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TRANSLATIONAL RELEVANCE

Tivantinib is being currently under phase 3 evaluation in advanced HCC patients with MET-high expressing tumors, assuming that it was a highly selective MET inhibitor. However, results presented here in a large collection of liver cancer cell lines provide evidence that anti-proliferative effect of tivantinib has no relation with functional MET targeting but tivantinib behaves as an antimitotic drug more efficient in highly proliferative cells. In human primary HCC we found a large overlap between tumors overexpressing MET and proliferation markers. While the association was not complete, this overlap could explain tivantinib therapeutic responses previously reported in MET-high HCC patients while MET is not the proper target. However, we suggest that a surrogate marker of cell proliferation, such as Ki67, should be tested in tivantinib clinical trials to assess its predictive value in tumor response compared with MET expression.

ABSTRACT

Purpose: Tivantinib was initially reported as a selective MET inhibitor and is under phase 3 evaluation in "MET-high" hepatocellular carcinoma (HCC) patients. However, it has been also proposed as an antimitotic agent. We aimed to evaluate the anti-tumor effect of tivantinib in HCC cells by combining pharmacological and molecular profiling.

Experimental design: Sensitivity to tivantinib, JNJ-38877605, PHA-665752, vinblastine and paclitaxel was tested in a panel of 35 liver cancer cell lines analyzed with exome sequencing, mRNA expression of 188 genes and protein expression. Drug effect was investigated by western blot and mitotic index quantification. Expression of candidate biomarkers predicting drug response was analyzed in 310 HCC.

Results: Tivantinib sensitivity profiles in the 35 cell lines were similar to those obtained with antimitotic drugs. It induced blockage of cell mitosis and high cell proliferation was associated with sensitivity to tivantinib, vinblastine and placitaxel. In contrast, tivantinib did not suppress MET signaling and selective MET inhibitors demonstrated an anti-proliferative effect only in MHCC97H, the unique cell line displaying *MET* gene amplification. HCC tumors with high expression of cell proliferation genes defined a group of patients with poor survival. Interestingly, highly proliferative tumors also demonstrated high MET expression likely explaining better therapeutic response of MET-high HCC patients to tivantinib.

Conclusions: Tivantinib acts as an antimitotic compound and cell proliferation markers are the best predictors of its antitumor efficacy in cell lines. Ki67 expression should be tested in clinical trials to predict tivantinib response.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and a major cause of cancer death worldwide (1). HCC is a highly heterogeneous disease both at the clinical and molecular levels. Despite recent progress in treatment, the prognosis for HCC patients with advanced disease remains poor. The multikinase inhibitor sorafenib is currently the only approved standard first-line systemic therapy for advanced HCC, however, survival benefit is modest (2,3). Very recently, the multikinase inhibitor regorafenib has shown survival benefit in second-line in HCC patients progressing on sorafenib treatment (4). However, many of new agents tested in phase 3 clinical trials have failed to show improvement in patient clinical outcome. Most of these studies were conducted in unselected population of patients and have not taken into account the molecular diversity of HCC. Therefore, evaluation of biomarkers predictive of drug response in preclinical models and at an early stage of clinical development is crucial for the design of more efficient phase 3 trials, increasing chance of positive results.

Tivantinib (ARQ197) was initially described as a selective, non-ATP competitive, oral inhibitor of the MET tyrosine kinase receptor (5). In HCC, MET is activated by overexpression or in rare cases (1-4%) by gene amplification (6-8) and this aberrant expression/activation has been associated with poor prognosis (9). In addition, various preclinical studies in cell lines and animal models have provided evidence for the implication of MET in the pathogenesis of HCC (6). Consequently, MET has been regarded as a promising therapeutic target in HCC patients. Recently, tivantinib has demonstrated improved progression-free and overall survival in a randomize phase 2 second line study in a subgroup of advanced HCC patients with high expression of MET whereas no clinical benefit was observed in the low MET-expressing group (9). These encouraging results led to the development of the first biomarker-based phase 3 clinical trial in HCC and there are currently,

two phase 3 ongoing clinical trials (NCT01755767, METIV-HCC; NCT02029157, JET-HCC) evaluating tivantinib efficacy in a selected population of HCC patients with high MET-expressing tumors identified using immunohistochemistry. However, several studies have questioned the mechanism of action of the drug, as they provided strong evidence that tivantinib acts on microtubule dynamics independently of MET and behaves as an antimitotic agent (10-13). Consequently, these findings raise some concerns about the rational to use MET as a reliable predictive biomarker of tivantinib response and as a criteria for the inclusion of patients in clinical trials. Moreover, they lead to reconsider the role of selective MET inhibitors in the treatment of HCC.

The aims of the present study were 1) to better characterize tivantinib pharmacological activity and its relationship with MET signaling 2) to assess the role of selective MET inhibitors in growth inhibition of liver tumor cells 3) to identify biomarkers that may predict antitumor effect of tivantinib, selective MET inhibitors and antimitotic compounds. For this purpose we combined pharmacological and molecular profiling of a large collection of 35 human liver cancer cell lines and we validated potential biomakers predictive of drug response in a series of 310 primary HCC tumors including 281 resected HCC and 29 advanced HCC.

MATERIALS AND METHODS

Cell lines and tumors

We collected a series of 35 human liver cancer cell lines obtained from commercial sources (n=31) or from Bettina Grasl-Kraupp's laboratory (n=4) (14), derived from HCC (n=33) or hepatoblastoma (HepG2 and Huh6) (Supplementay Table 1). All the cells were adapted and grown in Dulbecco's modified Eagle's medium (DMEM) except JHH5 and JHH6 that were grown in William's E medium. Culture media were supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin and cells were maintained at 37 °C in a humidified incubator in 5% CO2. Cell lines were authenticated by exome sequencing and all the cells were mycoplasma-free, as tested using the MycoAlert Mycoplasma detection kit (Lonza).

A series of 310 HCC tumors associated with various etiologies were provided by the French network of hepatic tumor biobanks (BB-0033-00085) and informed consent was obtained from all subjects in accordance with French legislation. All clinical data are described in Supplementary Table 2. They included 281 patients surgically treated in France and previously analysed by whole exome sequencing (n=170) or targeted re-sequencing on at least 2 genes (*CTNNB1* and *TP53*, n=111) and these characterizations were previously described (7,15). An additional series of 29 partly frozen and partly formalin fixed paraffin embedded biopsies of advanced HCC, provided by the "liver disease biobank" (FR_BB-0033-00027) was analyzed in the present study (see below). Advanced HCC were defined as patients who were not eligible for curative therapies and who received only palliative treatments (100% of the cases were BCLC B or C, Supplementary Table 2). By contrast, resected HCC included only patients subjected to curative treatments (69% of the cases were BCLC O or A, Supplementary Table 2)

Drugs and cell viability assay

Tivantinib, JNJ-38877605, PHA-665752, vinblastine and paclitaxel were purchased from Selleck Chemicals and dissolved in DMSO at 10 mM concentration. Cells were seeded in 96-well plates at an optimal density (1500 to 3000 cells/well) to ensure that they were in exponential growth phase at the end of the experiment. After overnight incubation, cells were treated with 5 concentrations of each compound (10-fold dilution from 0.001 to 10 μM in duplicates) using the HP D300 digital dispenser (Tecan). Cell viability was measured 48 hours after drug treatment by colorimetric MTS assay following the supplier's recommendations (Promega). Each experiment was repeated at least two times for each cell line and results were normalized on untreated cells. Curve fitting of dose-response data was performed using GraphPad Prism 6 Software and the two following classical parameters representative of drug sensitivity were derived: 1) the GI50 corresponding to the concentration of drug that inhibits 50% of cell viability and 2) the AUC corresponding to the area under the dose-response curve that provides an overall measure of cumulative response. When the GI50 was not reached, the values were set to the highest concentration tested (10 μM).

Western-blot analysis

Cell protein extracts were prepared using RIPA lysis buffer containing protease and phosphatase inhibitors and quantified using the BCA Protein Assay kit (Pierce). Western-blot analyses were conducted using the following primary antibodies: MET (#8198), phospho-MET (Tyr1234/1235) (#3129), ERK1/2 (#9102), phospho-ERK1/2 (Thr202/Tyr204) (#9101), AKT (#9272), phospho-AKT (Ser473) (#9271) and β-actin (#4967) was used as loading control. Protein of interest were detected using an anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody (#7074) and the ECL Chemiluminescence Western Blotting

Detection Kit (GE Healthcare), according to the provided protocol. Signal detection was performed using the ChemiDoc XRS system and the Image Lab software (BioRad). All antibodies were purchased from Cell Signaling Technology and used at 1:1000 dilution except secondary antibody which was used at 1:2000.

Reverse-phase protein array

RPPA technology was used to quantify MET, phospho-MET Tyr1234-1235 and Ki67 protein level in the 35 liver cancer cell lines and 202 resected HCC as previously described (16). Briefly, equal amounts of protein lysates were printed onto nitrocellulose covered slides. Five serial dilutions and two technical replicates per dilution were deposited for each sample. Arrays were revealed with anti-MET (Sc-10), anti-phospho-MET Tyr1234-1235 (CST3129) and anti-Ki67 (Dako M7240) antibodies. Quantification and normalization of RPPA data were performed using the NormaCurve method (16).

Mitotic index analysis

Mitotic index was determined by fluorescent imaging microscopy using an anti-histone H3 phospho-ser10 antibody conjugated to the fluorescent dye Alexa488 (ab151282, abcam) and the nucleic stain DAPI. Before staining, cells were fixed in paraformaldehyde 4%, blocked and permeabilized according to the the supplier's recommendations. Mitotic index was calculated as the percentage of histone H3 (phospho Ser 10) positive cells relative to the total number of cells. A minimum of 100 cells was counted for each condition.

Quantitative RT-PCR

We analyzed total mRNA from 35 liver cell lines, 310 resected and advanced HCC and we assessed quality as previously described (17). mRNA levels were determined by analyzing 500 ng of total RNA reverse transcribed using the High Capacity Transcription kit (Life

technologies) and TaqMan predesigned assays (Life technologies), on Fluidigm 96.96 (San Francisco) dynamic arrays using the BioMark Real-Time PCR system. A panel of 188 genes was analysed (Supplementary Table 3). Expression data (Ct values) were calculated using the Fluidigm Real-Time PCR Analysis software (4.1.3). Gene expression data was expressed with the $2^{-\Delta\Delta CT}$ method relative to Ribosomal 18S (*R18S*) and the mean expression level of the corresponding gene in normal liver samples.

Mutation and copy-number analysis

The 35 liver cancer cell lines were analysed by whole-exome sequencing as previously described (7,18). Identification of putative somatic variants and copy number aberrations were identified as described in the Supplementary Material and methods in 12 genes frequently (>5%) altered in human HCC tumors (*TERT* promoter, *CTNNB1*, *TP53*, *ARID1A*, *AXIN1*, *CDKN2A*, *ARID2*, *RPS6KA3*, *NFE2L2*, *KEAP1*, *ALB* and *APOB*) (7,18) and in *MET*.

Immunohistochemistry

Expression of Ki-67 and MET was assessed by immunohistochemistry on paraffin-embedded tumor tissue sections using respectively, a MIB-1 antibody (Dako, Glostrup, Denmark, 1/100 dilution) and the CONFIRM anti-total MET (SP44) rabbit monoclonal primary antibody (Ventana Medical Systems, Tucson, AZ, USA, prediluted) directed against a membranous and/or cytoplasmic epitope present in human normal epithelial or tumor cells. The Ki-67 proliferation index was determined by counting a minimum of 100 tumor cells. MET membranous staining was assessed according to staining intensity (0, 1+, 2+, 3+) and percentage of cells stained. As previously described (9), samples that scored at least 2+ in at least 50% of tumor cells were regarded as having high MET expression (MET-high). When present, associated with membranous staining, cytoplasmic staining was recorded and was

considered positive when 2+ or 3+ staining was observed in more than 10% of tumor cells (19).

Survival

Disease-specific survival was defined as time from primary tumor resection to death from cancer progression and within 5 years of follow-up. We excluded patients with non-curative resections or liver transplantations and patients who died less than 2 months after surgery. Survival rates were determined using the Kaplan-Meier method and any difference in survival between groups was assessed by the log-rank test.

Statistical analysis

Continuous variables were compared using Mann-Whitney test for pairwise comparison or Kruskal-Wallis test for comparison of multiple groups. All reported *P*-values were two-tailed, and differences were considered significant when the *P*-value was <0.05.

RESULTS

Tivantinib does not target MET signalling

We analyzed tivantinib sensitivity in a panel of 35 liver cancer cell lines including 33 cell lines derived from HCC and 2 cell lines derived from hepatoblastoma (Supplementary Table 1). Cell lines were categorized according to clinical definition, as sensitive when the GI50 was below 6 µM, corresponding to the maximum clinically tolerated dose (20,21) and as resistant when the GI50 was higher or equal to 6 µM (Fig. 1A). Using this definition, tivantinib inhibited efficiently cell viability in 25 of the 35 liver cancer cell lines. The GI50 and AUC values for tivantinib showed strong correlation across the panel of cell lines (Spearman r=0.95, $P<0.0001$) (Fig. 1A). Of note, one hepatoblastoma (Huh6) was sensitive to tivantinib whereas the second (HepG2) was resistant.

No relationship between MET mRNA expression/activation or copy-number and tivantinib sensitivity was identified among the cell lines while only mild association was found with MET protein expression (Fig. 1A). Only one cell line (MHCC97H) demonstrated a *MET* gene amplification associated with the highest mRNA, protein expression and activation but showed similar sensitivity as the non-amplified sensitive cell lines (Fig. 1A). For comparison, in our panel of 35 liver cancer cell lines, we also investigated the ability of two selective MET inhibitors (PHA-665752 and JNJ-38877605) to reduce cell proliferation. Profiles of growth inhibition with the two selective MET inhibitors were highly correlated together (r=0.54; $P=0.0007$, Fig. 1B) but totally different when compared to tivantinib profile (Fig. 1B). The two selective MET inhibitors demonstrated strong inhibition of cell viability (GI50 around 0.1 µM) only in MHCC97H, the unique cell line harboring *MET* amplification whereas they had little or no effects on the other non-amplified cell lines, except HCC-3 that showed an intermediate sensitivity with PHA-665752 (GI50=1.2 µM) (Supplementary Fig.1). Of note,

HCC-3 cell line showed a 2-fold increased *MET* gene copy-number (Supplementary Fig. 1B). Accordingly, in the two HCC cell lines (MHCC97H and HCC-3) most sensitive to MET inhibitors, we showed by western-blot that tivantinib was not able to suppress MET signaling, while the two selective MET inhibitors (PHA-665752 and JNJ-38877605) decreased MET phosphorylation as well as phosphorylation of the downsteam signaling effectors AKT and ERK1/2 in a dose-dependant manner (Fig. 1C).

Tivantinib acts as antimitotic agents

As previous studies showed that tivantinib interfered with microtubule dynamics similarly to antimitotic drugs, we compared sensitivity profiles of tivantinib with those of two antimitotic compounds: paclitaxel and vinblastine. As expected, the profiles of sensitivity across the 35 liver cancer cell lines were highly correlated among the two antimitotic compounds (paclitaxel/vinblastine, r=0.8; $P<0.0001$) (Fig. 2A). Strinkingly, tivantinib sensitivity patterns were very similar to those of antimitotic drugs (r=0.57, $P=0.0003$ for paclitaxel and r=0.64, $P<0.0001$ for vinblastine Fig. 2A), suggesting close relationship between tivantinib and antimitotic drug mechanism of action. Then, as antimitotic drugs are known to induce a mitotic arrest, we investigated the effect of tivantinib on the mitotic index by immunofluorescence, using an anti-phospho histone H3 antibody that specifically stained cells in mitosis. As expected, mitotic index was markedly increased (around 15-fold) following treatment with the two mitotic inhibitors paclitaxel and vinblastine compared to the untreated control cells, and we observed similar increase when cells were treated with tivantinib (Fig. 2B). Collectively, our results strongly support the recent findings that tivantinib antitumor effect is mediated through antimitotic activity and not through the inhibition of MET signalling in liver tumor cell lines.

Expression of cell proliferation markers are associated with tivantinib sensitivity

In order to identify potential biomarkers predictive of tivantinib sensitivity we performed molecular profiling of key genes involved in hepatocarcinogenesis in our panel of 35 liver cancer cell lines. We analyzed mRNA expression of 188 genes by quantitative RT-PCR focusing on genes related to the main cancer hallmark processes such as proliferation, apoptosis and drug resistance, and on more specific genes frequently altered in hepatocellular tumors (Supplementary Table 3). In addition, we searched for mutations and copy-number variation by sanger and exome sequencing in the 12 genes most frequently (>5%) altered in human HCC tumors and in *MET* (7) (Supplementary Table 4). We only found a mild association between *ARID1A* mutations and lower tivantinib sensitivity among the 13 analyzed genes (Supplementary Table 5). Then, among the 188 genes tested in qRT-PCR, we identified 12 genes significantly differentially expressed, including 6 genes underexpressed and 6 genes overexpressed in the group of sensitive cell lines (n=25) compared to the group of resistant cell lines (n=10) (Fig 3A). Calculation of pairwise correlation coefficient between these 12 genes delineated a main group of five co-regulated genes overexpressed in the sensitive cell lines (Fig. 3B and Supplementary Fig. 2A). Strikingly, four of these five genes, (*CDC20*, *RRM2*, *GMNN* and *RAN*) were directly involved in the regulation of cell cycle progression at different phases (Fig. 3B). When using the AUC as response metric we confirmed the significant association between mRNA expression of *CDC20*, *RRM2* and *GMNN* and tivantinib sensitivity (Fig. 3C and Supplementary Fig. 2B) while the association did not reach significance for *TAF9* (Spearman r=-0.29, *P*=0.09) and *RAN* (Spearman r=-0.26, *P*=0.13) (Supplementary Fig. 2B). Altogether, by combining results obtained with the two dose-response parameters GI50 and AUC, high mRNA expression of the three cell proliferation genes *CDC20*, *RRM2* and *GMNN* emerged as the best predictors of tivantinib sensitivity. Interestingly, mRNA levels of these three genes also showed good correlation

with sensitivity to the mitotic inhibitors paclitaxel and vinblastine while they had no predictive value for effectiveness of the two selective MET inhibitors (Fig. 3C). These findings reinforce the link between tivantinib and antimitotic mechanism of action and led us to hypothesize that the proliferation rate could predict tivantinib sensitivity in liver tumor cells. To test this hypothesis, we modulated growth rate of two HCC cell lines (HLE and SNU878) highly sensitive to tivantinib using three concentrations of fetal bovin serum (FBS) in culture medium (from 10% to 0.1%). As expected, when reducing FBS concentration we showed a decrease in cell proliferation associated with underexpression of the three cell proliferation genes *CDC20*, *RRM2* and *GMNN* as well as *MKI67* another classical proliferation marker (Fig. 3D). According to our hypothesis, tivantinib sensitivity was completely reversed at the lowest proliferation rates in both cell lines with GI50 increasing around 25-fold between the basal condition (10% FBS) and the lowest FBS concentration (0.1%) (Fig. 3D). We observed similar shift in GI50 when cells were treated with the antimitotic drug paclitaxel (Fig. 3D). However, cell proliferation rate did not impact sensitivity to the MET inhibitor PHA-665752. Taken together, our results suggested that tivantinib sensitivity is highly dependent on the rate of cell proliferation similarly to antimitotic drugs and mRNA expression of proliferation markers could be good predictors of its antitumor efficacy.

Proliferation genes and MET are coregulated in HCC primary tumors

As we identified proliferation genes as the best predictors of tivantinib sensitivity in cell lines, we hypothesized that MET behaved as a proliferation marker in human primary HCC tumors, likely explaining the positive association found in HCC patients between MET expression and tivantinib antitumor activity, although MET is not the target of tivantinib. To test this hypothesis, we analyzed a series of 281 resected HCC and 29 biopsies of advanced stage

HCC. As identified in cell lines, in the two series of HCC, we showed that *CDC20*, *RRM2*, *GMNN* and *MKI67* proliferation genes were coregulated (Fig. 4A). Moreover, in both resected and advanced HCC, we identified a close correlation between mRNA expression of each of the four proliferation markers and *MET* (Fig. 4A). Accordingly, protein expression analysis of MET and Ki67 by RPPA in 202 resected HCC confirmed the higher expression of Ki67 in the group of high-MET expressing HCC compared to the MET-low subgroup (Fig. 4A). Interestingly, stratifying HCC according to the low or high mRNA expression of proliferation markers, revealed significant association between high expression and shorter disease-specific survival in resected HCC patients (Fig. 4B). Moreover, highly proliferative tumors also demonstrated significant higher MET expression (Fig. 4B).

Then, we used immunohistochemistry to study the relationship between proliferation index assessed by Ki67 staining and MET expression in our series of advanced HCC. Tumors were categorized as MET-high or MET-low using the same criteria as previously defined in the second-line tivantinib phase 2 trial of advanced HCC from (9). In accordance with this study, in our series of 29 advanced HCC, we found 48% (14/29) of patients with MET-high tumors. Among them, 4 cases (14%) showed strong cytoplasmic expression associated with high membranous MET staining and define a subgroup of HCC with higher Ki67 proliferation index (Fig. 5).

Collectively, our results confirmed in HCC primary tumors the link between the overexpression of MET and proliferation markers, likely explaining better therapeutic response of MET-high HCC patients to tivantinib.

DISCUSSION

In the present study, we examined a large collection of liver cancer cell lines to better characterize tivantinib antitumor activity and identify biomarkers predictive of its sensitivity. Using this panel, we demonstrated that the pharmacologic activity of tivantinib did not involve MET inhibition but was rather mediated through antimitotic effect. Several lines of evidence converge towards this conclusion. First, we showed that sensitivity patterns of tivantinib among cell lines were unrelated to those of authentic selective MET inhibitors, while they closely resembled to those of antimitotic compounds. Secondly, tivantinib was not able to suppress MET kinase activity and dowstream signaling and induced mitotic blockade similarly to antimitotics, while MET inhibitors are known to preferentially induce G0-G1 arrest (22-24). Thus, our findings strengthen those of previous studies conducted in various cell-based assays and tumor xenografts showing that tivantinib inhibited cell proliferation and tumor growth independantly of MET by disturbing microtubule dynamics (10-13). Moreover, a recent case report described one patient with a MET-mutated papillary renal cell cancer who responded to the MET inhibitor crizotinib while tivantinib treatment resulted in rapid disease progression (25). This observation suggested that tivantinib may not be an effective MET inhibitor also in the clinical setting and sustain our results obtained in preclinical models as well as previous ones.

Initially, tivantinib was identified as a MET inhibitor in a kinase assay (5), but subsequent work showed that it was able to bind only to the inactive unphosphorylated-MET and has no direct effect on the MET kinase activity (26). Next, several studies (5,27-30), performed *in cellulo*, have shown a decreased phosphorylation of MET after a long-time exposure (24h) to tivantinib contrasting with our results and others that were obtained with a short-time (4-6h) exposure in hepatocellular or other types of tumor cells (11-13). Indeed, after 24 hours of tivantinib exposure, a decreased in both phospho- and total-MET proteins was shown by

western blot in most of the cell models (27-29). This observation could be non-specifically related to tivantinib but interpreted as a non-specific consequence of the decreased cell viability. In the same line, in tumor biopsies of patients treated with tivantinib (21), decreased MET and phospho-MET could be indirectly related to the anti-tumor effect of tivantinib.

Here, our study provides new evidence that anti-proliferative effect of tivantinib has no relation with functional MET targeting. Furthermore, interestingly, we also showed that expression of proliferation markers were the best predictors of tivantinib response in our cell line models and we demontrated that high proliferation rates were associated with greater sensitivity, while lower proliferation rates rendered liver tumor cells more resistant to the drug. We found similar association when compared with the mitotic inhibitors paclitaxel and vinblastine which is consistent with the well-known ability of microtubule-targeted agents to preferentially target rapidly proliferating cells.

Thus, our results contrast with the recent clinical findings of Santoro *et al.* suggesting that immunohistochemical overexpression of MET was a good predictor of tivantinib therapeutic efficacy in advanced stage HCC (9). Interestingly, by analysing a large collection of human primary HCC, we showed that tumors with high expression of cell proliferation markers also exhibited higher expression of MET both at mRNA and protein level. We confirmed this association using immunohistochemistry in a series of avanced HCC. Although, in our series, there was no relationship between membranous expression of MET and Ki67 proliferation index, we found a significant association between MET membranous and cytoplasmic staining and a high Ki67 mitotic index. While MET cytoplasmic staining was not taken into account in Santoro's study, a recent work in gastric carcinoma demonstrated that interpretation of both membranous and cytoplasmic MET staining was more accurate to assess MET overexpression (19).

Taken together, our findings could explain the better tivantinib therapeutic response previously reported in MET-high HCC patients while MET is not the proper target (9).

However, even if our study revealed a significant overlap between HCC overexpressing MET and proliferation markers, association was not complete. Thereby, because our study identified proliferation markers as the best predictors of tivantinib sensitivity, we suggest that Ki67, a routinely used immunohistochemical proliferation marker, could be more accurate than MET to predict tivantinib sensitivity and should be evaluated in the ongoing phase 3 clinical trials. We also showed that high expression of cell proliferation genes defined a subgroup of HCC patients with poor survival. These results may have also important clinical implications as tivantinib would be more efficient in more aggressive HCC.

Ki67 expression has not been used so far to predict therapeutic response in HCC. However, numerous studies have shown that high expression of Ki67 was a good predictor of sensitivity to neoadjuvant chemotherapy in breast cancer, suggesting that Ki67 could be also a reliable biomarker to predict tivantinib response in HCC (31-33).

HCC is known as a relatively chemoresistant tumor and classical systemic agents targeting microtubules such as paclitaxel have shown high toxicity and absence of antitumor effect in clinical trials (34). It has been reported that overexpression of ABC transporters could be responsible for acquired resistance to chemotherapy in HCC (35,36). In particular, overexpression of MDR1/P-glycoprotein has been shown to confer resistance to various microtubule inhibitors by facilitating drug efflux (37). Interestingly, two studies demonstrated that tivantinib sensitivity was not affected by MDR1 overexpression likely explaining why tivantinib may be clinically more efficient than commonly used antimitotic drugs (13,38). Thus, tivantinib appears as a promising new chemotherapy for the treatment of HCC as it is well tolerated with limited neurotoxicity (39) and may overcome resistance caused by the overexpression of ABC transporters, compared to conventional antimitotic agents.

An other aim of our study was to evaluate and redefine the role of autentic selective MET inhibitors in the treatment of HCC. In our panel of 35 liver cancer cell lines the only cell line that demonstrated sensitivity to selective MET inhibitors was *MET*-amplified while the non-amplified cell lines were unresponsive. Accordingly, in other cancer types such as gastric and lung cancers MET oncogenic addiction and susceptibility to MET inhibitors, were reported only in tumor cells harboring *MET* gene amplification (22,40,41). In HCC, *MET* amplification is an infrequent event accounting for 1-4% of the cases depending on the studies (7,8). Thus, *MET* amplification may identify a subset of rare HCC patients that may benefit from anti-MET therapy. Of note, the selective MET inhibitor JNJ-38877605 has been recently tested in phase 1 clinical trial and showed renal toxicity precluding further clinical development (42).

In conclusion, this work enabled to clarify the antitumor activity of tivantinib and selective MET inhibitors in HCC. We suggest that tivantinib should be definitely reclassified as an antimitotic agent and should no longer be considered as a MET inhibitor. Moreover, we identified Ki67 as a potential new biomarker predictive of tivantinib response that may help to refine selection of patients who may benefit from tivantinib treatment. However, the predictive value of Ki67 remains to be evaluated in clinical trials.

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FIGURES LEGENDS

Figure 1. Tivantinib does not inhibit MET function. A, Sensitivity of 35 liver cancer cell lines to tivantinib. The heatmaps below represent for each cell line (columns) tivantinib sensitivity using the AUC, and MET status at the mRNA (q-RT-PCR), protein (RPPA) and genomic (copy number analyzed by exome sequencing) levels. AUC of 1 represents no drug response. Copy-number, mRNA and protein levels for each cell line are expressed relative to the mean value of normal non-cirrhotic liver tissues. Associations between tivantinib AUC and MET status were analyzed using Spearman's test. Represented below the heatmap, is chromosomal aberrations identified in the MHCC97H cell line showing focal amplification of *MET* gene. B, Scatter plots showing correlations between sensitivity patterns assessed by the AUC of two selective MET inhibitors (PHA-665752 and JNJ-38877605) and tivantinib among 35 liver cancer cell lines. Correlation for each drug pair was assessed using Spearman's test. C, Western-blot analysis of MET phosphorylation and its downstream effectors AKT and ERK1/2 in two MET-dependant cell lines (MHCC97H and HCC-3) treated 4 hours with increasing doses of PHA-665752, JNJ-38877605 or tivantinib.

Figure 2. Tivantinib behaves as an antimitotic agent. A, Scatter plots showing correlations between sensitivity patterns assessed by the AUC of two antimitotic drugs (paclitaxel and vinblastine) and tivantinib among 35 liver cancer cell lines. B, Effect of tivantinib on the mitotic index was compared with the antimitotic drugs paclitaxel and vinblastine after overnight treatment of the HLE cell line with two different concentrations of each drug.

Figure 3. Cell proliferation rate and expression of proliferation markers predict tivantinib sensitivity in liver cancer cell lines. A, Volcano plot of mRNA expression of 188 genes comparing tivantinib sensitive (n=25) and resistant (n=10) cell lines according to the GI50. Red and blue dotes indicate respectively genes significantly overexpressed and

underexpressed in cell lines sensitive to tivantinib. The horizontal dashed line indicates the negative logarithm of *P*-value threshold (0.05). B, (Left panel) Group of five coregulated genes overexpressed at the mRNA level in tivantinib sensitive cell lines, correlations between pairs of genes were assessed using Spearman's test. (Right lower panel) The heatmap represents standardized mRNA expression values (z-score) (row) for the five genes across the 35 liver cancer cell lines panel (column). (Right upper panel) Four of the five genes are involved in cell cycle regulation. C, Scatter plots representing correlation between mRNA expression of the three proliferation genes *CDC20*, *RRM2* and *GMNN* and AUC sensitivity parameter for tivantinib, 2 antimitotic drugs and 2 selective MET inhibitors, across the panel of 35 liver cancer cell lines. Correlation significance was assessed using Spearman's test. D, Effect of the proliferation rate on tivantinib, paclitaxel (antimitotic) and PHA-665752 (anti-MET) sensitivity in two HCC cell lines grown in culture medium containing decreasing concentrations of FBS. For each concentration of FBS three parameters are shown: 1) cell viability assessed by MTS assay (bar chart left axis, lower panel, 9 replicates per FBS concentration), 2) mRNA expression of four proliferation markers quantified by qRT-PCR 3) drug sensitivity measured by the GI50 (dots right axis, lower panel). Shown is one representative experiment out of two independant experiments.

Figure 4. Expression of proliferation markers correlates with MET expression and survival in HCC. A, (Upper panel) Spearman's pairwise correlations between mRNA expression of 5 genes including 4 proliferation genes and MET was analyzed in 281 resected HCC (left panel) and in a series of 29 biopsies of advanced HCC (right panel). Scatter plots below show correlation between *MKI67* and *MET* mRNA. Ki67 protein expression was compared between the low (n=101) and high (n=101) MET protein expressing groups of resected HCC, as defined by the median protein level of MET in the whole series. B, Kaplan-Meier curves for disease-specific survival (DSS) in 250 patients with R0 resected HCC

stratified by the median mRNA expression level of four different proliferation genes. Corresponding MET expression according to this stratification is shown on the left of each survival plot. Comparisons between groups were assessed using a Mann-Whitney test. mRNA expression levels were quantified by qRT-PCR and protein by RPPA, results for each tumor (T) were normalized on the mean expression value of normal liver tissues (N).

Figure 5. Immunohistochemical expression of Ki67 and MET are associated in advanced HCC. A, Representative immunostaining patterns of MET and Ki67 in advanced HCC biopsy samples. Upper panel: a well differentiated HCC without MET staining (membranous and cytoplasmic score 0). Ki67 proliferation index was low (6%). Middle panel: a MET-high well differentiated HCC showing MET membranous staining scored 2 in more than 50% of tumor cells, without cytoplasmic staining. Ki67 proliferation index was intermediate 19%. Lower panel: a MET-high poorly differentiated HCC showed cytoplasmic and membranous MET staining of tumor cells. Ki67 proliferation index was high (46%). B, Ki67 proliferation index according to MET expression assessed by immunohistochemistry in biopsies of 29 advanced HCC. MET expression was categorized in 3 groups with low or high membranous staining alone or with both high membranous and cytoplasmic staining. Comparison between groups was assessed using a Kruskall-Wallis test.

Figure 1

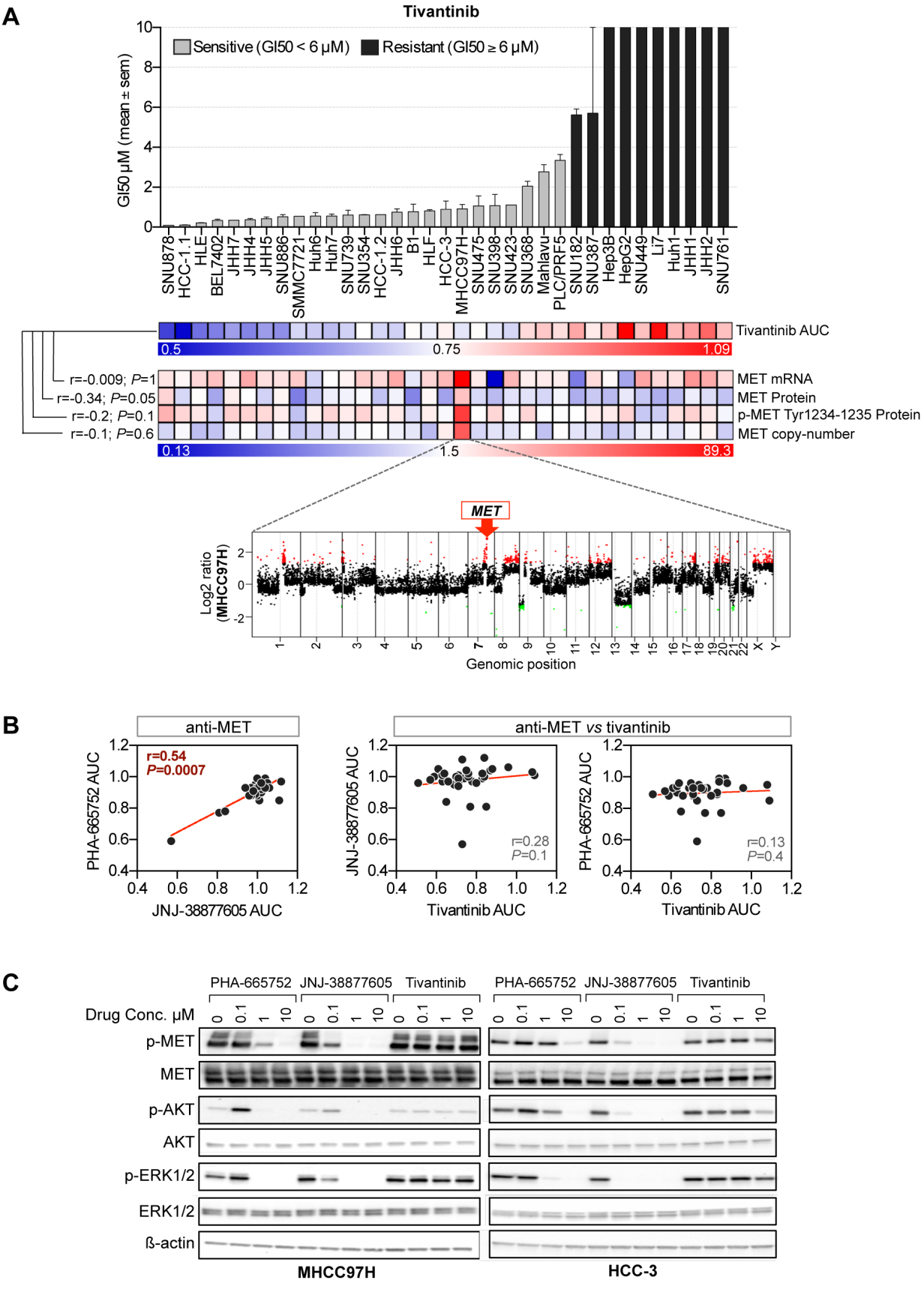

A
Tivantinib
Sensitive (GI50 < 6 µM)
Resistant (GI50 ≥ 6 µM)
GI50 µM (mean ± sem)
SNU878
HCC-1.1
HLE
BEL7402
JHH7
JHH4
JHH5
SNU886
SMMC7721
Huh6
Huh7
SNU739
SNU354
HCC-1.2
JHH6
B1
HLF
HCC-3
MHCC97H
SNU475
SNU398
SNU423
SNU368
Mahlavu
PLC/PRF5
SNU182
SNU387
Hep3B
HepG2
SNU449
Li7
Huh1
JHH1
JHH2
SNU761
Tivantinib AUC
0.5
0.75
1.09
r=-0.009; P=1
r=-0.34; P=0.05
r=-0.2; P=0.1
r=-0.1; P=0.6
MET mRNA
MET Protein
p-MET Tyr1234-1235 Protein
MET copy-number
0.13
1.5
89.3
MET
Log2 ratio (MHCC97H)
Genomic position
B
anti-MET
anti-MET vs tivantinib
PHA-665752 AUC
JNJ-38877605 AUC
r=0.54
P=0.0007
JNJ-38877605 AUC
Tivantinib AUC
r=0.28
P=0.1
PHA-665752 AUC
Tivantinib AUC
r=0.13
P=0.4
C
PHA-665752
JNJ-38877605
Tivantinib
Drug Conc. µM
p-MET
MET
p-AKT
AKT
p-ERK1/2
ERK1/2
ß-actin
MHCC97H
HCC-3

Figure 2

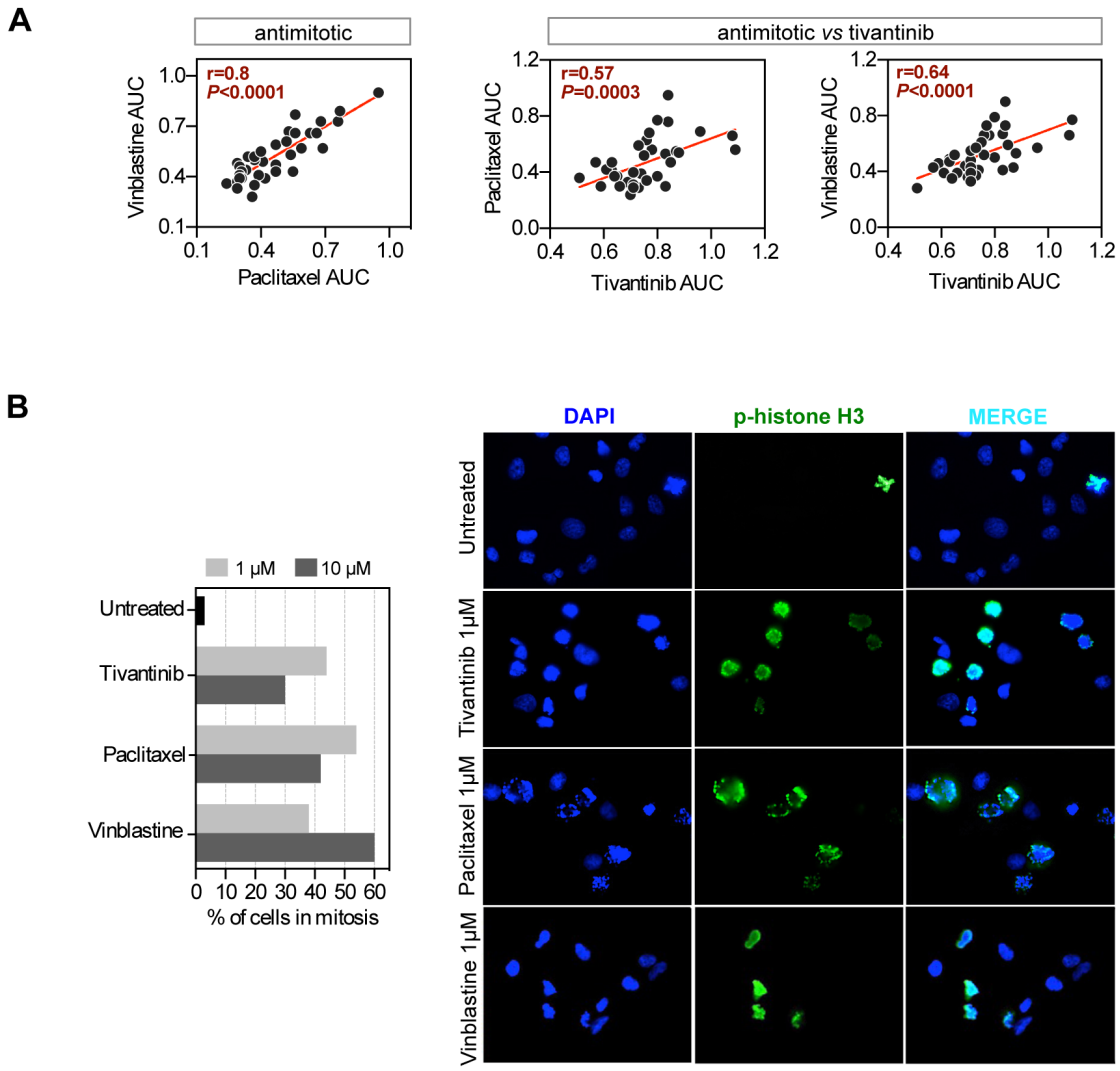

A
antimitotic
antimitotic vs tivantinib
r=0.8
P<0.0001
Vinblastine AUC
Paclitaxel AUC
r=0.57
P=0.0003
Paclitaxel AUC
Tivantinib AUC
r=0.64
P<0.0001
Vinblastine AUC
Tivantinib AUC
B
1 μM
10 μM
Untreated
Tivantinib
Paclitaxel
Vinblastine
% of cells in mitosis
DAPI
p-histone H3
MERGE
Untreated
Tivantinib 1μM
Paclitaxel 1μM
Vinblastine 1μM

Figure 3

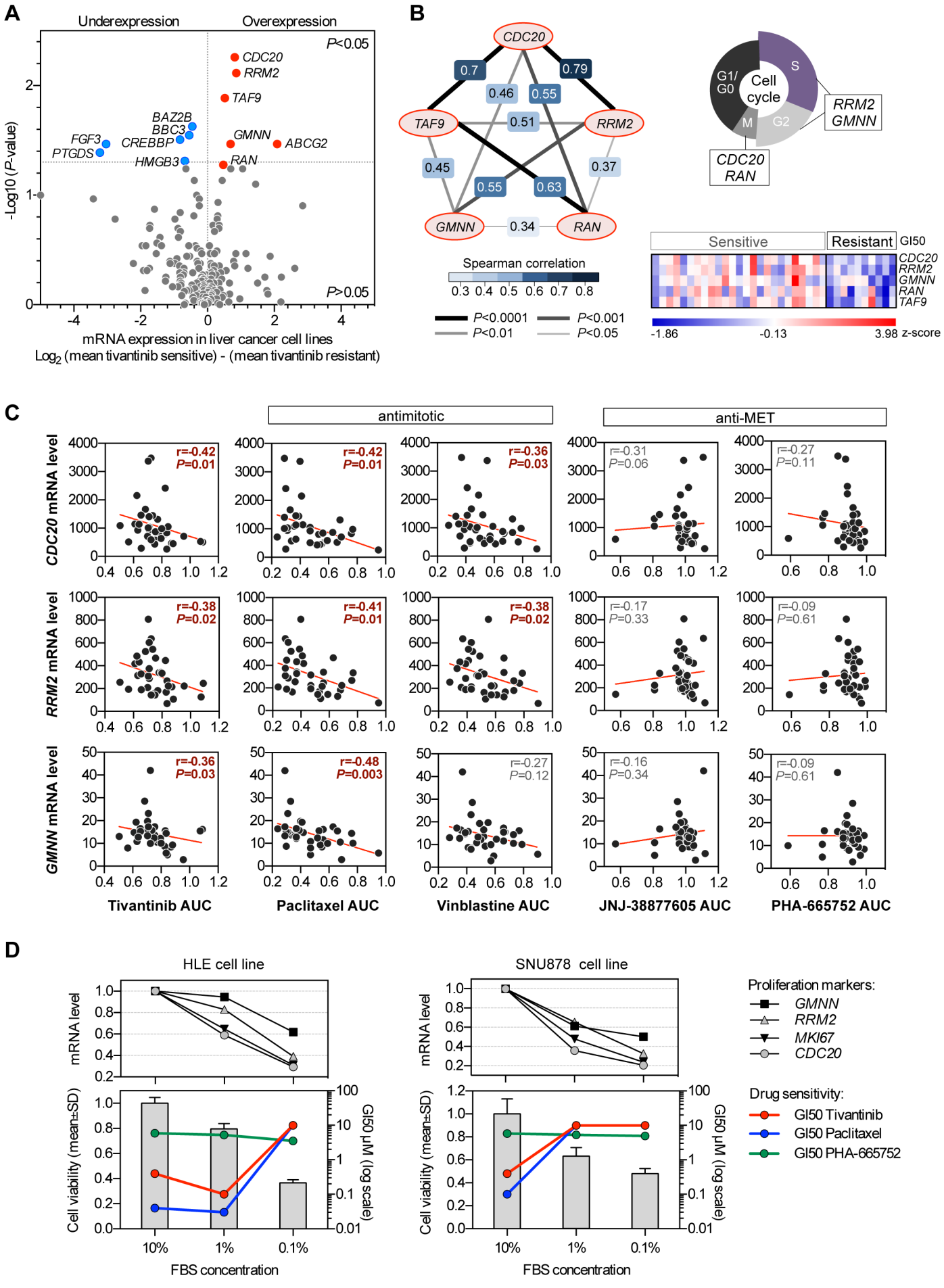
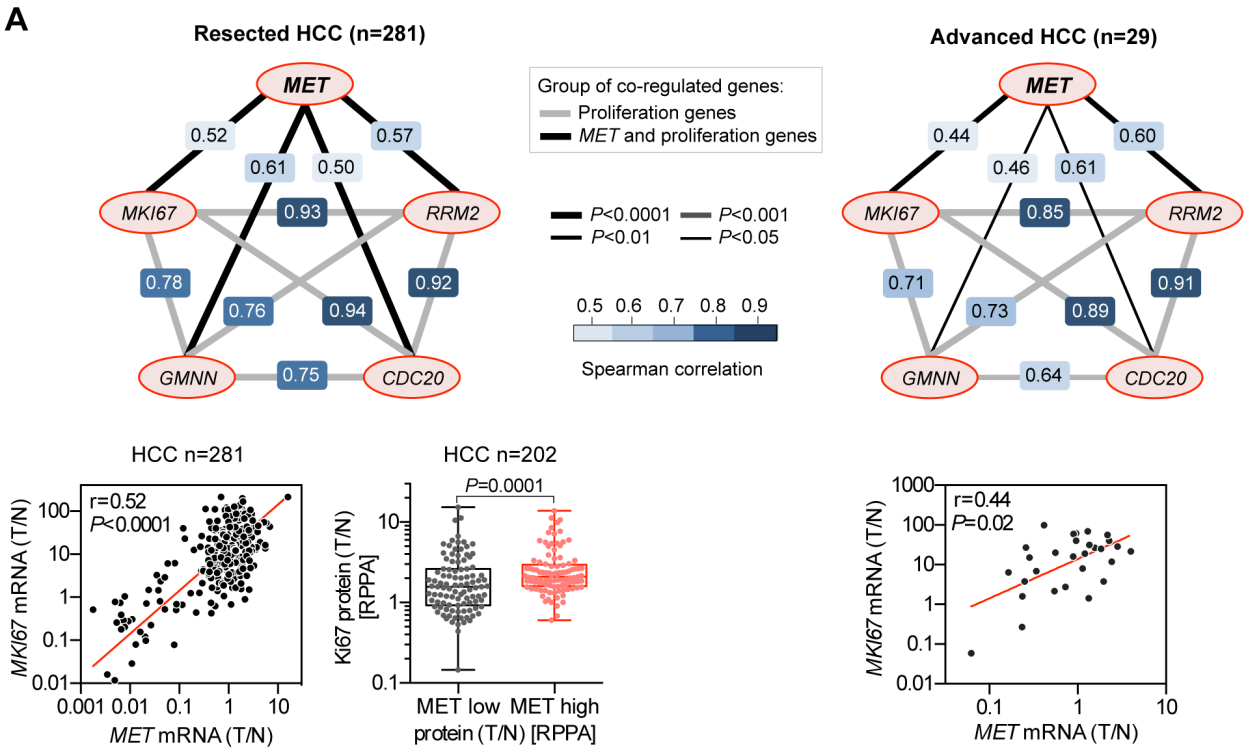

A
Underexpression
Overexpression
P<0.05
P>0.05
CDC20
RRM2
TAF9
BAZ2B
BBC3
FGF3
CREBBP
GMNN
ABCG2
PTGDS
HMGB3
RAN
-Log10 (P-value)
mRNA expression in liver cancer cell lines
Log2 (mean tivantinib sensitive) - (mean tivantinib resistant)
B
CDC20
TAF9
RRM2
GMNN
RAN
0.7
0.79
0.46
0.55
0.51
0.45
0.37
0.55
0.63
0.34
Spearman correlation
0.3 0.4 0.5 0.6 0.7 0.8
P<0.0001
P<0.001
P<0.01
P<0.05
G1/ G0
S
Cell cycle
M
G2
RRM2 GMNN
CDC20 RAN
Sensitive
Resistant
GI50
CDC20
RRM2
GMNN
RAN
TAF9
-1.86
-0.13
3.98 z-score
C
antimitotic
anti-MET
CDC20 mRNA level
RRM2 mRNA level
GMNN mRNA level
r=-0.42 P=0.01
r=-0.42 P=0.01
r=-0.36 P=0.03
r=-0.31 P=0.06
r=-0.27 P=0.11
r=-0.38 P=0.02
r=-0.41 P=0.01
r=-0.38 P=0.02
r=-0.17 P=0.33
r=-0.09 P=0.61
r=-0.36 P=0.03
r=-0.48 P=0.003
r=-0.27 P=0.12
r=-0.16 P=0.34
r=-0.09 P=0.61
Tivantinib AUC
Paclitaxel AUC
Vinblastine AUC
JNJ-38877605 AUC
PHA-665752 AUC
D
HLE cell line
SNU878 cell line
mRNA level
Cell viability (mean±SD)
GI50 µM (log scale)
10%
1%
0.1%
FBS concentration
Proliferation markers:
GMNN
RRM2
MKI67
CDC20
Drug sensitivity:
GI50 Tivantinib
GI50 Paclitaxel
GI50 PHA-665752

Figure 4


A
Resected HCC (n=281)
Advanced HCC (n=29)
MET
MKI67
RRM2
GMNN
CDC20
0.52
0.57
0.61
0.50
0.93
0.78
0.92
0.76
0.94
0.75
0.44
0.60
0.46
0.61
0.85
0.71
0.91
0.73
0.89
0.64
Group of co-regulated genes:
Proliferation genes
MET and proliferation genes
P<0.0001
P<0.001
P<0.01
P<0.05
0.5 0.6 0.7 0.8 0.9
Spearman correlation
HCC n=281
r=0.52
P<0.0001
MKI67 mRNA (T/N)
MET mRNA (T/N)
HCC n=202
P=0.0001
Ki67 protein (T/N) [RPPA]
MET low MET high
protein (T/N) [RPPA]
r=0.44
P=0.02
MKI67 mRNA (T/N)
MET mRNA (T/N)

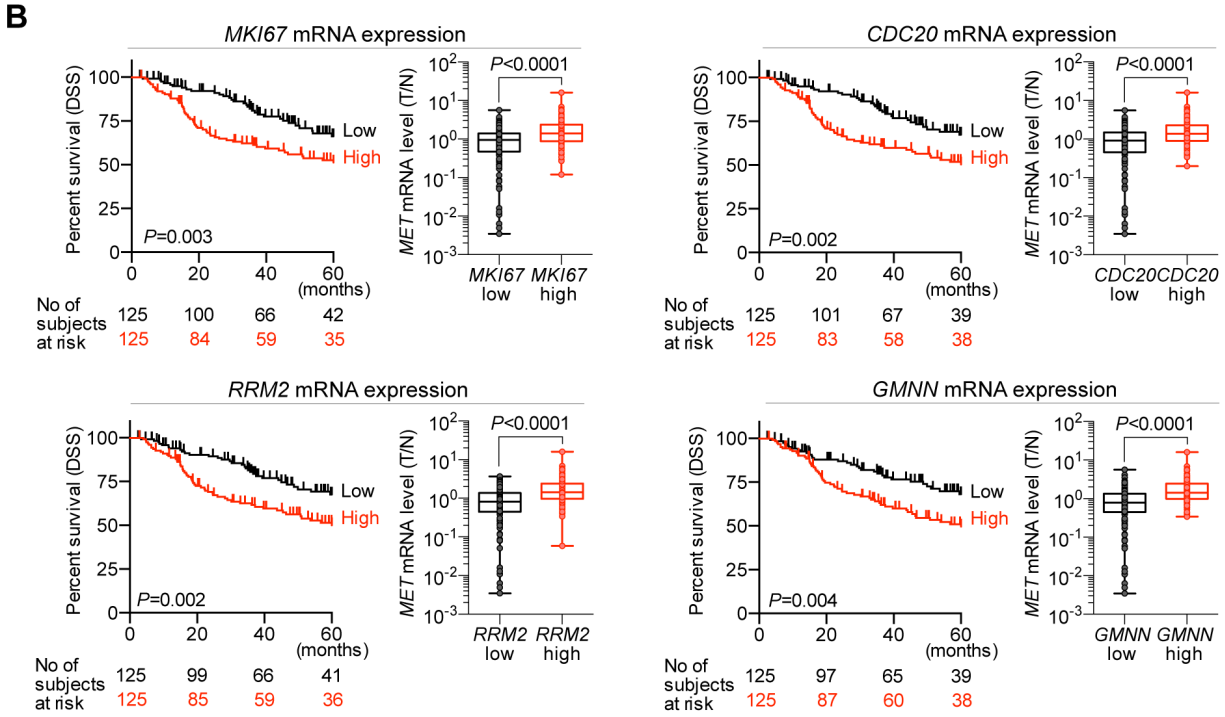
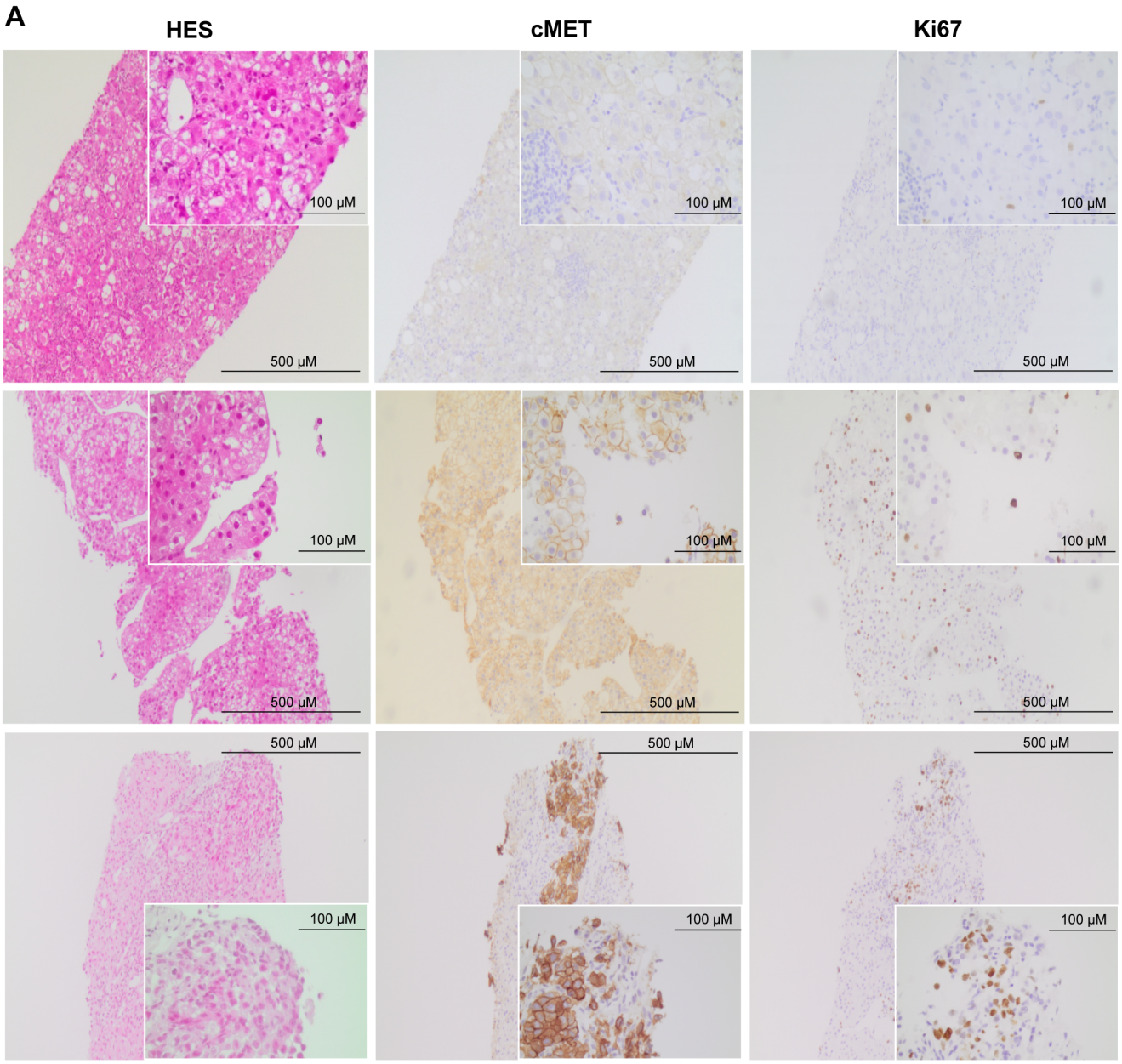
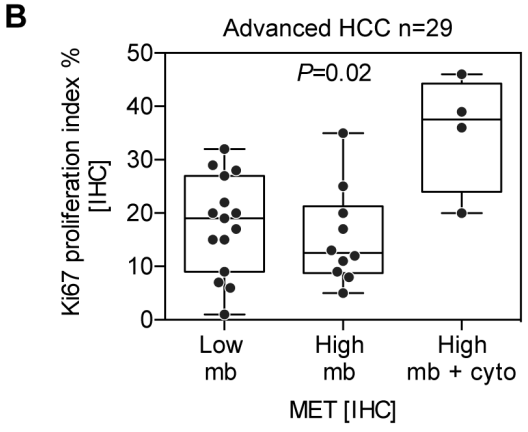

B
MKI67 mRNA expression
Percent survival (DSS)
Low
High
P=0.003
(months)
No of subjects at risk
125 100 66 42
125 84 59 35
MET mRNA level (T/N)
P<0.0001
MKI67 low
MKI67 high
CDC20 mRNA expression
P=0.002
125 101 67 39
125 83 58 38
CDC20 low
CDC20 high
RRM2 mRNA expression
P=0.002
125 99 66 41
125 85 59 36
RRM2 low
RRM2 high
GMNN mRNA expression
P=0.004
125 97 65 39
125 87 60 38
GMNN low
GMNN high

Figure 5


A
HES
cMET
Ki67
100 µM
500 µM


B
Advanced HCC n=29
P=0.02
Ki67 proliferation index % [IHC]
50
40
30
20
10
0
Low mb
High mb
High mb + cyto
MET [IHC]