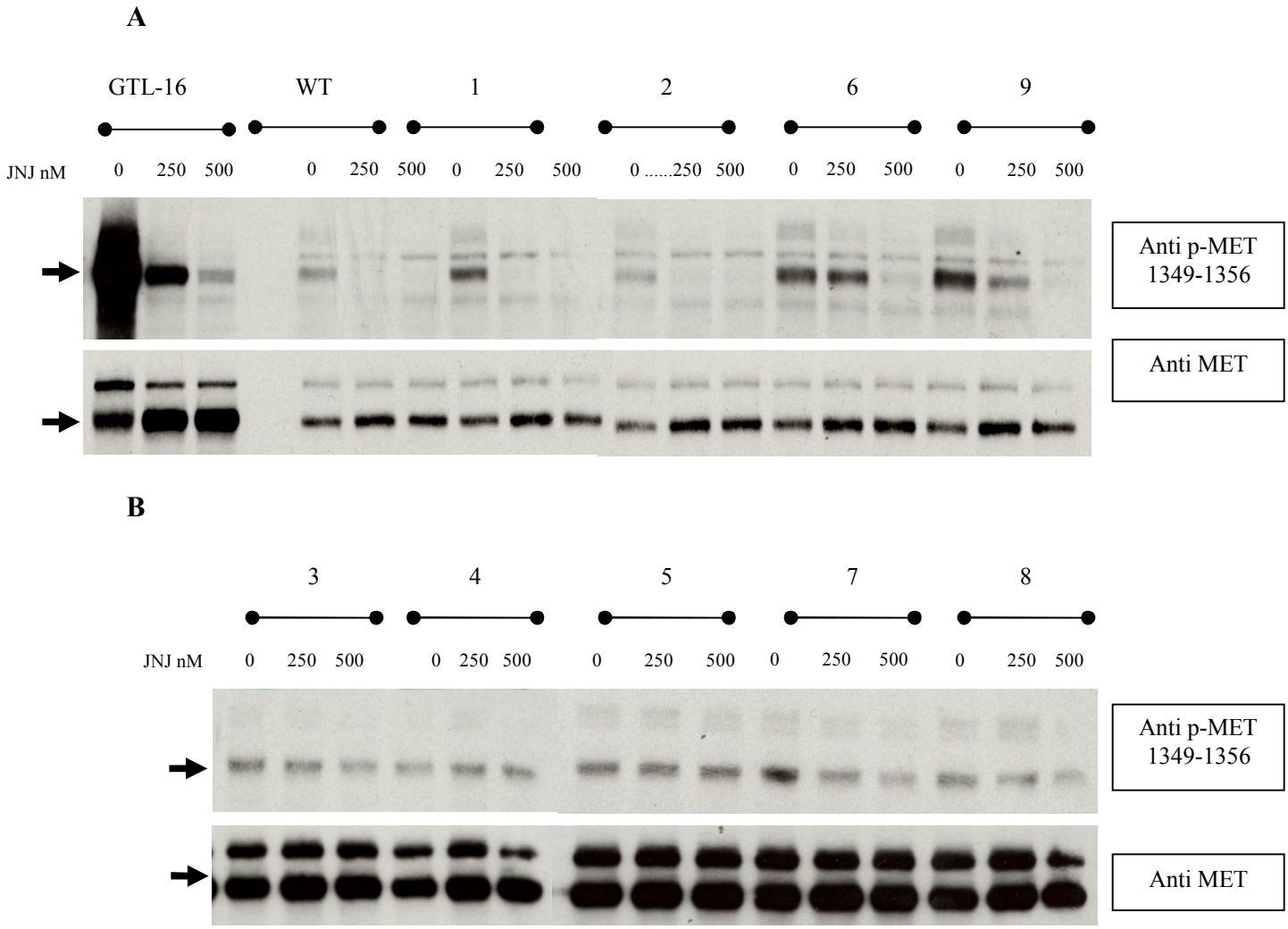


Fig 3.5

COS-7 cells transiently transfected with MET cDNAs (WT or mutated), were treated with the small molecule TKI JNJ-38877605. WB analysis confirmed that mutants responsive to PHA-665752 were sensitive also to JNJ-38877605 (A) while mutants resistant to PHA-665752 were resistant also to JNJ-38877605. GTL16 cells (A) were used as a positive control for the TKI inhibition and antibody detection.

Generation of stably transfected cell lines expressing the MET^{PRC} mutants

As shown by Jeffers et al., MET receptors containing the different PRC point mutations display different abilities to induce transformation in NIH 3T3 fibroblasts (Jeffers M, et al., 1997). In fact, only mutations that affect residues located in the kinase activation loop efficiently transformed NIH 3T3 mouse fibroblasts (Fig 3.6).

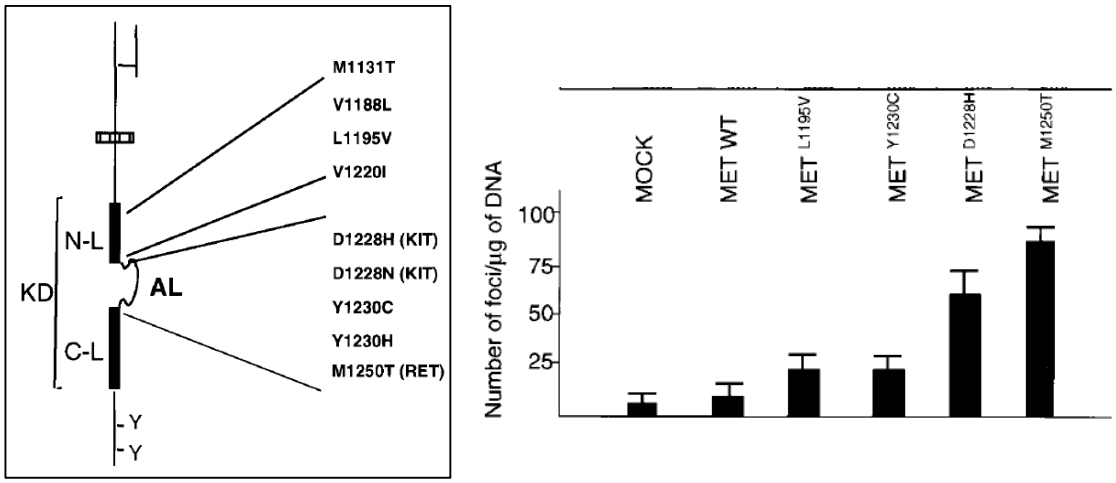


Fig 3.6

Left part: Map of MET mutations found in Hereditary Papillary renal carcinomas. Schematic representation of functional domains of MET tyrosine kinase. The black box depicts the tyrosine kinase domain (KD), which can be subdivided into amino- and carboxyl-terminal lobes (N-L and C-L, respectively), separated by a large cleft referred to as the activation loop (AL). YY represents the receptor multifunctional docking site. Mutations found in PRCs are listed and the homology with residues mutated in *RET* and *KIT* receptors is indicated.

Right part: Transforming ability of MET ^{PRC} mutants evaluated using the focus formation assay. Values reported represent the average of three independent experiments.

[**Data and pictures adapted from the following article:** Giordano S. *et al.*, Different point mutations in the met oncogene elicit distinct biological properties. *FASEB J.* 2000 Feb;14(2):399-406.]

Furthermore, the MET^{PRC} mutant endowed with the highest transforming ability (namely MET M1250T) also displayed the highest catalytic activity (Giordano, S. et al., 2000).

In order to evaluate if the cells expressing the MET^{PRC} mutants display a different biological behavior in the presence or in the absence of PHA-665752, we aimed at generating stably transduced NIH 3T3 cells (that express very low levels of endogenous MET). We thus chose two representative MET^{PRC} mutants: MET^{PRC} 8 (MET D1228N), resistant to PHA-665752, and MET^{PRC} 9 (MET M1250T), responsive to the drug treatment.

To optimize the transduction efficiency, we decided to express the MET mutants in lentiviral vectors. We thus mutagenized the MET cDNA cloned in the pRLL2 lentiviral vector, already available in the lab. The two PRC mutants were thus obtained performing an *in vitro* site-directed mutagenesis (Strategene's QuickChange II XL Site-Directed Mutagenesis Kit) that allows to introduce site-specific mutations in the double-stranded plasmid pRLL2 containing the MET wild type cDNA. The obtained mutagenized cDNAs were validated by direct sequencing (Fig 3.7).

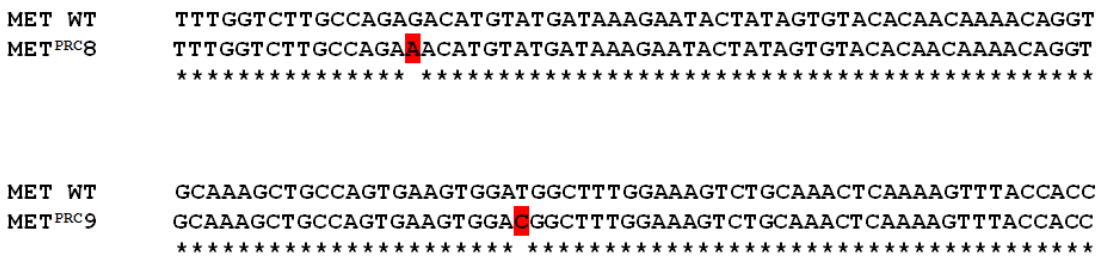


Fig 3.7

Alignment between the MET wild type cDNA sequence and the MET^{PRC} mutants number 8 or 9.

The point mutations are highlighted in red, the mutational analysis was performed using the Mutation Surveyor software, the represented alignment was obtained by the program "ClustalW Multiple Alignment" available in the web.

To produce stably expressing cells, we used the lentivirus expression systems based on HIV-1 that are becoming very popular for gene delivery into host cells, because they offer many advantages over both traditional retroviruses and adenoviruses.

First, we performed the multiplasmid transient transfection (the most widely used technique for generation of lentiviral vectors) with the traditional transient transfection protocol using 293T adherent cells and calcium phosphate/DNA co-precipitation followed by ultracentrifugation (Vigna, E. and Naldini, L., 2000).

The concentrated virus was quantified by the HIV-1 p24 Antigen ELISA (an enzyme linked immunoassay used to detect Human Immunodeficiency Virus Type 1 (HIV-1) p24 antigen in research specimens), and cell infections were performed using one µg/ml of concentrated virus for 16 hours in the presence of polybrene (increasing virus adhesion to cells).

Before performing biological assays, we confirmed the expression of the constructs in the stably transfected cells (Fig 3.8, bottom panel). We then carried out some biochemical experiments (response to HGF ligand and TKI inhibition) by stimulating

the cells with HGF (400U/ml for 15 minutes in serum free conditions), in the absence or in the presence of either PHA-665752 or JNJ-38877605. The results obtained with the two inhibitors were over imposable; figure 3.8 shows those obtained with JNJ-38877605. As it can be observed, while both MET wt and MET^{PRC} 9 were inhibited by the TKI, MET^{PRC} 8 remained tyrosine phosphorylated, as previously observed in COS-7 cells **(**see Fig 3.5**).**

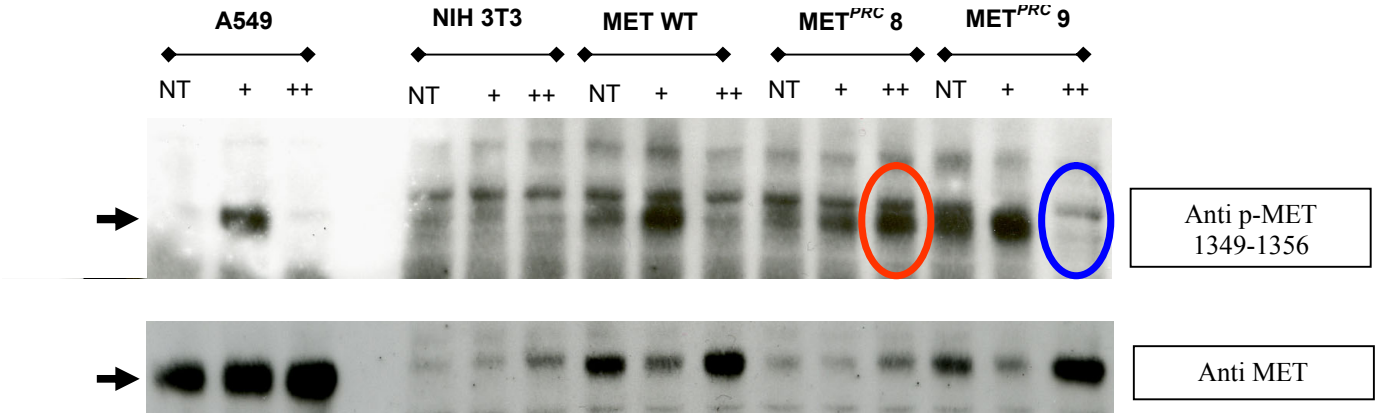


Fig 3.8

Cells expressing the different constructs (MET WT or mutated), were stimulated with HGF (400U/ml for 15 min) in the absence (+) or in the presence (++) of JNJ-38877605 [500]nM.

WB analysis showed that all cells expressing the MET cDNA responded to the ligand HGF; in presence of the TKI, the mutant number 8 was not inhibited (red circle) while cells expressing the responsive mutant 9 (blue circle) or MET WT lost their phosphorylation. NIH 3T3 cell line wt (untransduced) were used as negative control. A549 cells (an adenocarcinomic human alveolar basal epithelial cell line expressing an endogenous MET receptor quantitatively comparable to transduced NIH 3T3 cells) were used as positive control of the experiment and of anti p-MET antibody detection. Expression of the different constructs in stably transduced NIH 3T3 cells was previously evaluated using an anti MET antibody (bottom panel).

Biological properties of stably transfected cell lines expressing MET^{PRC} mutants

To evaluate if the different behavior of the MET^{PRC} mutants could impact also the biological properties of the expressing cells, we decided to analyze some MET-dependent activities such as cell migration and anchorage-independent growth. Concerning the biological responses, treatment with the TKI did not modify either receptor phosphorylation or MET-induced biological activities in cells expressing the resistant mutant D1228N (mutant 8): this was evident both in the soft agar assay (which evaluates the ability to grow in anchorage-independent conditions) and in migration/invasion assays, such as wound healing and transwell assays (see Fig 3.9).

In details, colony formation and viability (both measured by Alamar Blue quantification) were not impaired in the presence of JNJ-38877605 in cells expressing the mutant number 8, while they were strongly decreased in those expressing MET wt or the mutant 9. Untransduced NIH 3T3 cells were used as negative control: as shown, these cells were not able to form colonies in soft agar.

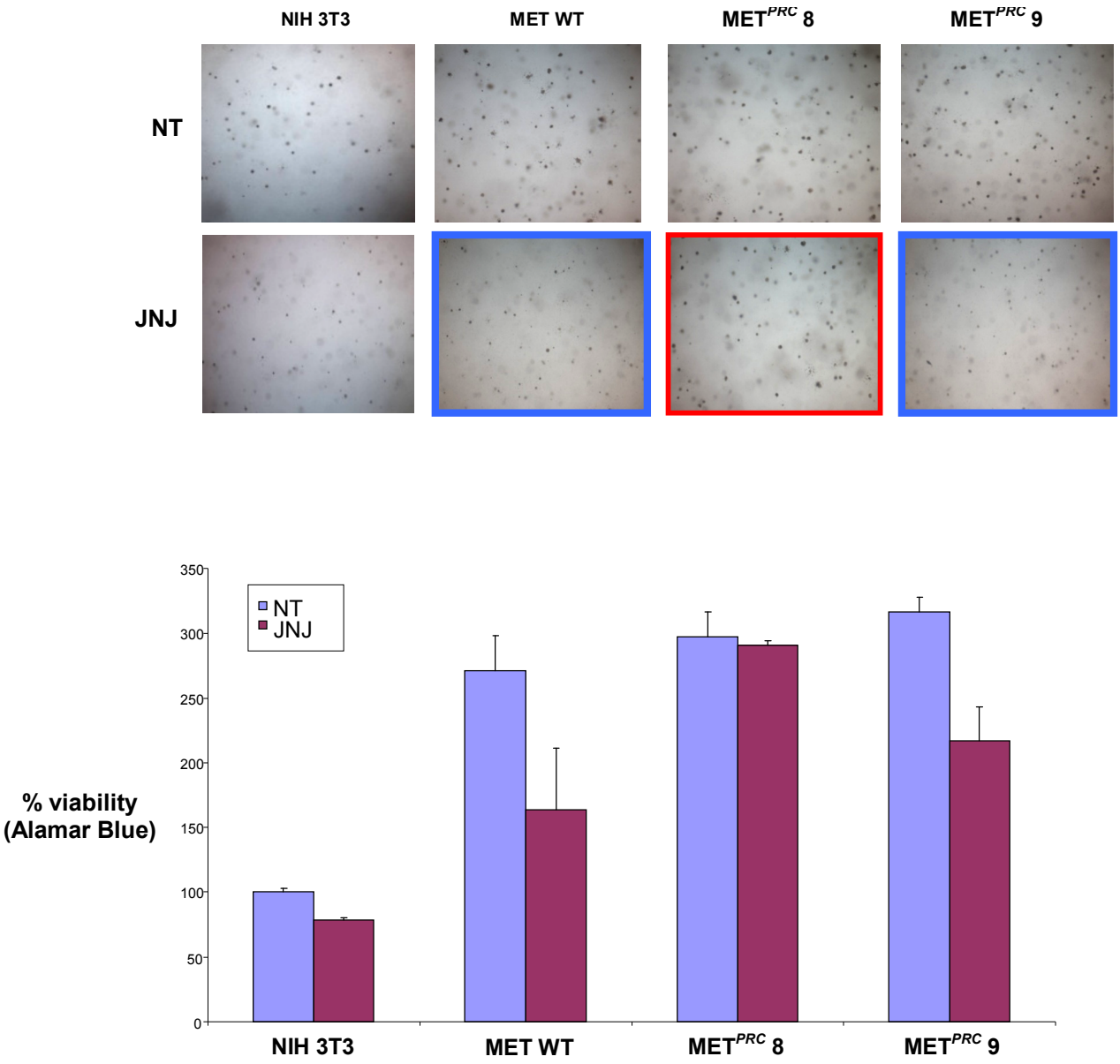


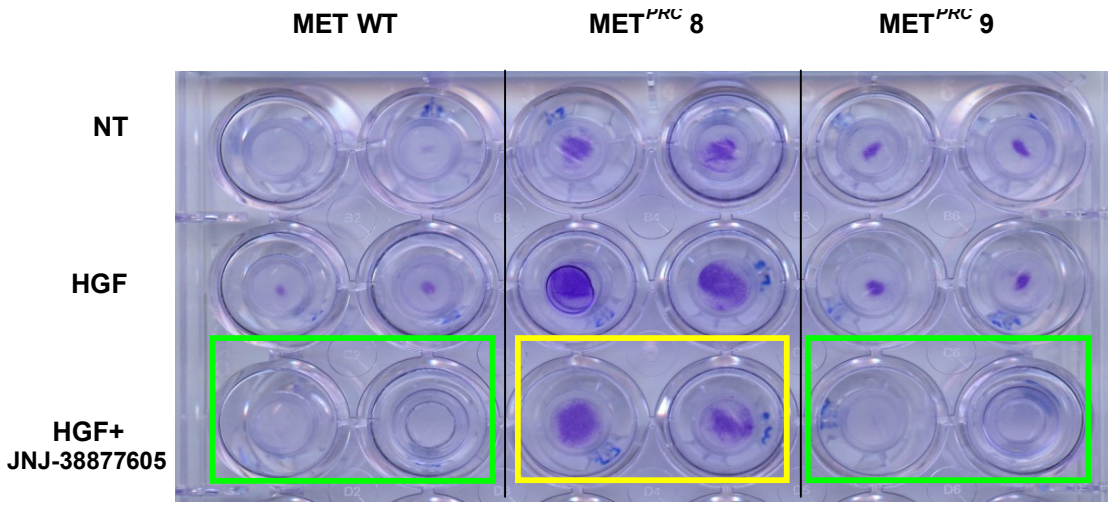
Fig 3.9

Anchorage-independent growth assays in soft agar and Alamar Blue quantification of cell viability. In the presence of the MET inhibitor, cells expressing either MET wt or the sensitive mutant number 9 (blue lines) were severely impaired in their ability to grow in anchorage independent conditions, while cells expressing the resistant mutant 8 (red line) were unaffected. NIH 3T3 cell line wt (not expressing the MET gene) were used as negative control. The experiment was performed in presence of HGF (20 ng/ml) and in presence or absence of JNJ-38877605.

We also performed *in vitro* biological assays to evaluate cell motility. In the invasion assay, cells were seeded in Transwell chamber, on the upper side of a porous polycarbonate membrane. The medium in both chambers was supplemented with low percentage of serum; the lower chamber was supplemented with HGF (20 ng/ml) in presence or absence of JNJ-38877605. After 16 h, cells attached on the upper side of the membrane were mechanically removed. Cells that migrated to the lower side were fixed with gluataraldehyde and stained with crystal violet. Stained cells were photographed (see Fig. 3.10 A).

To evaluate the ability to migrate and repair wounds, we performed a Wound Healing assay. This method mimics cell migration during wound healing *in vivo*. The basic steps involve creating manually a "wound" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration and comparing the images to quantify the migration rate of the cells. As shown in Fig. 3.10 B, all cells increased their ability to migrate in presence of HGF (20 ng/ml) compared to the counterpart not treated (NT), but cells transfected with MET wt or with the responsive mutant 9 were not able to close the wound in presence of JNJ-38877605. Once again, the resistant mutant 8, showed an opposite response and its ability to migrate and close the wound was not impaired by the inhibitor.

A



B

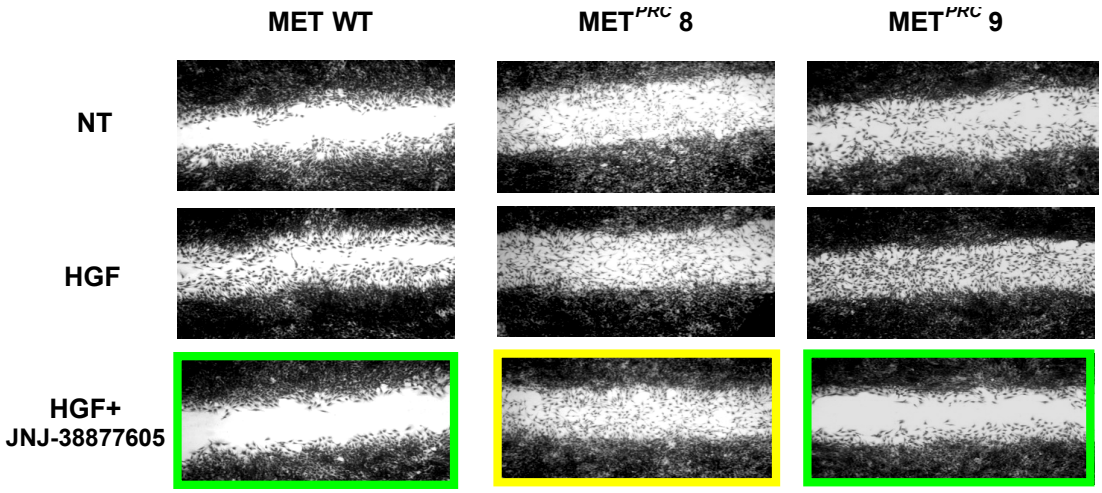


Fig 3.10

Cells expressing MET WT or the mutants 8 and 9 were used to evaluate the ability to invade and migrate.

The upper panel shows the results of a representative invasion assay in Transwells, in the lower part is represented the ability to migrate and repair the wound by Wound Healing Assay. Both assays were performed using low percentage of serum, plus (experimental point named HGF) or minus (named NT) HGF (20 ng/ml), or in presence of HGF (20 ng/ml) plus JNJ-38877605 [500] nM.

In both assays these two mutants showed an opposite response: while cells expressing MET wild type or the responsive mutant 9 (green rectangles) were inhibited, the resistant mutant 8 (yellow rectangles) was able to migrate also in the presence of inhibitor in both assays.

Analysis of lung tumors for the presence of MET mutations

Nowadays pivotal studies in NSCLC (Non-Small Cell Lung Cancer) are ongoing, using specific chemical and biological anti-MET inhibitors; three of them (Met MAb, Crizotinib, ArQule-197) are in phase III clinical trials. The used drugs fall in two different categories: small kinase inhibitors (TKIs) and monoclonal antibodies (mAbs). They act with different mechanisms, since small TKIs interact with the receptor intracellular portion while mAbs bind to the extracellular domain. It is thus very likely that mutations present in diverse parts of the receptor can differentially impact on the ability to respond to either of the drugs. For these reasons and since MET^{PRC} mutations have been found not only in the germline of patients but also in sporadic tumors, we decided to analyze the sequence of the MET tyrosine kinase in human surgically resected lung cancers.

As shown in the Tab 3.2, we collected resected lung tumors in collaboration with the Oncologic Hospital "A. Businco" of Cagliari (Dr. R. Versace) and Hospital "S.Giovanni Battista" of Turin (Prof. E. Ruffini), and we gathered the follow up of all patients (unfortunately not complete in all cases). The Classification of Malignant Tumors (TNM) is one of the most widely used staging systems. The TNM system is based on the extent of the tumor (T)**,** the extent of spread to the lymph nodes (N)**,** and the presence of distant metastases (M)**.** A number is added to each letter to indicate the size or extent of the primary tumor and the extent of cancer spread. Tumor grade is a system used to classify cancer cells in terms of how abnormal they look under a microscope and how quickly the tumor is likely to grow and spread. Tumor grade should not be confused with the stage of a cancer. Cancer stage refers to the extent or severity of the cancer, based on factors such as the location of the primary tumor,

tumor size, number of tumors, and lymph node involvement (spread of cancer into lymph nodes).

NUMBER OF SAMPLE	ISTOLOGY	STAGE	GRADING	T	N	M
3118	Ca pavimentoso G2 pT1aN0	I A	G2	T1	N0	
2279	Mts polmonari di ca retto G2	IV	G2			
119	adenocarcinoma con estese aree di necrosi	IB	G2	2	0	0
280	carcinoma non a piccole cellule	IIA		1	1	0
285	carcinoma a grandi cellule con attività neuroendocrina	IB		2	0	0
286	Adenocarcinoma					
288	carcinoma epidermoide			1	0	0
290	carcinoma bronchiolo alveolare mucinoso					

Tab 3.2

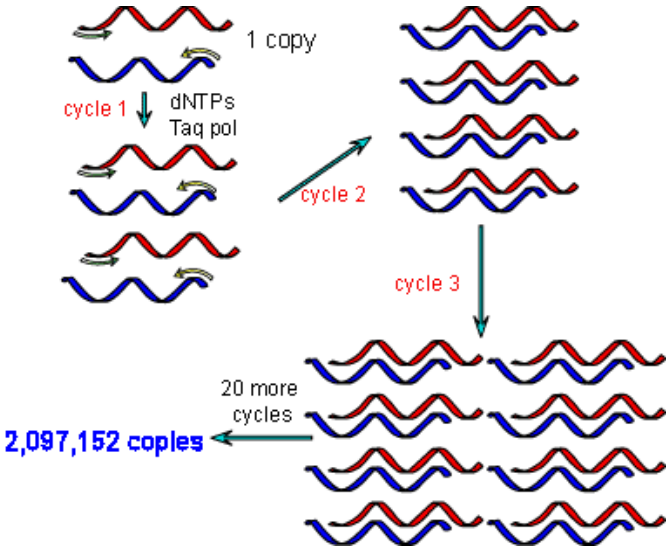
List of samples used for mutational analysis of the MET gene and corresponding follow up. Classification is based on the Classification of Malignant Tumors (TNM), a cancer staging system that describes the extent of cancer in a patient's body: T stands for tumor size and invasiveness. The T number can range from T1 to T4. T1 and T2 are differentiated primarily on size (<3 cm = T1, >3 cm = T2) and if the tumor is visible within a lobar bronchus (T2). T3 tumors involve the chest wall, but may

be resectable (operable). T4 tumors are not surgically resectable because they have invaded the mediastinum (the area and organs between the lungs) and involve the heart, great vessels, trachea or esophagus, or because they involve the pleura (lining of the lung) with a malignant pleural effusion (accumulation of fluid around the lining of the lung). N stands for Nodal involvement (lymph nodes) and is staged from N1 to N3. M stands for the presence (1) or absence (0) of metastases (spread to a distant site). Grading (1–4) refers to the differentiation of the cancer cells (i.e. they are "low grade" if they appear similar to normal cells, and "high grade" if they appear poorly differentiated). About staging, Non-small cell lung carcinoma is usually staged from IA (best prognosis) to IV (worst prognosis).

We purified total RNA from 16 samples (8 tumors and the corresponding peritumoral normal tissues). Upon RNA retrotranscription, we PCR amplified overlapping portions of the MET intracellular domain; amplified segments were then purified using the chemical method named Solid Phase Reversible Immobilization (SPRI) based on speed beads (Agencourt Ampure Xp Kit), and finally sequenced. (see Fig 3.11).

In one tumoral sample (but not in the corresponding peritumoral normal tissue) we found the presence of a missense mutation (Y1230H) resulting in aminoacid substitution (see Fig 3.12 A). The mutated aminoacid corresponds to Y1230H, which belongs to the MET^{PRC} mutants resistant to treatment with small kinase inhibitors. (Fig 3.12 B). Moreover, a very recent study (Qi J., et al., 2011) has shown that this mutation destabilizes the conformation of the MET TK domain and contributes to the development of acquired resistance to MET inhibitors.

A



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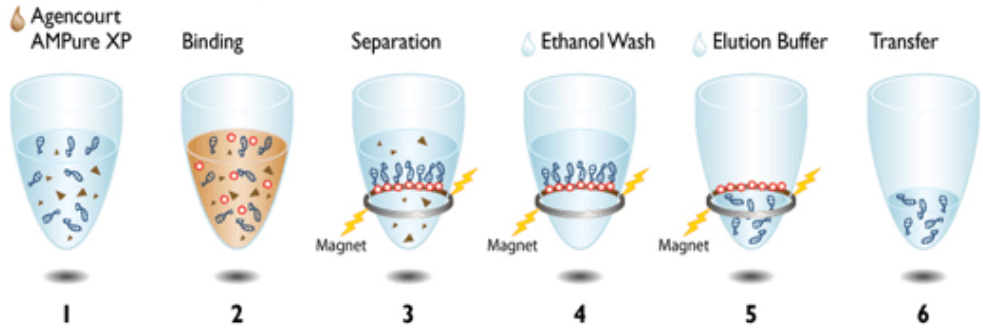


C

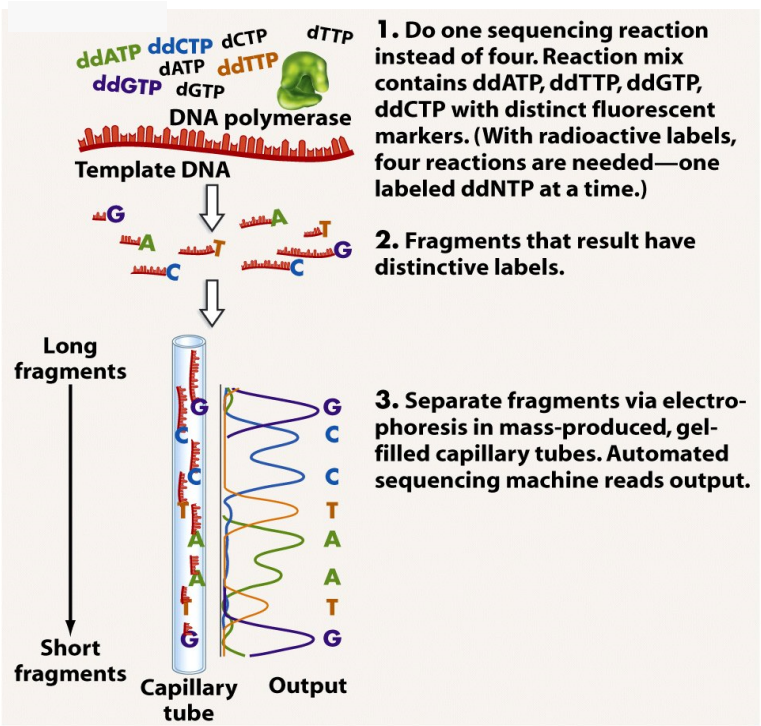
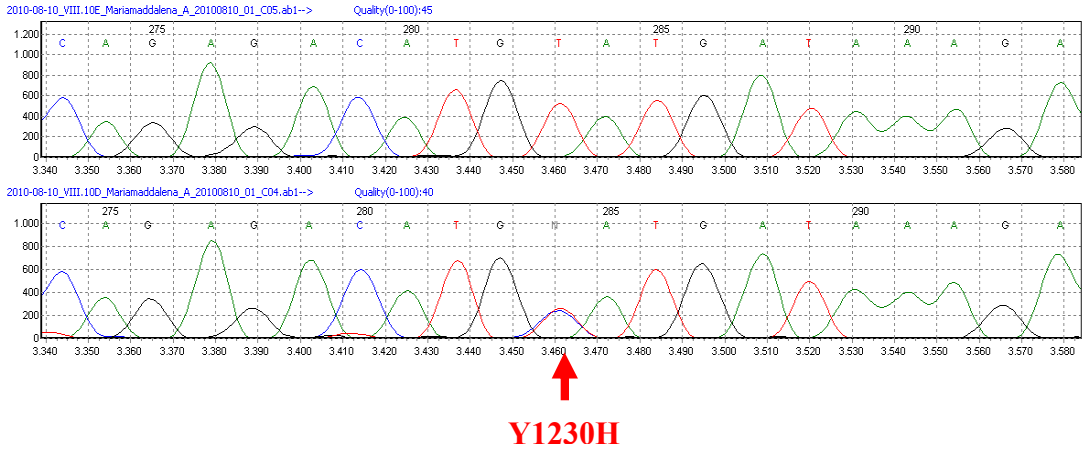


Fig 3.11

RNA obtained from the different samples was retro-transcribed; sequences comprising the TK domain of MET were amplified by PCR (A), purified with the Agencourt Ampure Xp Kit (B) and finally sequenced by Sanger's method (C).

A



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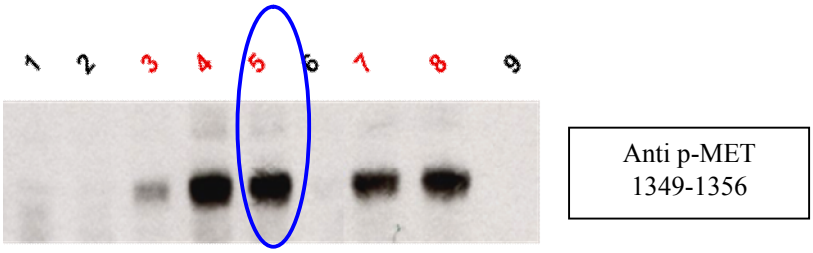


Fig 3.12

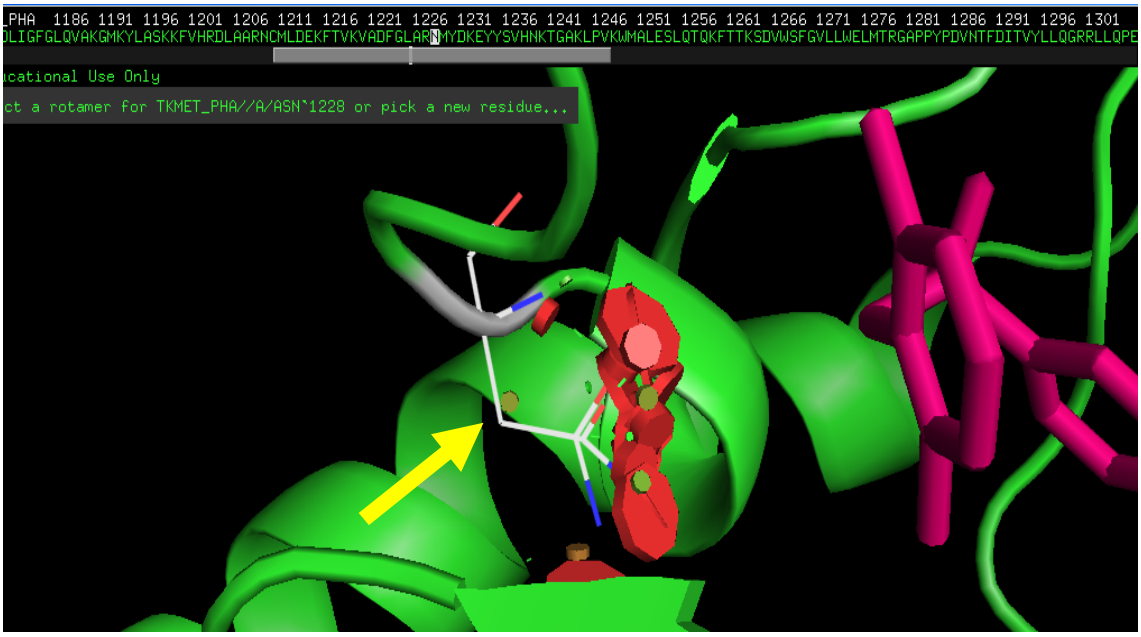
The sequence analysis of MET tyrosine kinase domain in human resected lung cancer samples, was performed on RNA derived from both the tumors and corresponding normal tissues. In one sample (sample number 119 listed in the previous tab) the tumoral counterpart revealed the presence of the MET^{PRC} mutation Y1230H (the number 5) which was not present in the corresponding peritumoral tissue (A). This mutation, as previously reported, induces resistance to PHA-665752 . As shown the mutated receptor maintains a persistent phosphorylation also in presence of inhibitor, as highlighted by the blue circle in the lower part of the panel (B).

***In silico* structural analysis of the MET tyrosine kinase domain**

Since pharmacological resistance can be due to impaired interaction between the drug and the RTK, we performed an *in silico* analysis of the MET tyrosine kinase domain Structures were mutagenized with the PyMol Software starting from the crystal structure of the MET TK domain available in the NCBI structure site (NCBI STRUCTURE_ PDB 2WKM "X-Ray Structure Of PHA-00665752 Bound To The Kinase Domain Of c-MET http://www.rcsb.org/pdb/explore/explore.do?pdbId=2WKM). From this analysis it was evident that the mutations D1228N (MET^{PRC} 8) and Y1230H (MET^{PRC} 5, found in one lung cancer sample) conferring resistance to TKIs, were located very close to the ATP-binding site (Fig 3.13 A, B), while M1250T (MET^{PRC} 9), which did not induce resistance, was placed away from this site (Fig 3.13 C). For this reason, it is likely that -for steric hindrance- the ATP-competitive inhibitors (PHA-665752 and JNJ- 38877605) are unable to interact with their binding sites in the context of these mutated receptors.

Analyzing also all the other PRC mutations (Fig 3.13 E), we observed that all the mutations conferring resistance were located in the proximity of the PHA-665752 binding site, while mutated aminoacidic residues of the responsive mutants were far from this region. Concerning the MET^{PRC} mutant 3, that was only partially inhibited in presence of PHA-665752, its localization is outside the ATP binding site (see Fig 3.13 D), suggesting the existence of a different mechanism of drug resistance.

A

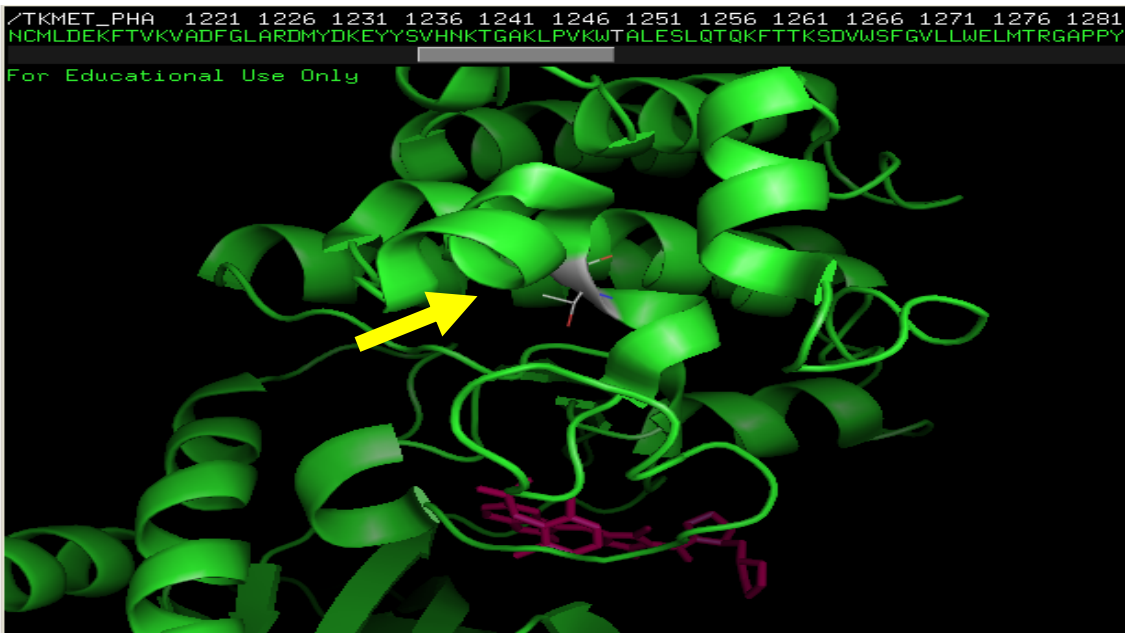


D1228N
(MET^{PRC} 8)

B

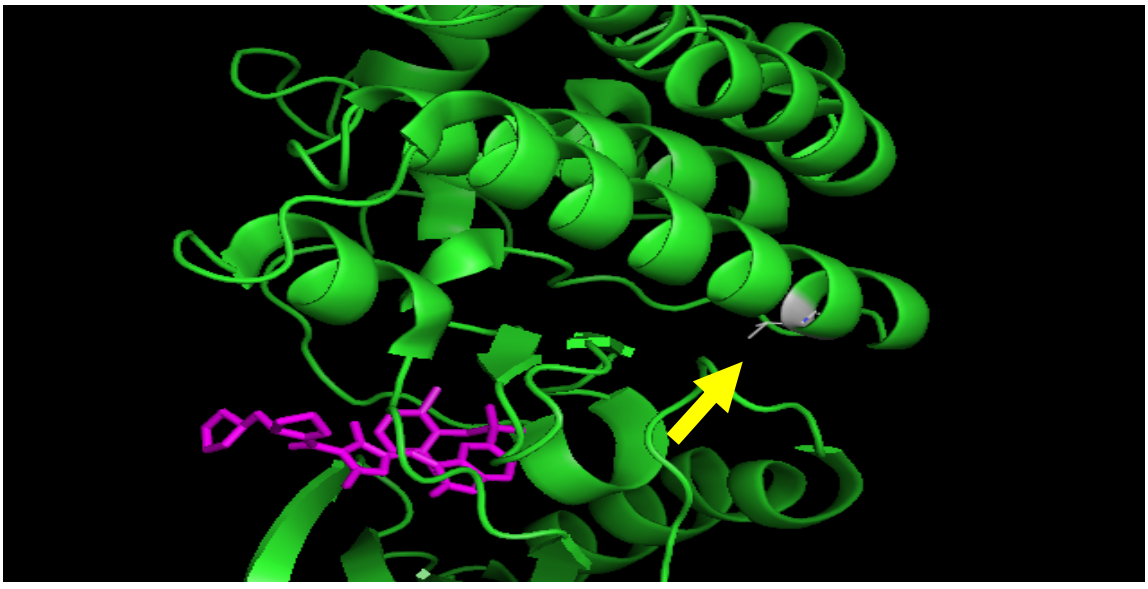
Y1230H
(MET^{PRC} 5)

C



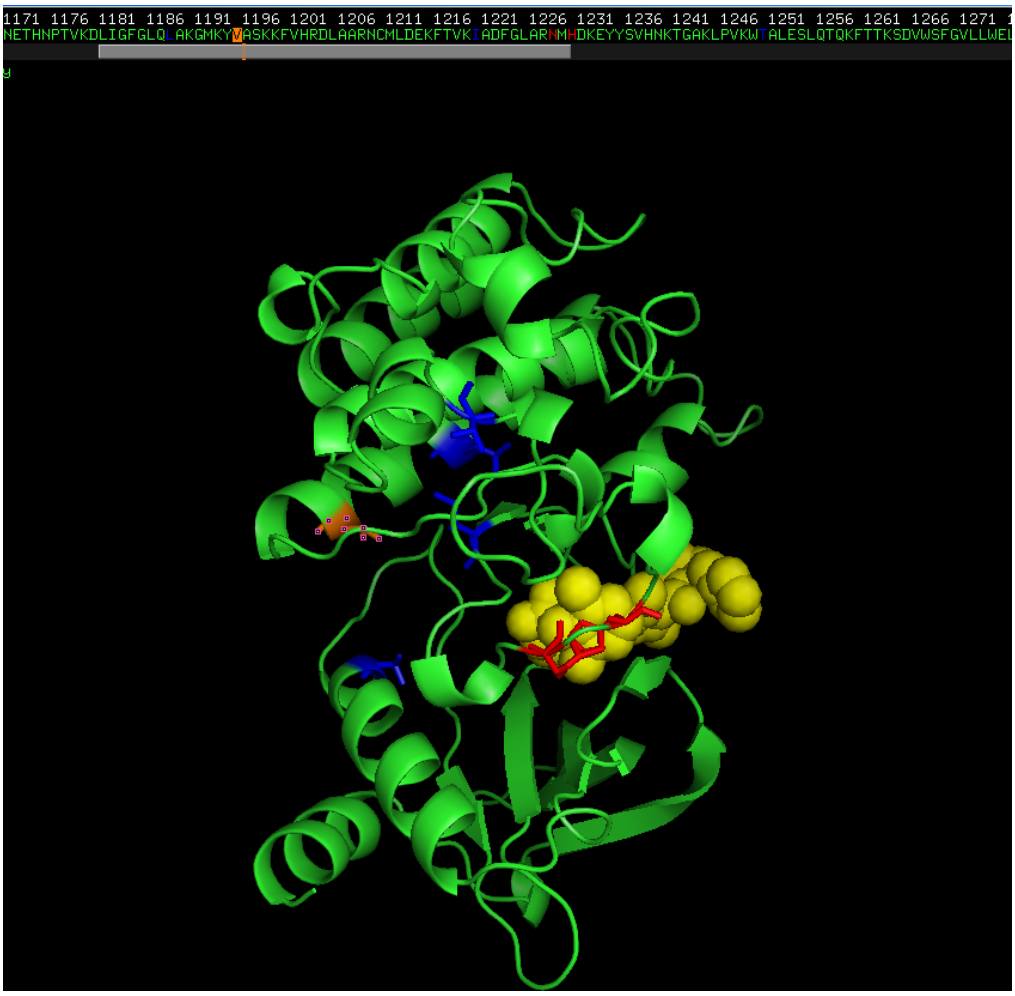
M1250T
(MET^{PRC} 9)

D



L1195V
(MET^{PRC} 3)

E



ALL MET^{PRC} MUTANTS

Fig 3.13

In silico analysis of MET TK domain in presence of the ATP-competitive inhibitor PHA-665752.

In all the pictures the secondary structure of MET TK domain is green. In A, B, C, D the mutated aminoacidic residue is indicated by the yellow arrow and the PHA-665752 molecule is magenta. In A and B, red balls indicate the steric hindrance. Panel E shows the TK domain with all the PRC mutants: the red ones are the resistant, while the blue ones are those responsive to small molecules PHA-665752 (the yellow structure, represented by bubbles); the partially resistant PRC 3 is orange.

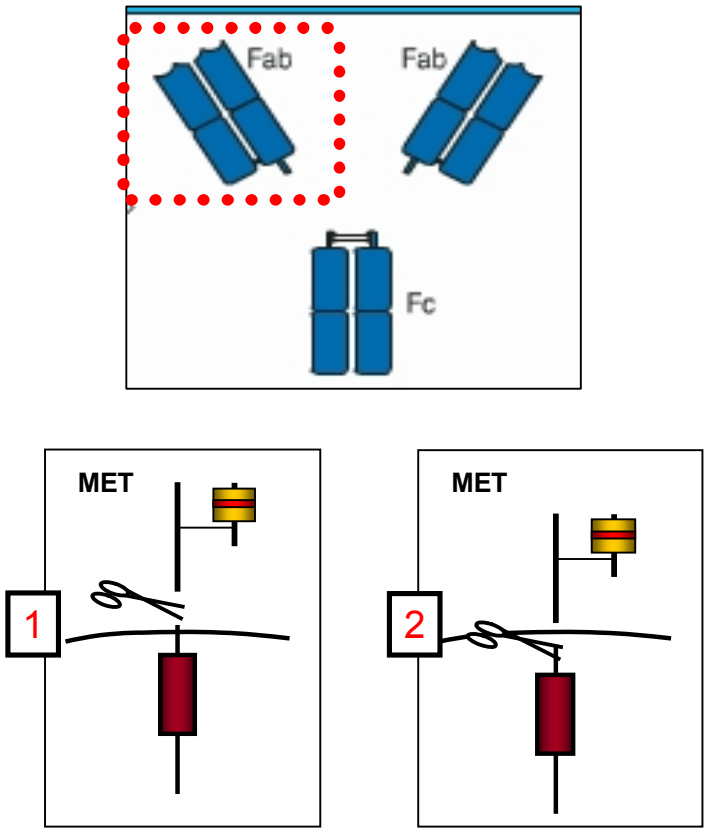
Bypassing resistance: use of an anti-MET specific monoclonal antibody

As previously mentioned, while the presence of mutations in the intracellular TK domain can impair the ability to respond to small kinase inhibitors, it is likely that the mutated receptors are still able to respond to the inhibitory activity of monoclonal antibodies directed against the extracellular portion.

One of such mAbs, named DN30, was previously produced and studied in the laboratory (Pietronave S, et al., 2010). This monoclonal antibody is directed against the extracellular portion of the receptor and behaves as a partial agonist (Prat, M. et al., 1998). In fact, it induces MET activation, although at low levels, followed by promotion of MET down-regulation through a molecular mechanism which involves cleavage of the extracellular portion (also called "shedding") of the receptor and proteasomal degradation of the intracellular portion (Petrelli, A. et al., 2006), (fig 3.14 A). To eliminate the partial agonistic activity of this mAb, some colleagues at the IRCC have ingegnerized the antibody in order to produce a monovalent form (DN30 FAb), which maintains only the antagonistic activity (Pacchiana G., et al., 2010).

To evaluate the inhibitory activity of the DN30 FAb, we grew the transfected COS7 cells for 72 hours in the presence of this molecule. We then examined the supernatant of the cells to look for the presence of the released MET extracellular portion. As we observed that FAb DN30 treatment caused "shedding" of extracellular domain in all MET^{PRC} mutants (fig 3.14 B) we could conclude that the DN30 FAb was indeed active on all the mutants. When we analyzed the activation status of the different mutants, we found indeed that FAb DN30 treatment resulted in their inhibition, independently from their sensitivity to TKIs. An example is shown in figure 3.15 where all the TKI-resistant mutants were inhibited (and thus lost their tyrosine phosphorylation) and the total amount of MET decreased upon DN30 FAb treatment.

A



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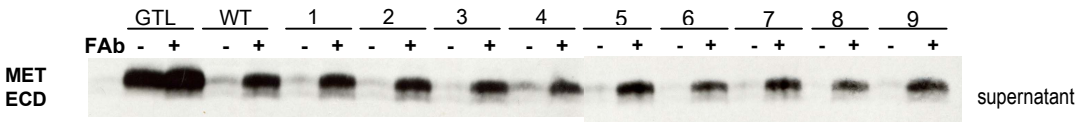

Fig 3.14

Mechanism of action of the monoclonal antibody DN30 **(A)**. This monoclonal antibody (DN-30) is directed against the extracellular portion of MET and binds to MET at subnanomolar affinity, inducing proteolytic cleavage of the extracellular portion close to the cell membrane and release of a soluble receptor in the extracellular space (Petrelli, A. et al., 2006). Following ectodomain shedding, operated by a metalloprotease of the ADAM family, the remaining transmembrane fragment becomes substrate of a second protease (γ-secretase) that detaches the kinase-containing portion from the membrane and rapidly addresses it toward the proteasome degradation pathway (Foveau B, et al. 2009). Therefore, the net result of DN-30 binding to MET is (*a*) the generation of a soluble "decoy" MET that neutralizes HGF and forms heterodimers with *bona fide* MET (Michieli, P. et al., 2004) and (*b*) the proteolytic degradation of the MET kinase domain. This translates into neutralization of HGF/MET-mediated biological activities. In picture 3.10 B is reported the ectodomain shedding of all PRC mutants and MET wt after Fab DN30 treatment. Upon 72h of Fab DN30 treatment (24μg/ml), cells were starved for 16hrs, then the medium collected and loaded for the western blot analysis. As shown, FAb DN30 treatment caused the ectodomain shedding in all mutants, including the TKI-resistant group. In these experiments, GTL16 cell line was used as positive control.

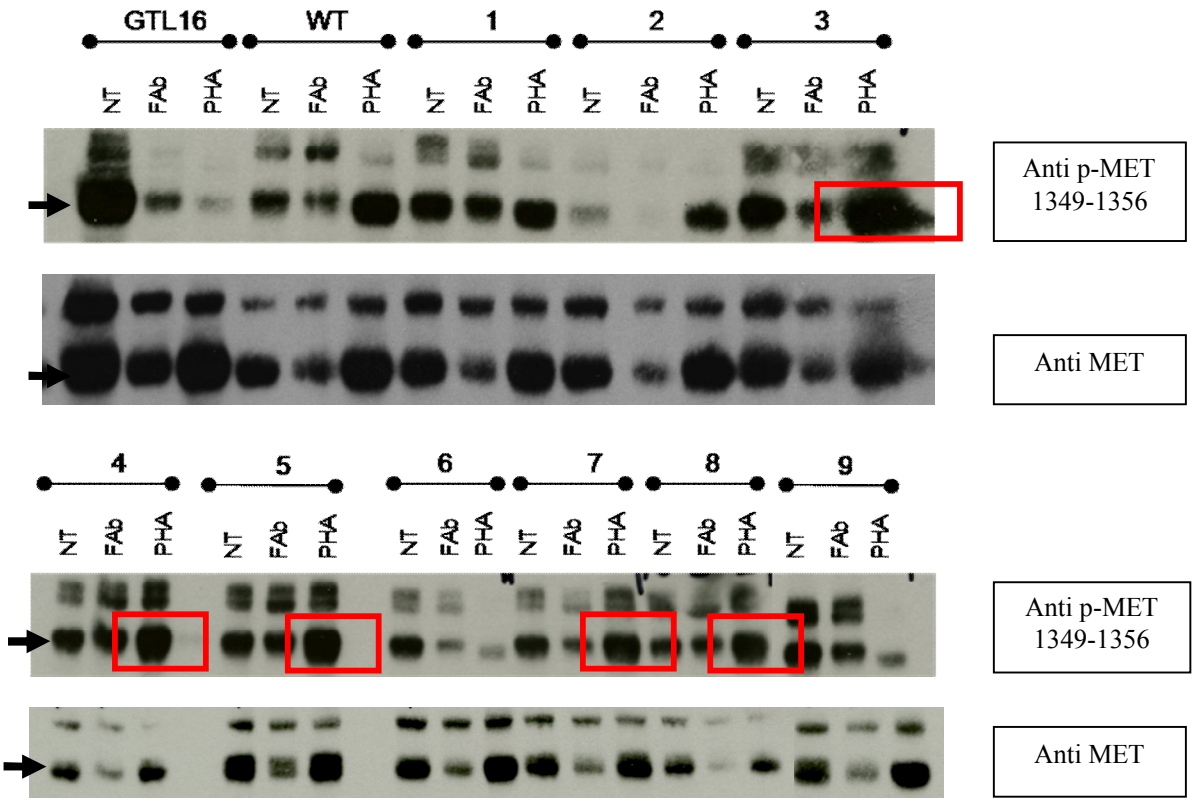

Fig 3.15

WB analysis on COS-7 cells expressing MET WT and the MET^{PRC} mutants.

Red rectangles highlight that FAb DN30 (28 µg/ml) treatment significantly decreased the phosphorylation all the resistant mutants (reference numbers 3, 4, 5, 7, 8) . Also the total amount of MET, as a direct consequence of ectodomain shedding, was significantly decreased in cells treated with the antibody respect the counterpart not treated (NT) or inhibited by PHA-665752. GTL16 cells were used as positive control of the experiment.

DISCUSSION

Selective inhibition of protein tyrosine kinases is gaining importance as an effective therapeutic approach for the treatment of a wide range of human cancers.

The fact that the inhibition of a single oncogene can cause the death of cancer cells (referred as oncogene addiction) supported the idea of using highly specific inhibitors directed against the oncogenic proteins. The paradigm of the clinical success of *targeted therapies* based on *oncogenic addiction* is represented by the use of Imatinib (a small kinase inhibitor directed against the cytoplasmic tyrosine kinase ABL) for the treatment of CML (Chronic Myeloid Leukemia) in patients bearing BCR-ABL translocation. The *oncogenic addiction* has been proven also in different biological contests and on different targets: Trastuzmab in HER2 over-expressing breast cancers, Cetuximab (anti-HER1) in CRC (Colorectal Cancer) and HNSCC (Head and Neck Squamous Cell Carcinoma) and Gefitinib and Erlotinib (HER1 inhibitors) in NSCLC (Non-Small Cell Lung Cancer) (Petrelli, A. & Giordano, S., 2008).

However, as extensively documented, initially successful therapy is often hampered by acquired resistance to the drug and subsequent relapse and this could be caused by different mechanisms. Nowadays, given that many patients are starting to benefit from the discovery of monoclonal antibodies and of small molecules targeting tyrosine kinases, the investigators are now trying to understand and unveil the mechanisms through which neoplastic cells lose their ability to respond to these drugs (also named secondary resistance or acquired resistance). Luckily, it appears that the majority of the resistance models developed *in vitro* are predictive of what is observed *in vivo* and can thus help researchers in identifying and studying this crucial clinical problem.

Many different mechanisms have been demonstrated to sustain resistance to targeted therapies. The most common mechanism of resistance, in terms of genetic alterations of the target, is the presence or appearance of point mutations impairing or preventing the interaction between the target and the drug. The most frequent types of mutations are those decreasing the affinity of the drug for the target kinase domain, while maintaining the catalytic activity. Mutations that alter the aminoacids surrounding the binding site of the drug decrease the availability of the target region towards the inhibitor, without interfering with the ATP binding (Zhang et al., 2009). Other reported mutations increase the affinity of the kinase for the ATP, decreasing the effectiveness of the ATP-competitive inhibitors (Tanaka R, Kimura S., 2008).

Some reports support the idea that the appearance of mutations in tumors after treatment with a specific TKI is the result of a process of selection of a pre-existing cell population. Such theory supports the idea that a small population of the tumor bulk *a priori* contains the mutation, which confers a primary resistance to these cells, therefore giving them a selective advantage in the presence of the inhibitor. The bulk tumor mass is thus killed by the drug, allowing a short period of response, lasting until the cells resistant to the TKI become the majority. This theory is supported by the fact that some of these "resistance-related mutations" can be found in a small percentage of tumor cells in patients that have not undergone targeted therapy (Bachleitner-Hofmann T., et al., 2008; Kreuzer KA, et al., 2003; Roche-Lestienne C, et al., 2002).

On the other hand, other investigators believe that the high dependence of a cell on a specific oncogenic survival pathway forces genomic instability, allowing the induction of mutations that confer resistance to the inhibitor. This genomic instability can induce mutations either in the drug target or in other signal transducers that activate alternative pathways able to sustain cell viability (Ricci C, et al., 2002).

About the MET gene, as previously mentioned, activating mutations have been described in sporadic tumors such as childhood hepatocellular carcinomas, sporadic papillary renal carcinomas, gastric carcinomas, lung carcinomas and head and neck squamous cell carcinomas. The main proof of the direct involvement of MET in tumorigenesis was given by the identification of germ-line activating mutations in patients with hereditary renal papillary carcinoma (HPRC).

Nowadays pivotal studies in NSCLC (Non-Small Cell Lung Cancer) are ongoing using specific chemical and biological anti-MET inhibitors; three of them (MET MAb, Crizotinib, ArQule-197) are in phase III clinical trials. They act with different mechanisms and are directed against different portions of the MET receptor: small TKIs (crizotinib and ArQule-197) interact with the intracellular portion, while mAbs bind to the extracellular domain. It is thus very likely that mutations present in different parts of the receptor can differentially impact on the ability to respond to either of the drugs.

Recently, MET mutations have been identified within the sema domain, juxtamembrane domain, and intrcellular regions in small cell and non-small cell lung cancers, lung adenocarcinomas, gastric cancer, renal carcinomas, and mesotheliomas (Ma PC, et al., 2003; Kong-Beltran M, et al. 2006; Ma PC, et al., 2008; Jagadeeswaran R, et al., 2006; Lee JH, et al., 2000). Thus, mutational activation of MET is not restricted to renal cancer and may be a more common mechanism by which MET is aberrantly activated during tumorigenesis. A few studies have shown that some of these mutations induce resistance to MET kinase inhibitors (Timofeevski SL, et al., 2009; Berthou S, et al., 2004; Bellon SF, et al., 2008). Therefore, additional studies are required to understand the effect of MET mutations in tumor progression and resistance to therapy.

From this scenario it is clear the need of choosing the most suitable therapeutic approaches in order to avoid the phenomenon of pharmacologic resistance. This requires tailoring of the therapy using drugs able to act also in the presence of mutations, which could determine pharmacologic resistance to the treatment. On these bases, my PhD work was aimed at evaluating the activity of some available anti-MET therapies targeting the MET receptor mutated in the kinase domain.

First of all, our results demonstrated that it is possible to categorize the MET^{PRC} mutants in two different groups: the "responsive" group whose phosphorylation was inhibited in presence of PHA-665752, and the "resistant" group in which receptor phosphorylation and activation are not affected by the inhibitor.

Second, the lack of receptor inhibition was not a dose-dependent effect, but a real inability to respond to the drug treatment. In fact, using PHA-665752 at two different concentrations: [250] nM and [500] nM (ten times higher than the IC50), tyrosine phosphorylation of some MET^{PRC} mutants was not inhibited even at these high doses. One mutant (MET^{PCR} mutant 3) was only partially inhibited, suggesting the existence of a different mechanism of drug resistance.

We then demonstrated that the phenomenon of resistance is not restricted to PHA-665752, but is shared also by other ATP-competitive MET inhibitors, such as the JNJ-38877605.

To evaluate the biological meaning of these observation, we engineered NIH 3T3 cells (that express very low levels of endogenous MET) to express MET^{PRC} mutated forms. We found that while -induced biological activities (such as migration, invasion and growth) were impaired by TKIs in cells expressing wt MET or MET^{PRC} "responsive mutants", they were not affected in cells MET expressing the "resistant

mutants". These results show that loss of biochemical inhibition was paralleled by loss of MET-dependent biological activities.

We then asked how these mutations can impair or prevent the response to TKIs. Our results, obtained by *in silico* analysis, demonstrated that all the mutations belonging to the "resistant" group are located very close to ATP binding site. It is likely that, for the steric hindrance due to the conformational change in the kinase domain (as a result of the aminoacidic change), the ATP-competitive inhibitors -such as PHA-665752 and JNJ-38877605- are unable to interact with their binding sites. One other possible mechanism of resistance in a mutated receptor can be due to the fact that the mutation alters the domain conformation and leads to a decrease in the affinity for the drug: this is likely to happen in the case of the mutant number 3, (which showed dose-dependent resistance) in which the mutation is localized outside the drug binding site.

In the era of *targeted therapies*, the phenomenon of resistance related to target's genetic mutations is extremely important in order to better treat tumors containing mutations. In the few lung tumors we examined, we found indeed a case presenting a MET^{PRC} mutation. Most importantly, the identified mutation (Y1230H) belongs to the "resistant" group. It is thus likely that treatment with a TKI of a patient bearing such a mutant receptor will not end with a favorable outcome.

At this point, we wondered if we could figure a therapeutic approach which could represent an alternative treatment for patients with tumors carrying MET mutants resistant to TKIs. Such an approach could be the use of a monoclonal antibody directed again the extracellular portion of the receptor, which is in the wild type conformation also in the MET^{PRC} mutants.

We thus treated cells expressing the MET^{PRC} mutants with the monomeric form of an anti-MET monoclonal antibody (FAb DN30). Indeed, we found that the antibody was

able to inhibit all TKI mutants, independently from their sensitivity to small TKIs. These results indicate that despite some mutated forms are unable to respond to small molecule TKIs, they still remain responsive to treatment with antibodies directed against the MET extracellular portion, likely because the extracellular domain is in the wild type conformation. In clinical terms, these data show that we can "bypass" resistance to TKIs by use of mAbs directed against the MET extracellular portion**.** In conclusion, our results indicate that in tumors harboring MET tyrosine kinase mutations that prevent or impair the interaction between the ATP-competitive TKIs and the receptor, the use of small molecule TKI could be inappropriate.

We propose that the use of specific anti-MET monoclonal antibodies (such as FAb DN30) can represent, a therapeutic alternative to treat TKIs-resistant tumors harboring mutations in the MET tyrosine kinase domain, Our data could thus help in better tailoring the anti-MET targeted therapies, thus contributing to increasing their effectiveness.

MATERIALS AND METHODS

Plasmid constructs and mutagenesis

pCEV29.1 expression vector was available in the lab wt MET cDNA was cloned in the vector as described (Giordano et al., 2000) and PRC mutations were introduced in by polymerase chain reaction (Bardelli, A. et al., 1998). Human MET residues are numbered according to Gene Bank# X54559 (Ponzetto, C. et al., 1991).

The human MET cDNA cloned in the plasmidic construct pRLL2 was available in the lab, then mutagenized by using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Thechnologies). The QuikChange II site-directed mutagenesis kit is used to make point mutations, replace amino acids, and delete or insert single or multiple adjacent amino acids. This mutagenesis' method was performed using PfuUltra high-fidelity (HF) DNA polymerase for mutagenic primer-directed replication of both plasmid strands with the highest fidelity. The basic procedure utilizes a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, both containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by PfuUltra HF DNA polymerase, without primer displacement. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with Dpn I. The Dpn I endonuclease (target sequence: 5′-Gm6ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. The nicked vector DNA containing the desired mutations is then transformed into competent cells.

Transfection, infection

COS-7 cells were transfected by the DEAE-dextran method. Briefly, plasmid DNA (2 μg/ml) was resuspended in DMEM containing DEAE-dextran and added to a 100-mm dish of subconfluent COS 7 cells. Dimethyl sulfoxide shock was performed after 4 h of incubation at 37°C.

Lentiviruses were produced by transient transfection of 293T cells whit the calcium-phosphate procedure containing the DNA to be transfected, as described elsewhere (Vigna and Naldini, 2000). Cell infection was performed over-night and in the presence of polybrene (hexadimethrine bromide). Polybrene is a relatively non-toxic polymer, that was shown to enhance the adsorption of virus complex onto cells in culture (Coelen et al., 1983).

Cell culture

293T, GTL16, A549, COS-7 and NIH 3T3 cell lines from ATCC were cultivated in DMEM (293T, NIH 3T3) or RPMI (GTL16 and A549) supplemented with 1% Q, 0.1% penicillin (5000U/ml, Faber), 0.1% streptomycin (5mg/ml, Squibb) and with 10% FBS or Calf Serum deactivated by heating (NIH 3T3), at 37°C in 5% CO_2.

Protein extraction and Western blot

For Western blot analysis, cells were lysed in boiling LB buffer [2% SDS, 0.5 mol/L Tris-HCl (pH 6.8)]. Protein concentration of whole-cell lysates was evaluated with the BCA Protein Assay kit (Pierce) and equal amounts of total proteins were analyzed by SDS-PAGE and Western blotting. Western blots were performed according to standard methods. The antibodies used were as follows: anti-MET antibody DL21 (Prat, M. et al. 1998) and anti-MET Zymed (Invitrogen), anti-phospho MET

Tyr1349/1356 (Cell Signaling Technology). Final detection was done with enhanced chemiluminescence (ECL) system (Amersham).

Biological assays

For invasion assays, cells were seeded in Transwell chamber, on the upper side of a porous polycarbonate membrane. The medium in both chambers was supplemented with low percentage serum; the lower chamber was supplemented with HGF (20 ng/ml) alone or in presence of JNJ-38877605 (Johnson and Johnson) [500] nM. After 16 h, cells attached on the upper side of the membrane were mechanically removed. Cells that migrated to the lower side were fixed with gluataraldehyde and stained with crystal violet. Stained cells were photographed.

For analysis of colony formation in soft agar, cells were diluted to a concentration of $7x10^3$ cells/ml in DMEM containing 10% FBS and 0.5% Seaplaque agar, with HGF (20 ng/ml), in presence or absence of JNJ-38877605. Cells were seeded in 12-well plates (1 ml per well) containing a 1% agar underlay and supplemented twice a week with DMEM containing 10% FBS and, where indicated, HGF and JNJ-38877605. Colonies were stained with tetrazolium salts three weeks after seeding and the viability was evaluated using the alamarBlue® cell viability reagent (Invitrogen). This reagent is used to assess cell viability by adding the 10X, ready-to-use solution to cells in culture media, followed by a 1–4 hours incubation at 37ºC (to allow cells to convert resazurin to resorufin). The resulting fluorescence was read on a 96-well plate reader.

For the wound healing assay, NIH 3T3 cells were plated to create a confluent monolayer. After scraping the cell monolayer with a p200 pipet tip, cells were washed

and the media replaced with DMEM 10% serum alone, or HGF (20 ng/ml), or HGF (20 ng/ml) in presence of JNJ-38877605. Cells dishes were incubated for 24-48 hrs. The migration assay was stopped when the wound was repaired, the cells were fixed in glutheraldeide then stained with crystal violet and photographed.

Tumor samples collection

Tumor samples were obtained in accordance with consent procedures approved by the Ethic Committee of Hospital San Giovanni Battista (Turin) and the University Of Cagliari. The follow-up reports, was adapted to the new guidelines in the 7th Edition of TNM in Lung Cancer of the International Association for the Study of Lung Cancer (IASLC).

RNA extraction, RT-PCR , PCR and sequencing

Total RNA was extracted from lung tumors using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA (500 ng) was retrotranscribed into cDNA using the High Capacity cDNA Reverse Transcription Kit containing the Multiscribe Reverse Transcriptase (Applied Biosystem).

The cDNA coding for the MET intracellular domain was amplified by polymerase chain reaction (PCR). To increase the PCR specificity, sensitivity and yield, without the need for lengthy optimizations and/or the redesigning of primers specificity, we performed the Touch Down PCR (TD-PCR) using the TaqGold polymerase (Applied). TD-PCR employs an initial annealing temperature above the projected melting temperature (Tm) of the primers being used, then progressively transitions to a lower, more permissive annealing temperature over the course of successive cycles.

Any difference in Tm between correct and incorrect annealing will produce an exponential advantage of twofold per cycle (Korbie & Mattick, 2008).

Then amplified segments were purified using the chemical method named Solid Phase Reversible Immobilization (SPRI) based on speed beads (Agencourt Ampure Xp Kit), and finally sequenced by the Sanger's method (Sanger F, Coulson AR , 1975).

The mutational analysis, was performed by using the Mutation Surveyor® DNA variant analysis of Sanger Sequencing software, manufactured by SoftGenetics (Pennsylvania, USA).

***In silico* analysis**

The *in silico* analysis of the MET tyrosine kinase domain, was performed using the software PyMOL. PyMOL is one of a few open source visualization tools available for use in structural biology (http://pymol.org/). The Py portion of the software's name refers to the fact that it extends, and is extensible by the Python programming language. Using PyMOL, the MET TK structure was mutagenized from: "X-Ray Structure Of PHA-00665752 Bound To The Kinase Domain Of c-Met", Source: NCBI Structure_ Protein Date Bank (PDB) 2WKM (see http://www.rcsb.org/pdb/explore.do?structureId=2wkm).

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