Clinical Cancer Research

Proliferation Markers Are Associated with MET Expression in Hepatocellular Carcinoma and Predict Tivantinib Sensitivity *In Vitro*

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Abstract

Purpose: Tivantinib was initially reported as a selective MET inhibitor and is under phase III evaluation in "MET-high" hepatocellular carcinoma (HCC) patients. However, it has been also proposed as an antimitotic agent. We aimed to evaluate the antitumor effect of tivantinib in HCC cells by combining pharmacologic 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 analysis and mitotic index quantification. Expression of candidate biomarkers predicting drug response was analyzed in 310 HCCs.

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 paclitaxel. In contrast, tivantinib did not suppress MET signaling, and selective MET inhibitors demonstrated an antiproliferative 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. *Clin Cancer Res; 23(15); 4364–75. ©2017 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and a major cause of cancer-related 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 the new agents tested in phase III clinical trials have failed to show an 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 III 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

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Translational Relevance

Tivantinib is being currently under phase III evaluation in advanced hepatocellular carcinoma (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 antiproliferative 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. Although 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.

and overall survival in a randomize phase II 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 III clinical trial in HCC, and there are currently, two phase III 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 IHC. 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 rationale to use MET as a reliable predictive biomarker of tivantinib response and as a criterion 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 this study were (i) to better characterize the pharmacologic activity of tivantinib and its relationship with MET signaling; (ii) to assess the role of selective MET inhibitors in growth inhibition of liver tumor cells; and (iii) to identify biomarkers that may predict antitumor effect of tivantinib, selective MET inhibitors and antimitotic compounds. For this purpose, we combined pharmacologic and molecular profiling of a large collection of 35 human liver cancer cell lines, and we validated potential biomarkers 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 B. Grasl-Kraupp's laboratory ($n = 4$; ref. 14), derived from HCC ($n = 33$) or hepatoblastoma (HepG2 and Huh6; Supplementary Table S1). All the cells were adapted and grown in DMEM except JHH5 and JHH6 that were grown in William's E medium. Culture media were supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, and cells were maintained at 37°C in a humidified incubator in 5% CO_2. 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 S2. They included 281 patients surgically treated in France and previously analyzed by whole-exome sequencing ($n = 170$) or targeted resequencing on at least two genes (*CTNNB1* and *TP53*, $n = 111$), and these characterizations were described previously (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 this study (see below). Advanced HCCs were defined as patients who were not eligible for curative therapies and who received only palliative treatments [100% of the cases were Barcelona Clinic Liver Cancer (BCLC) B or C, Supplementary Table S2]. In contrast, resected HCC included only patients subjected to curative treatments (69% of the cases were BCLC O or A, Supplementary Table S2).

Drugs and cell viability assay

Tivantinib, JNJ-38877605, PHA-665752, vinblastine, and paclitaxel were purchased from Selleck Chemicals and dissolved in DMSO at 10 mmol/L concentration. Cells were seeded in 96-well plates at an optimal density (1,500 to 3,000 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 μmol/L 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 twice 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: (i) the GI_{50} corresponding to the concentration of drug that inhibits 50% of cell viability and (ii) the AUC corresponding to the area under the dose–response curve that provides an overall measure of cumulative response. When the GI_{50} was not reached, the values were set to the highest concentration tested (10 μmol/L).

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), and phospho-AKT (Ser473; #9271), and β-actin (#4967) was used as the loading control. Proteins of interest were detected using an anti-rabbit IgG horseradish peroxidase–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 (Bio-Rad). All antibodies were purchased from Cell Signaling Technology

and used at 1:1,000 dilution except secondary antibody, which was used at 1:2,000.

Reverse-phase protein array

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 described previously (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 Norma-Curve 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 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.

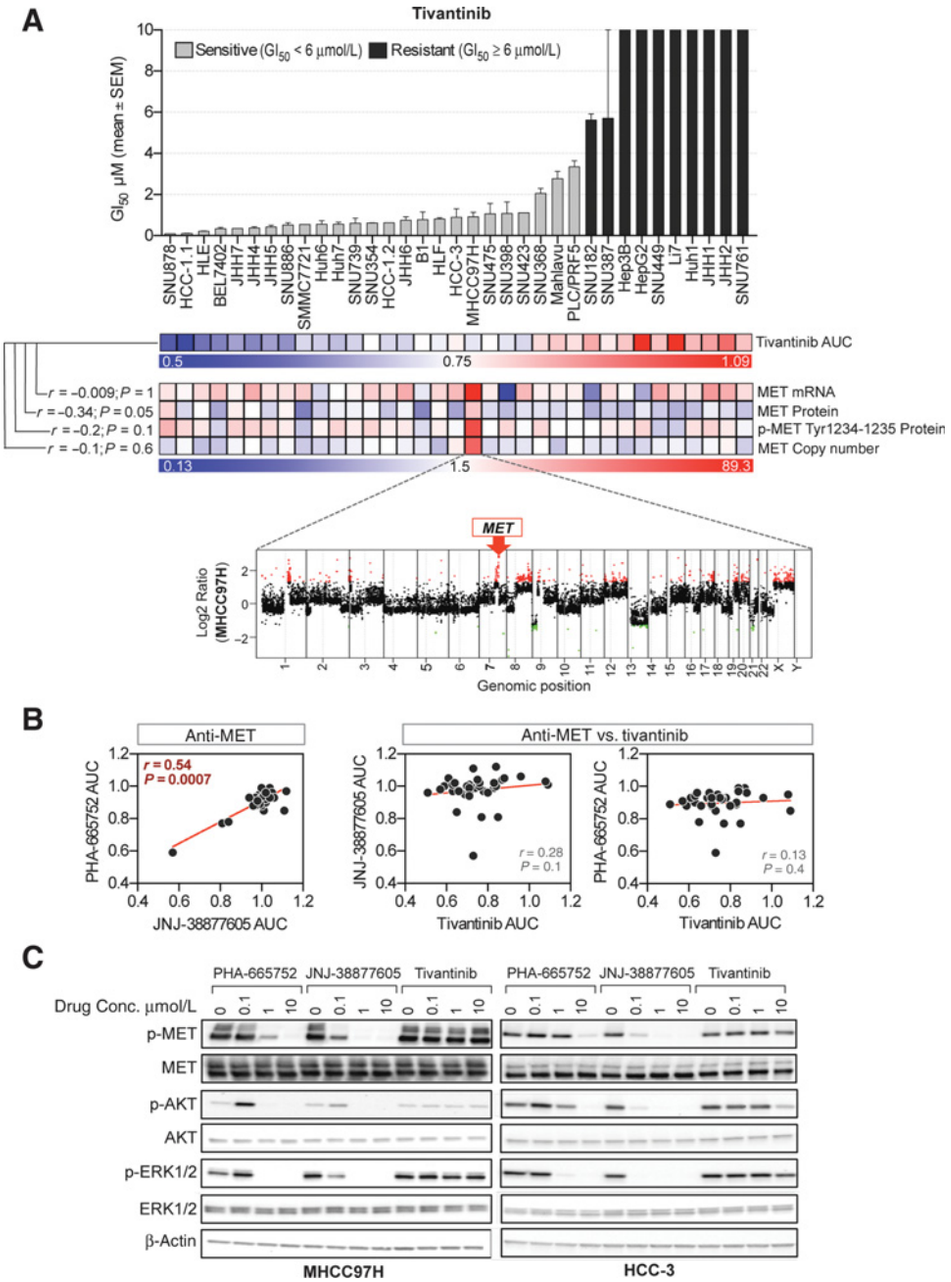


Figure 1.
Tivantinib does not inhibit MET function. **A,** Sensitivity of 35 liver cancer cell lines to tivantinib. The heatmaps below represent for each cell line (columns) tivantinib sensitivity using the AUC and MET status at the mRNA (qRT-PCR), protein (RPPA), and genomic (copy number analyzed by exome sequencing) levels. AUC of 1, no drug response. Copy number, mRNA, and protein levels for each cell line are expressed relative to the mean value of normal noncirrhotic liver tissues. Associations between tivantinib AUC and MET status were analyzed using Spearman test. Represented below the heatmap are 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 test. **C,** Western blot analysis of MET phosphorylation and its downstream effectors AKT and ERK1/2 in two MET-dependent cell lines (MHCC97H and HCC-3) treated 4 hours with increasing doses of PHA-665752, JNJ-38877605, or tivantinib.

Quantitative RT-PCR

We analyzed total mRNA from 35 liver cell lines, 310 resected and advanced HCC, and we assessed quality as described previously (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 dynamic arrays using the BioMark Real-Time PCR system. A panel of 188 genes was analyzed (Supplementary Table S3). Expression data (C_t values) were calculated using the Fluidigm Real-Time PCR Analysis software (4.1.3). Gene expression data were expressed with the $2^{-\Delta\Delta C_t}$ 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 analyzed by whole-exome sequencing as described previously (7, 18). Putative somatic variants and copy-number aberrations were identified as described in the Supplementary Materials and Methods in 12 genes (>5%) frequently altered in human HCC tumors (*TERT* promoter, *CTNNB1*, *TP53*, *ARID1A*, *AXIN1*, *CDKN2A*, *ARID2*, *RPS6KA3*, *NFE2L2*, *KEAP1*, *ALB*, and *APOB*; refs. 7, 18) and in *MET*.

IHC

Expression of Ki-67 and MET was assessed by IHC on paraffin-embedded tumor tissue sections using, respectively, an MIB-1 antibody (Dako, 1/100 dilution) and the CONFIRM anti-total MET (SP44) rabbit monoclonal primary antibody (Ventana Medical Systems, prediluted) directed against a membranous and/or cytoplasmic epitope present in human normal epithelial or tumor cells. The Ki67 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 the time from primary tumor resection to death from cancer progression and within 5 years of follow-up. We excluded patients with noncurative 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

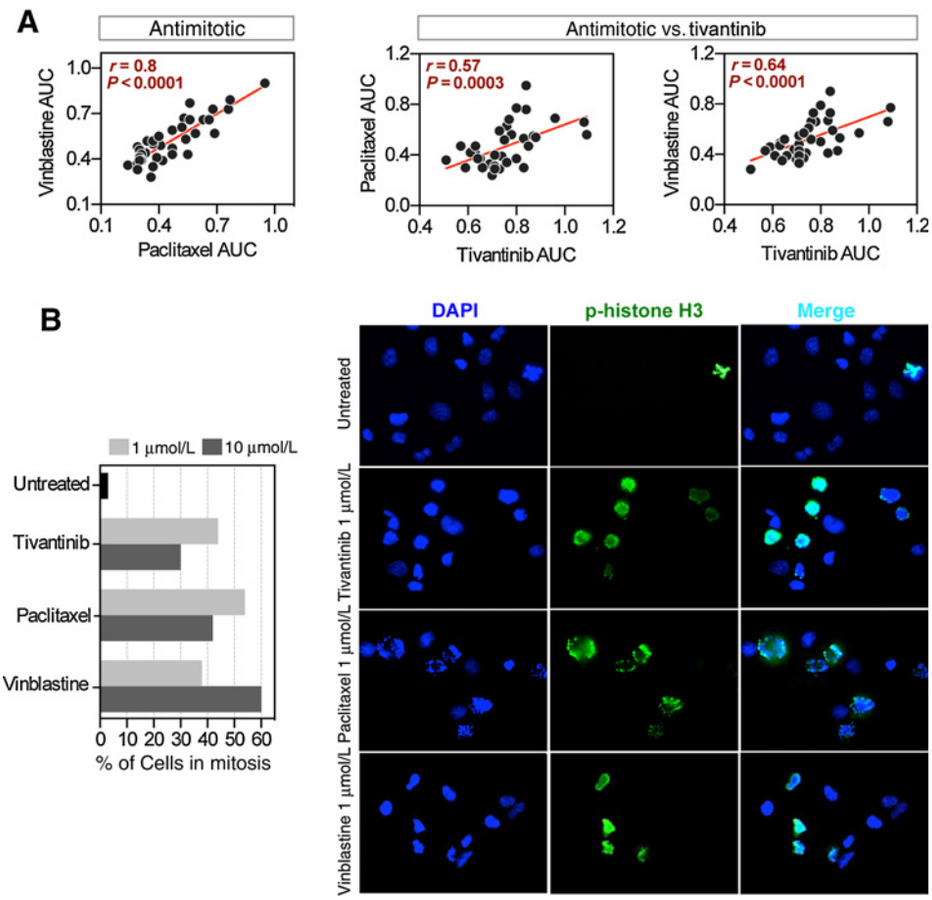
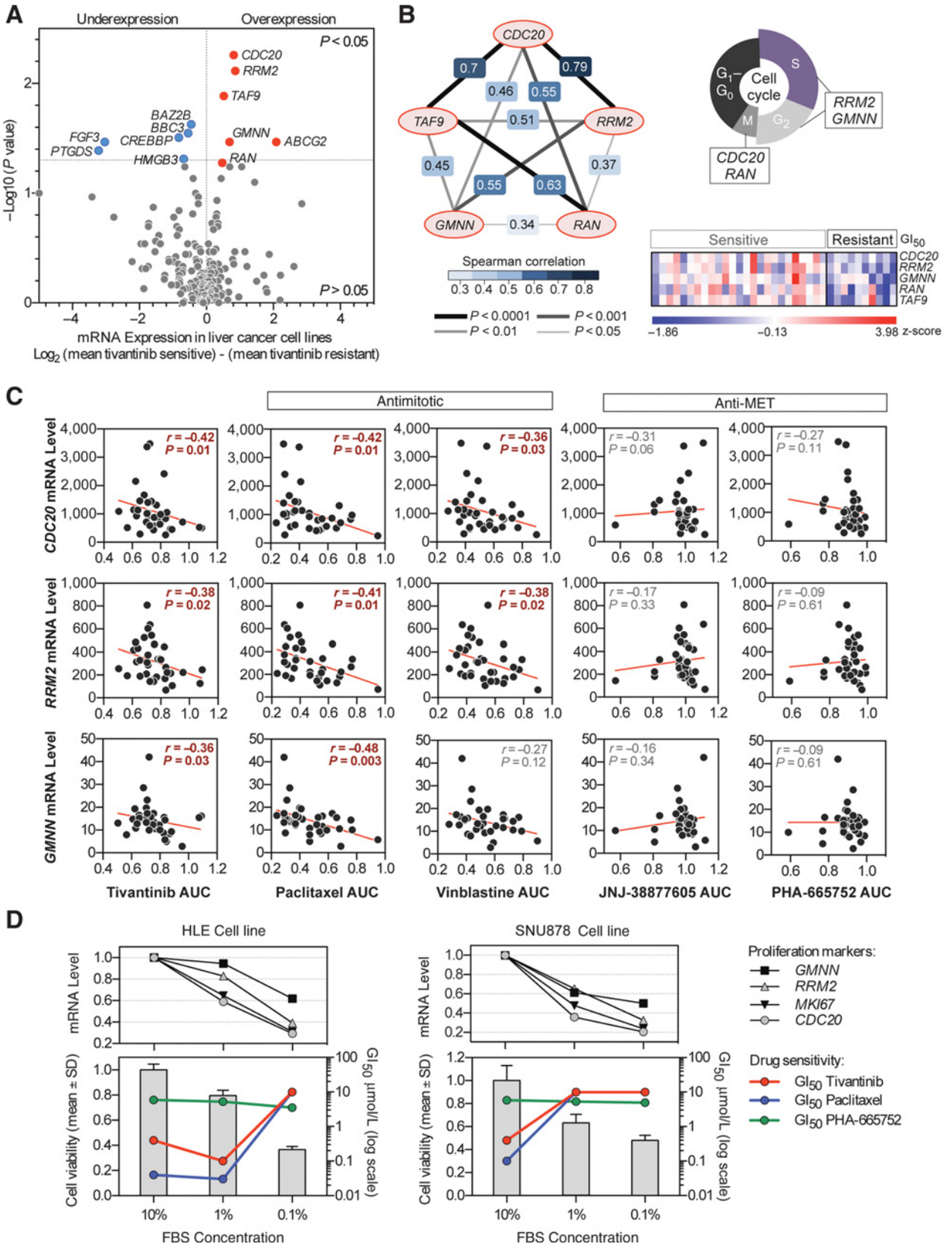


Figure 2.
Tivantinib behaves as an antimitotic agent. **A,** Scatter plots showing correlations between sensitivity patterns assessed by the AUC of two antimitotic drugs (paclitaxel and vinblastine) and tivantinib among 35 liver cancer cell lines. **B,** Effect of tivantinib on the mitotic index was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



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 signaling

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 S1). Cell lines were categorized according to clinical definition, as sensitive when the GI_{50} was below 6 μmol/L, corresponding to the maximum clinically tolerated dose (20, 21) and as resistant when the GI_{50} was higher or equal to 6 μmol/L (Fig. 1A). Using this definition, tivantinib inhibited efficiently cell viability in 25 of the 35 liver cancer cell lines. The GI_{50} 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 nonamplified 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 with tivantinib profile (Fig. 1B). The two selective MET inhibitors demonstrated strong inhibition of cell viability (GI_{50} around 0.1 μmol/L) only in MHCC97H, the unique cell line harboring *MET* amplification, whereas they had little or no effects on the other nonamplified cell lines, except HCC-3 that showed an intermediate sensitivity with PHA-665752 (GI_{50} =1.2 μmol/L; Supplementary Fig. S1). Of note, HCC-3 cell line showed a 2-fold increased *MET* gene copy number (Supplementary Fig. S1B). Accordingly, in the two HCC cell lines (MHCC97H and HCC-3) most sensitive to MET inhibitors, we showed by Western blot analysis 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 downstream signaling effectors AKT and ERK1/2 in a dose-dependent manner (Fig. 1C).

Tivantinib acts as an antimitotic agent

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). Strikingly, 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 with the untreated control cells, and we observed a 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 signaling in liver tumor cell lines.

Expression of cell proliferation markers is associated with tivantinib sensitivity

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 S3). 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* (Supplementary Table S4; ref. 7). We only found a mild association between *ARID1A* mutations and lower tivantinib sensitivity among the 13 analyzed genes (Supplementary Table S5). 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 with 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 coregulated genes overexpressed in the sensitive cell lines (Fig. 3B; Supplementary Fig. S2A). Strikingly, four of these five genes

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 GI_{50}. Red and blue dots indicate, respectively, genes significantly overexpressed and underexpressed in cell lines sensitive to tivantinib. Horizontal dashed line, negative logarithm of *P* value threshold (0.05). **B,** Left, group of five coregulated genes overexpressed at the mRNA level in tivantinib-sensitive cell lines; correlations between pairs of genes were assessed using Spearman test; bottom right, the heatmap represents standardized mRNA expression values (z-score; row) for the five genes across the 35 liver cancer cell lines panel (column); top right, 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 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: (i) cell viability assessed by MTS assay (bar chart left axis, bottom, 9 replicates per FBS concentration); (ii) mRNA expression of four proliferation markers quantified by qRT-PCR; (iii) drug sensitivity measured by the GI_{50} (dots right axis, bottom). Shown is one representative experiment out of two independent experiments.

(*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; Supplementary Fig. S2B), 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. S2B). Altogether, by combining results obtained with the two dose–response parameters GI_{50} and AUC, high mRNA expression of the three cell proliferation genes, *CDC20*, *RRM2*, and *GMNN*, emerged as the best predictor 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 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 GI_{50} increasing around 25-fold between the basal condition (10% FBS) and the lowest FBS concentration (0.1%; Fig. 3D). We observed similar shift in GI_{50} 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 a good predictor 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 with 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 IHC 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 II trial of advanced HCC from ref. 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 this 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 toward 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. Second, tivantinib was not able to suppress MET kinase activity and downstream signaling and induced mitotic blockade similarly to antimitotics, while MET inhibitors are known to preferentially induce G_0–G_1 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 independently 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 sustains our results obtained in preclinical models as well as previous ones.

Initially, tivantinib was identified as an 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 (24 hours) to tivantinib contrasting with our results and others that were obtained with a short-time (4–6 hours) exposure in hepatocellular or other types of tumor cells (11–13). Indeed, after 24 hours of tivantinib exposure, a decrease in both phospho- and total MET proteins was shown by Western blot analysis in most of the cell models (27–29). This observation could be nonspecifically related to tivantinib but interpreted as a nonspecific 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 antitumor effect of tivantinib.

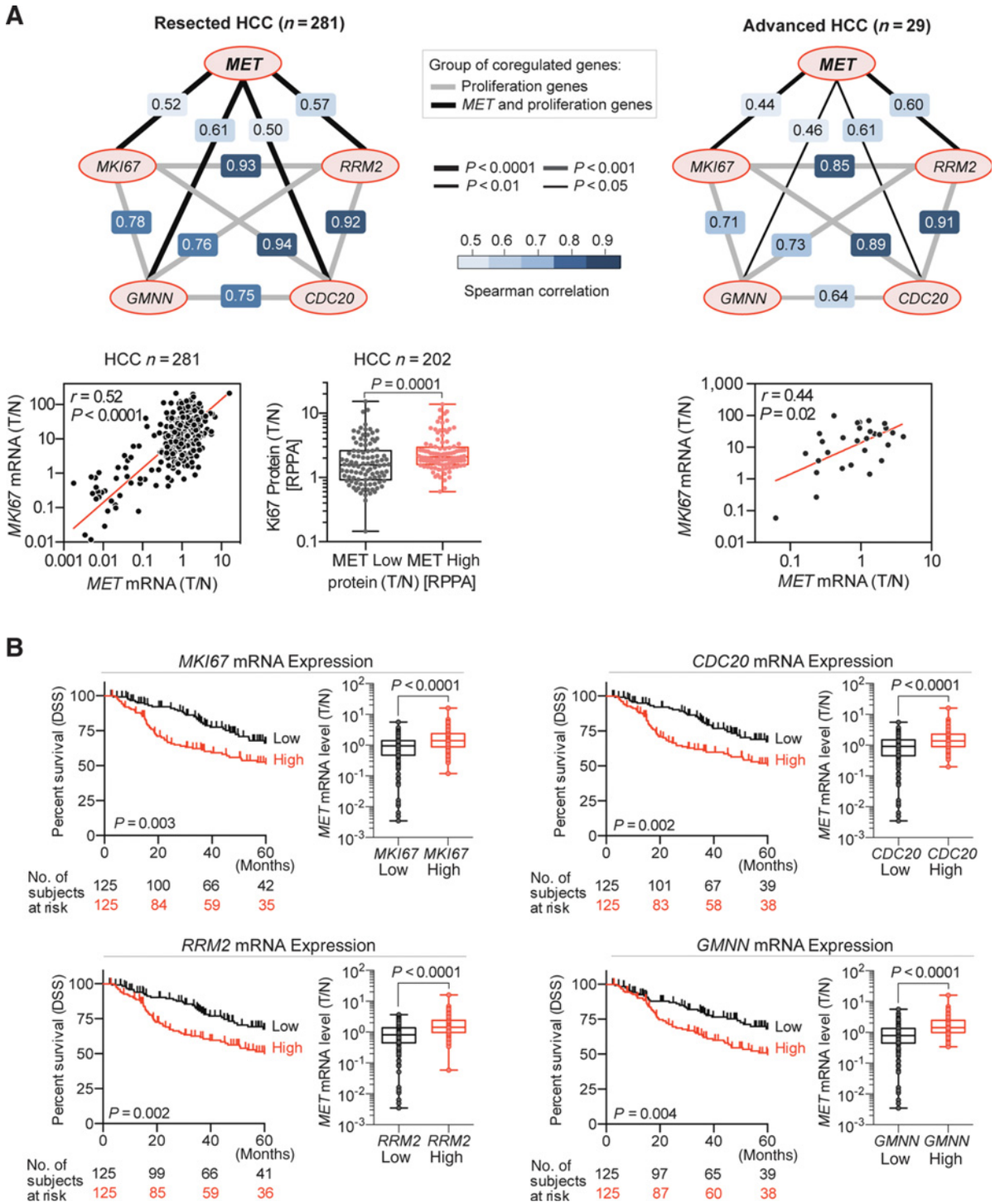


Figure 4.

Expression of proliferation markers correlates with MET expression and survival in HCC. **A,** Top, Spearman pairwise correlations between mRNA expression of 5 genes, including 4 proliferation genes and MET, were analyzed in 281 resected HCC (left) and in a series of 29 biopsies of advanced HCC (right). 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).

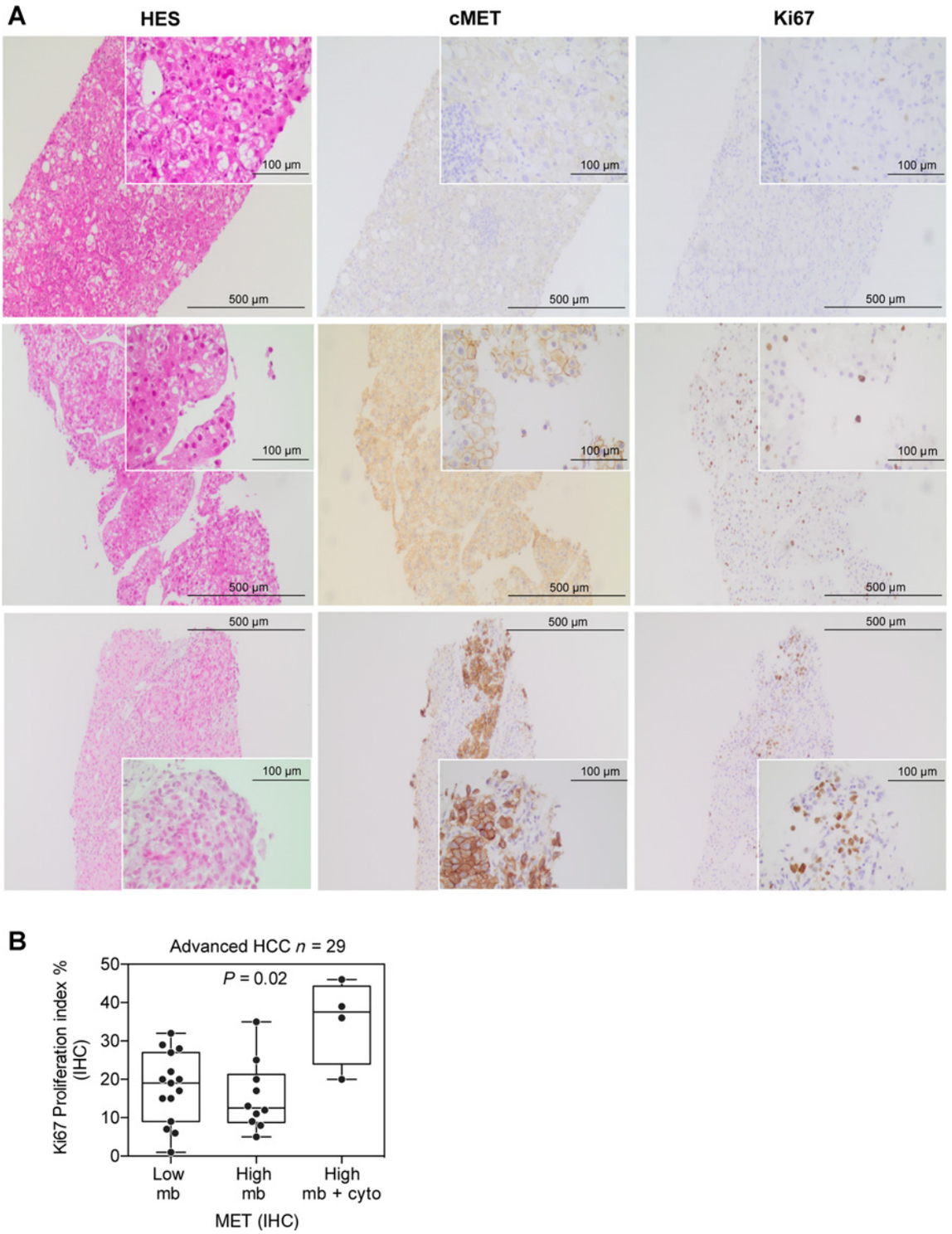


Figure 5.

IHC expression of Ki67 and MET is associated in advanced HCC. **A,** Representative immunostaining patterns of MET and Ki67 in advanced HCC biopsy samples. Top, a well-differentiated HCC without MET staining (membranous and cytoplasmic score 0). Ki67 proliferation index was low (6%). Middle, an 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%. Bottom, an 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 IHC in biopsies of 29 advanced HCC. MET expression was categorized in three groups with low or high membranous staining alone or with both high membranous (mb) and cytoplasmic (cyto) staining. Comparison between groups was assessed using a Kruskal–Wallis test.

Here, our study provides new evidence that antiproliferative effect of tivantinib has no relation with functional MET targeting. Furthermore, interestingly, we also showed that expressions of proliferation markers were the best predictors of tivantinib response in our cell line models, and we demonstrated that high proliferation rates were associated with greater sensitivity, whereas 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 and colleagues suggesting that IHC overexpression of MET was a good predictor of tivantinib therapeutic efficacy in advanced stage HCC (9). Interestingly, by analyzing 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 IHC in a series of advanced 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. Although 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 IHC proliferation marker, could be more accurate than MET to predict tivantinib sensitivity and should be evaluated in the ongoing phase III clinical trials. We also showed that high expression of cell proliferation genes defined a subgroup of HCC patients with poor survival. These results may also have 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 also be 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 with conventional antimitotic agents.

Another aim of our study was to evaluate and redefine the role of authentic 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, whereas the nonamplified 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% to 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 I 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 an MET inhibitor. Moreover, we identified Ki67 as a potential new biomarker predictive of tivantinib response that may help refine selection of patients who may benefit from tivantinib treatment. However, the predictive value of Ki67 remains to be evaluated in clinical trials.

Disclosure of Potential Conflicts of Interest

M. Ziol reports receiving speakers bureau honoraria from Gilead. No potential conflicts of interest were disclosed by the other authors.

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