Purpose: Oncogenic mutations in the KRAS/PI3K/AKT pathway are one of the most frequent alterations in cancer. Although PI3K or AKT inhibitors show promising results in clinical trials, drug resistance frequently emerges. We previously revealed Wnt/β-catenin signaling hyperactivation as responsible for such resistance in colorectal cancer. Here we investigate Wnt-mediated resistance in patients treated with PI3K or AKT inhibitors in clinical trials and evaluate the efficacy of a new Wnt/tankyrase inhibitor, NVP-TNKS656, to overcome such resistance.

Experimental Design: Colorectal cancer patient-derived sphere cultures and mouse tumor xenografts were treated with NVP-TNKS656, in combination with PI3K or AKT inhibitors. We analyzed progression-free survival of patients treated with different PI3K/AKT/mTOR inhibitors in correlation with Wnt/β-catenin pathway activation, oncogenic mutations, clinicopathological traits, and gene expression patterns in 40 colorectal cancer baseline tumors.

Results: Combination with NVP-TNKS656 promoted apoptosis in PI3K or AKT inhibitor-resistant cells with high nuclear β-catenin content. High FOXO3A activity conferred sensitivity to NVP-TNKS656 treatment. Thirteen of 40 patients presented high nuclear β-catenin content and progressed earlier upon PI3K/AKT/mTOR inhibition. Nuclear β-catenin levels predicted drug response, whereas clinicopathologic traits, gene expression profiles, or frequent mutations (KRAS, TP53, or PIK3CA) did not.

Conclusions: High nuclear β-catenin content independently predicts resistance to PI3K and AKT inhibitors. Combined treatment with a Wnt/tankyrase inhibitor reduces nuclear β-catenin, reverses such resistance, and represses tumor growth. FOXO3A content and activity predicts response to Wnt/β-catenin inhibition and together with high β-catenin may be predictive biomarkers of drug response providing a rationale to stratify colorectal cancer patients to be treated with PI3K/AKT/mTOR and Wnt/β-catenin inhibitors.

Introduction
Colorectal cancer is a leading cause of death worldwide (1), mostly because conventional treatments or new target-directed drugs are ineffective in patients presenting late-stage metastatic disease (2). It is therefore crucial to unmask the molecular mechanisms responsible for such resistance and to provide new predictive biomarkers of drug response that could improve the selection of patients sensitive to treatment.

Activating mutations in genes encoding constituents of the KRAS/PI3K/AKT signaling pathway can be considered one of the most frequent cancer-causing genetic alterations in solid tumors, including colorectal cancer (3). Thus, a new generation of drugs targeting PI3K or AKT activity is being tested in numerous clinical trials with promising results in some tumor types. Unfortunately, colorectal cancer patients show an enhanced resistance to these drugs (4–8).

Active AKT phosphorylates FOXO proteins promoting their sequestration in the cytoplasm and blocking their capacity to induce the expression of target genes coding for proteins involved in cell-cycle arrest and apoptosis (9). Therefore, the efficacy of PI3K and AKT inhibitors can be mediated in part through nuclear
Apoptosis. We previously described that Wnt/β-catenin oncogenic signaling confers resistance to FOXO3A-dependent apoptosis promoted by PI3K or AKT-inhibitory drugs (10). Such resistance was driven by nuclear β-catenin that impaired the capacity of FOXO3A to execute its apoptotic program. Thus, we hypothesized that reducing nuclear β-catenin content by Wnt inhibitors would overcome the resistance to PI3K or AKT inhibitors and combined treatments could be beneficial for treating colorectal cancer patients.

Abnormal activation of the Wnt/β-catenin pathway by mutations in APC, CTNNB1/β-catenin, or AXIN2 is responsible for the initiation and progression of almost all colorectal cancers (11). These mutations reduce the capacity of the Wnt pathway destruction complex, formed by APC, AXIN, and GSK3β, to commit β-catenin to degradation. As a result, β-catenin accumulates in the nucleus, binds the TCF/LEF transcription factors, and induces the expression of Wnt target genes that play key roles in tumor progression (12). We and others have previously shown that binding of β-catenin to different transcription factors enhances the expression of alternative sets of target genes (13). FOXO3A is one of these transcription factors, for which β-catenin acts as a transcriptional coactivator (14).

As inappropriate activation of the Wnt/β-catenin pathway was first linked to colon cancer three decades ago, there has been intense interest in developing effective inhibitors (15, 16). It has been described that tankyrases promote AXIN1/2, enhancing the activity of the destruction complex and reducing free β-catenin. These inhibitors are showing promising preclinical results as Wnt/β-catenin inhibitors for the treatment of Wnt-addicted tumors (18–20).

Here, we present evidence that high nuclear β-catenin content is associated to resistance to PI3K and AKT inhibitors in the context of clinical trials, whereas frequent mutations or clinicopathologic traits implicated in colorectal cancer progression do not. We demonstrate that combining these drugs with NVP-TNKS656, a new therapeutic small-molecule inhibitor of the Wnt/tankyrase pathway that reduces nuclear β-catenin (21), overcomes such resistance and represses tumor growth in colorectal cancer patient-derived xenograft (PDX) models. We also identified FOXO3A as a determinant of response to Wnt/β-catenin inhibitors and FOXO3A/β-catenin target genes as better pharmacodynamic markers than the canonical TCF/β-catenin targets.

Our data indicate that nuclear FOXO3A and β-catenin content and activity could be valuable predictive biomarkers of drug response and we propose an experimental-based rationale to better guide the molecular selection of colorectal cancer patients entering new clinical trials with PI3K/AKT and/or Wnt/β-catenin pathway inhibitors.

Materials and Methods

Patients in clinical trials

Patients were enrolled in clinical trials with PI3K/AKT/mTOR inhibitors carried out in the Vall d’Hebron University Hospital (Barcelona, Spain; Clinical trial identifiers: 14-MC-JWAA, NCT01115751; B2151001, NCT00940498; CBEZ235A2101, NCT00620594; BK4120×2101, NCT01273800; CBYL719×2101, NCT01219699; INK1117-001, NCT01449370; PAM4743g, NCT01090960; X165-05; NCT00485719). Tumor response was assessed according to RECIST 1.0 or 1.1 (22, 23). We analyzed formalin-fixed paraffin-embedded (FFPE) tumor samples from colorectal cancer patients at baseline before entering clinical trials with PI3K/AKT/mTOR inhibitors.

Patient-derived cells

Written informed consent was signed by all patients. The project was approved by the Research Ethics Committee of the Vall d’Hebron University Hospital (Barcelona, Spain; Approval ID: PR(IR)79/2009). Patient-derived cells were obtained as previously described (24). Cells were injected subcutaneously in NOD-SCID mice or were seeded as sphere cultures.

Animals, xenotransplantation, and treatments

Experiments were conducted following the European Union’s animal care directive (2010/63/EU) and were approved by the Ethical Committee of Animal Experimentation of Vall d’Hebron Institute of Research (ID: 40/08 CEAA and 47/08/10 CEAA).

Xenografts were obtained as described in ref. (24). API2 xenografts were obtained from the Institute of Research (ID: 40/08 CEEA and 47/08/10 CEEA). The project was approved by the Research Ethics Committee of the Hospital of Badalona (Barcelona, Spain; Clinical trial identifier: NCT01090960; B2151001, NCT00940498; CBEZ235A2101, NCT00620594; BK4120×2101, NCT01273800; CBYL719×2101, NCT01219699; INK1117-001, NCT01449370; PAM4743g, NCT01090960; X165-05; NCT00485719). Tumor response was assessed according to RECIST 1.0 or 1.1 (22, 23). We analyzed formalin-fixed paraffin-embedded (FFPE) tumor samples from colorectal cancer patients at baseline before entering clinical trials with PI3K/AKT/mTOR inhibitors.

Gene expression

Gene expression of 292 selected genes was profiled in baseline tumors of patients treated with PI3K/AKT/mTOR inhibitors using the nCounter platform from Nanostring Technologies. Differentially expressed genes were identified in tumors presenting high or low nuclear β-catenin content using Partek Genomics Suite Software. Lists were cut-off at fold change of 1.2 and a P value < 0.075 (two-tailed one-way ANOVA test).

For microarray analyses, we used a genome wide Human Gene 1.0 ST Array (Affimetrix). Data were acquired using the Affimetrix GeneChip/GeneTitan platforms. Genes were considered differentially expressed in NVP-TNKS656 versus vehicle-treated tumors at 1.5 fold change and P < 0.05 using a two-tailed one-way ANOVA test. Microarray data are deposited at ArrayExpress database (E-MTAB-2446). To perform qRT-PCR, DNA from endpoint tumor xenografts was used to synthesize cDNA using Superscript-III reverse transcriptase with oligo-dT and random

Translational Relevance

To date, PI3K and AKT inhibitors are showing limited clinical benefit mostly due to unknown resistance mechanisms and the lack of predictive biomarkers of drug response. We demonstrate that Wnt inhibitors can overcome β-catenin–induced resistance to PI3K and AKT inhibitors in colorectal cancer tumors. We also provide the experimental evidence for a rational stratification of patients to be treated with PI3K/AKT and/or Wnt/β-catenin pathway inhibitors using β-catenin and FOXO3A as predictive biomarkers of drug response. Such refined molecular selection of patients could represent a significant improvement in response to treatment and an important step forward in advancing colorectal cancer therapy.
Genotyping

Tumor samples from colorectal cancer patients in clinical trials with PI3K/AKT/mTOR inhibitors were genotyped by sequencing the amplified product of a multiplexed PCR reaction (Amplicon sequencing) as described in Supplementary Materials and Methods. Frequent mutations in 57 oncogenes and tumor suppressor genes were interrogated (Supplementary Table S1). Microsatellite instability was analyzed using the MSI-Analysis System (Pro-genes). Frequent mutations in 57 oncogenes and tumor suppressor genes (Supplementary Table S2).

Three PDX models were genotyped by Exome sequencing. Patients provided written informed consent for somatic and germline DNA analysis. Mutations were called with VarScan2 software, either using the mpileup2snp or somatic commands, depending on the availability of normal tissue (26). Nontumoral tissue was not available for PDX-P2, thus, common SNPs were filtered according to the 1000 genome catalogue (27). SIFT and Polyphen-2 helped predicting functionality of the identified mutations. Complete Exome sequencing data from PDX-P2, P5, and P30 are available at the SRA database at NCBI (BioProject ID: PRJNA242531).

Immunohistochemistry and immunofluorescence

Samples from paraffin-embedded tissues were stained as described in ref. 24 using the following antibodies (Supplementary Table S3). Nuclei were stained with Hoechst 33342 (5 μg/mL; Sigma-Aldrich). Pictures of the immunofluorescent signal were captured using a NIKON C2+ confocal microscope and analyzed with MBF ImageJ software using criteria previously described (28, 29).

β-Catenin immunohistochemistry was done using the Dako Autostainer Plus Staining System. For visualization, EnVision FLEX detection system (DAKO) was used. Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted for examination. β-Catenin staining was evaluated by a pathologist as described in Supplementary Materials and Methods.

DLD1F and HT29F cells were seeded on glass coverslips and treated for 6 hours with 4-hydroxytamoxifen 100 nmol/L (4-OHT, Sigma-Aldrich). Cells were fixed in 4% para-formaldehyde (PFA) and immunofluorescent staining was performed as described previously (10).

Apoptosis assays

Patient-derived cells were seeded in suspension as sphere cultures on low attachment multiwell dishes, whereas cell lines were seeded in adherent multiwell culture dishes. Cells were pretreated with NVP-TNKS656 (100 nmol/L, Novartis) or DMSO for 48 hours and then with API2 (20 μmol/L, Tocris Bioscience) and/or NVP-BKM120 (2.4 μmol/L, Selleck Chemicals) for another 48 hours prior apoptosis analysis. Proportions of apoptotic cells were determined using the Annexin V-eGFP (BioVision) kit. Dead cells were detected as DAPI negative (1 μg/mL, Roche). Cells were analyzed by flow cytometry using a Navios Flow Cytometer (Beckman Coulter).

To measure apoptosis by immunofluorescence in sphere cultures, cells suspended in culture media were mixed 1:1 with Matrigel (BD Biosciences), fixed for 1 hour in 4% PFA, permeabilized with PBS/1% Triton X-100 at room temperature for 3 hours, and blocked overnight at 4°C in PBS/1% Triton X-100/3% BSA. Samples were incubated for 24 hours with primary antibodies (Supplementary Table S3). Secondary antibodies and Hoechst 33342 (5 μg/mL) were incubated overnight at room temperature.

Cell culture

Cell lines were cultured under standard conditions. DLD1F cells are DLD1 derivatives expressing pcDNA-FOXO3A(3A):ER (30). HT29F cells express pLHCX-HA-FOXO3A(3A):ER. All parental cell lines were originally obtained from ATCC. Cell lines were authenticated by short-tandem repeat analysis by the cell bank.

Western blot analysis

The detailed protocol for protein extraction is described in Supplementary Materials and Methods. Western blot analysis was performed as described in ref. (10) using specific antibodies (Supplementary Table S3).

TCF/LEF1 reporter assays

DLD1 cell line was stably transfected with a vector (7TGP, obtained at Addgene) expressing eGFP controlled by a promoter containing seven TCF/LEF transcription factor–binding sites (7xTOP; ref. 31). Cells were treated with NVP-TNKS656 100 nmol/L (Novartis) for 7 days and eGFP accumulation was measured by flow cytometry using a Navios Flow Cytometer (Beckman Coulter).

Statistical analysis

We analyzed progression-free survival (PFS) of patients by the Kaplan–Meier method and compared the curves using a log-rank ( Mantel–Cox) test. We used Pearson correlation test to compare time on previous line of treatment versus time on treatment with PI3K/AKT/mTOR inhibitors of patients analyzed for nuclear β-catenin content and to correlate apoptosis versus nuclear β-catenin or FOXO3A content in sphere cell cultures treated with NVP-TNKS656, API2, and NVP-BKM120. Pearson correlation test was also used to compare nuclear FOXO3A content in patients and corresponding PDX tumors, or in primary tumors versus liver metastases or to compare with SLC2A3 mRNA levels.

Differences in apoptosis of treated sphere cell cultures: levels of AXIN1, β-catenin, phosphoSer, Glu7, and cleaved caspase-3 expression in tumor xenografts; mRNA expression of NVP-TNKS656 target genes by qRT-PCR; and apoptosis of DLD1F or HT29F cell lines, were analyzed by an unpaired t test comparing the means of two groups of values. Fisher exact test served to analyze the differences in response among patients regarding nuclear β-catenin content, mutations affecting PIK3CA, KRAS,
TP53, APC, or tumor histologic TNM status. P values lower than 0.05 were considered significant in all tests.

Results

Effective pharmacologic inhibition of Wnt/β-catenin and PI3K/AKT pathways reduces tumor growth

We hypothesized that reducing nuclear β-catenin content could be sufficient to sensitize colorectal cancer tumors to the treatment with PI3K or AKT inhibitors. Consequently, we blocked the Wnt/β-catenin signaling using the tankyrase inhibitor NVP-TNKS656 (21) in colorectal cancer PDX models. We selected five PDX models with high (P2, P7, P19, P22, and P30) and five with low nuclear β-catenin content (P5, P6, P31, P33, and P34; Fig. 1A and B). Sphere cell cultures derived from xenograft tumors of each model were treated with API2 or NVP-BKM120, inhibiting AKT or PI3K activity, respectively, alone or in combination with NVP-TNKS656 (Fig. 1C; Supplementary Table S4). API2 or NVP-BKM120 induced significantly less apoptosis in cells with high rather than low nuclear β-catenin content by measuring the proportion of Annexin V-positive cells. Combination with NVP-TNKS656 significantly increases apoptosis in cells with high as opposed to low nuclear β-catenin content. Similar results were observed by using NVP-XAV939, another inhibitor of tankyrase activity (Supplementary Fig. S1). Alternative measurement of apoptosis by cleaved caspase-3 showed equivalent results (Supplementary Fig. S2). Furthermore, apoptosis induced by API2 or NVP-BKM120 treatment showed a significant inverse correlation with nuclear β-catenin content (Fig. 1D). Such correlation was lost when API2 or NVP-BKM120 was combined with NVP-TNKS656. Mutations in PIK3CA, KRAS, or TP53 genes or tumor site did not condition a differential response of sphere cell cultures to PI3K or AKT inhibition (Supplementary Fig. S3). Only one out of the 10 PDX models presented microsatellite instability (MSI), preventing the possibility to evaluate its impact on drug response (Supplementary Table S5).

Our data indicate that nuclear β-catenin content conditions drug response in patient-derived sphere cell cultures, whereas frequent mutations in colorectal cancer do not (Fig. 1C and D and Supplementary Fig. S3; Supplementary Table S5).

We further investigated these results in vivo. Cells from three PDX models with known nuclear β-catenin and FOXO3A status and with limited response to AKT or PI3K inhibition in vitro (P2, P5, and P30), were injected subcutaneously into NOD-SCID mice (Figs. 1B and D and 2A). Treatment with NVP-TNKS656 caused a systemic reduction of nuclear β-catenin content and function in skin and intestine, tissues where the Wnt pathway tightly controls homeostasis, but showed no major negative side effects (Supplementary Fig. S4).

API2 alone or in combination with NVP-TNKS656 did not repress tumor growth in PDX-P2, with low basal nuclear β-catenin and FOXO3A content, probably representing a case of a colorectal cancer tumor resistant to AKT inhibition due to mechanisms independent of nuclear β-catenin accumulation (Fig. 2A and B). Exome sequencing revealed a mutation in the AKT2 gene that could explain the lack of reduction of phospho-S6 or tumor growth upon API2 treatment in this model (Supplementary Fig. S5; Supplementary Table S6).

PDX-P2 presented high nuclear β-catenin and low FOXO3A amounts, was resistant to API2 alone, and yet tumor growth rate was reduced upon combination with NVP-TNKS656 (Fig. 2A and B). Tankyrase inhibition significantly reduced nuclear β-catenin, API2 decreased phospho-S6 content, and both diminished proliferation (Fig. 2C, Supplementary Fig. S5).

PDX-P30, derived from a liver metastasis, presented high amounts of both nuclear β-catenin and FOXO3A (Fig. 2A). It was also resistant to API2, but NVP-TNKS656 treatment alone reduced tumor growth rate equally to the drug combination (Fig. 2B). Tankyrase but not AKT inhibition promoted apoptosis, whereas the number of proliferative cells was not affected (Supplementary Fig. S5). This was the only model where NVP-TNKS656 alone showed an effect on tumor growth, probably due to high endogenous amounts of FOXO3A that might have induced apoptosis when nuclear β-catenin was reduced by NVP-TNKS656. NVP-TNKS656 increased AXIN1 protein levels in all subcutaneous tumors confirming its activity as a Wnt/tankyrase pathway inhibitor (ref. 21; Fig. 2D).

FOXO3A/β-catenin target genes are pharmacodynamic markers of response to Wnt/tankyrase-inhibitory drugs

Although NVP-TNKS656 reduced the high nuclear β-catenin content observed in both PDX-P2 and PDX-P30, treatment only reduced tumor growth rate in the latter model (Fig. 2 and Supplementary Fig. S5B). Contrarily, NVP-TNKS656 did not affect tumor growth in PDX-P5 model. We studied whether the high FOXO3A content observed in the metastatic PDX-P30 model (Fig. 2A) could determine the repression by NVP-TNKS656 of a distinct set of Wnt/β-catenin target genes and its enhanced sensitivity to treatment. RNA from tumors of PDX-P2, PDX-P30, and PDX-P5 models was analyzed at the endpoint of the in vitro experiments (Fig. 2B). The three models showed a distinct gene expression pattern that was modified by NVP-TNKS656 treatment (Fig. 3A; Supplementary Tables S7–S9). The prometastatic S100A4 gene was repressed in both PDX-P2 and PDX-P30 models (Fig. 3B). Four NVP-TNKS656-repressed genes in PDX-P2, two in PDX-P5, and yet none in PDX-P30 were direct TCF/β-catenin targets (Supplementary Table S10). Instead, two genes in PDX-P2, one in PDX-P5, and 9 in PDX-P30 were FOXO3A/β-catenin targets (Supplementary Table S11), many of them formally associated with metastasis (10, 32–34). The regulation of some of these NVP-TNKS656-repressed genes was further confirmed by qRT-PCR in the same tumor xenograft samples (Fig. 3C). Any gene evaluated in PDX-P5 model was significantly regulated by NVP-TNKS656, a result in line with its low nuclear β-catenin content and its lack of tumor growth response (Fig. 2B). Our data suggest that FOXO3A/β-catenin targets could be better pharmacodynamic markers than TCF/β-catenin target genes for evaluating therapeutic Wnt/tankyrase pathway inhibition.

FOXO3A determines the response to Wnt/β-catenin pathway inhibitors

NVP-TNKS656 treatment was particularly effective in a tumor with high endogenous nuclear β-catenin and FOXO3A content, promoting apoptosis, reducing tumor growth rate (Fig. 2) and preferentially repressing the expression of FOXO3A instead of TCF target genes (Fig. 3). The repression of the Wnt/β-catenin pathway by NVP-TNKS656 alone did not promote apoptosis in DLD1 or HT29 colon cancer cells but...
Colorectal cancer patient–derived cells with high amounts of nuclear β-catenin present high sensitivity to API2 or NVP-BKM120 in combination with NVP-TNKS656. A, representative pictures of immunofluorescence and confocal microscopy of histologic sections of the indicated PDX models with high (left) or low (right) nuclear β-catenin content. Inserts show magnification to better visualize β-catenin sub-cellular localization. Dashed lines delineate nuclei. Nuclei were stained with Hoechst 33342 (blue). Scale bars, 100 μm; magnifications 50 μm. B, column scatter plot showing the amount of nuclear β-catenin measured by immunofluorescence and confocal microscopy in 10 primary tumors and liver metastases from which sphere cell cultures were derived and used to test drug response. Horizontal lines indicate arithmetic mean values, and error bars show SD. C, column scatter plot showing the apoptosis induced in sphere cell cultures of these 10 patient-derived models treated as indicated. Data, fold change of apoptotic cells induced by the treatment compared with cells treated with vehicle. Horizontal lines indicate arithmetic mean values, and error bars show SEM. P values correspond to unpaired t tests. The original percentage of Annexin V–positive cells is shown in Supplementary Table S4. D, scatter plots representing the apoptosis induced by API2 (top left) and NVP-BKM120 (top right) or API2 + NVP-TNKS656 (bottom left) and NVP-BKM120 + NVP-TNKS656 (bottom left) in sphere cell cultures of these 10 patient-derived models versus the histologic amount of nuclear β-catenin in the original patient’s tumors. Data, fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. P values correspond to Pearson correlation test. B and D, β-catenin relative units (r.u.) were calculated as described in Materials and Methods.
enhanced the apoptosis induced by exogenous nuclear FOXO3A-ER (Fig. 4). These data confirm the capacity of nuclear β-catenin to confer resistance to FOXO3A-induced apoptosis (10) and, therefore, the therapeutic value of reducing nuclear β-catenin by tankyrase inhibitors in FOXO3A-active cancer cells.

Furthermore, NVP-TNKS656 promoted apoptosis in patient-derived sphere cell cultures proportionally to the amount of nuclear FOXO3A detected in their correspondent original patient samples (Supplementary Fig. S6). We also observed that nuclear FOXO3A content positively correlated with the expression of SLC2A3 mRNA, a FOXO3A/β-catenin target gene (10) that was repressed upon NVP-TNKS656 treatment in vivo (Fig. 3).

Interestingly, we observed that paired primary tumors and liver metastases accumulated similar nuclear FOXO3A amounts, showing that its activation could be durable and occur prior progression to metastatic stages (Supplementary Fig. S7).

Finally, by profiling 130 colorectal cancer cases, we identified a distinctive population of patients with tumors presenting high expression of FOXO3A/β-catenin target genes (Fig. 5C). The expression of TCF/β-catenin target genes also identified such a

Figure 2.
NVP-TNKS656 stabilizes AXIN1 and reduces both, nuclear β-catenin and tumor growth alone or in combination with the AKT inhibitor API2 in colorectal cancer PDX models. A, representative pictures of double immunofluorescent staining and confocal microscopy to detect β-catenin (red) and FOXO3A (green) in histologic sections of subcutaneous xenografted tumors from patients P2, P5, and P30. Right panels show magnifications to visualize the amounts of nuclear β-catenin and FOXO3A. Scale bar, 100 μm; magnifications, 20 μm. Nuclei were stained with Hoechst 33342 (blue). Arrowheads point to β-catenin localized exclusively in cell membranes in tumors from P5 model. B, tumor cells derived from the three indicated patients were injected subcutaneously in NOD-SCID mice and treated as indicated. A minimum of 5 mice with tumors in both flanks was treated in each group. The graphs represent the fold change calculated by comparing the tumor volume at each given time point to the volume at the first day of treatment. Error bars and ± SD are shown for tumor volume fold change of all tumors. Unpaired t tests were used to compare the area under the curve generated for each growing tumor along the experiment and grouped by treatment. Asterisks indicate significant differences (P < 0.05). C, representative pictures showing immunofluorescent staining of β-catenin in tumor xenografts from PDX-P2 model growing in mice treated with vehicle or NVP-TNKS656. Inserts show magnifications to better visualize β-catenin reduction from cytoplasm and nuclei upon tankyrase inhibition. Scale bars, 100 μm; inserts, 50 μm. D, three xenograft tumors per group of treated mice were processed for the analysis of AXIN1 by Western blot analysis (top). β-Tubulin was used as loading control. Blots were quantified using ImageJ software (bottom). Protein expression levels are represented as relative units (r.u.) versus vehicle treated samples. Bars indicate SD. P values correspond to unpaired t tests.
population but showed lower signal. Interestingly, FOXO3A/β-catenin and TCF/β-catenin target genes clustered separately among all cases profiled. Samples were also evaluated for their MSI and mutational status of KRAS, BRAF, and PIK3CA, all molecular features relevant for colorectal cancer tumors. Any obvious correlation was observed between them and gene expression signatures distinctive of active FOXO3A/β-catenin transcription (Fig. 5C; Supplementary Table S12).

These data suggest that patients with tumors that are active for FOXO3A/β-catenin transcription can be identified by transcriptional profiling, whereby reduction of nuclear β-catenin by Wnt inhibitors could promote FOXO3A-dependent apoptosis.

Comparative analysis of β-catenin as potential biomarker of resistance to PI3K and AKT-inhibitory drugs

We decided to compare the potency of nuclear β-catenin in predicting resistance to PI3K or AKT inhibitors with the most frequent mutations or histological traits observed in colorectal cancer tumors. Limited availability of samples from clinical trials only permitted the study of tumors at baseline from a cohort of 40 colorectal cancer patients treated with PI3K/AKT/mTOR pathway inhibitors in several phase I clinical trials (Fig. 6A, top; Supplementary Table S13). We selected tumors from patients treated with half the maximum tolerated dose aiming to homogenize the study cohort. There was no significant difference in PFS between patients treated with different PI3K, AKT, or dual PI3K/mTOR drug subtypes (Fig. 6A, bottom). We performed a double-blinded evaluation of nuclear β-catenin content by two independent pathologists using immunohistochemistry and immunofluorescence on all baseline tumor samples (Fig. 6B and Supplementary Fig. S8). We classified tumors into two histologic categories: high or low, depending on the number of cells positive for nuclear β-catenin accumulation. Out of 40 cases, 13 were high and 27 were low in content.

After the first clinical diagnosis of disease progression by computerized axial tomography (CT) scan, all 13 patients presenting tumors with high nuclear β-catenin content had progressed despite treatment with PI3K/AKT/mTOR pathway inhibitors. Contrarily, 9 of 27 patients with tumors presenting low β-catenin continued treatment after CT scan evaluation, showing some degree of stabilized disease (Fig. 6C, top). Such longer lasting response to PI3K/AKT/mTOR pathway inhibitors did not correlate with better response to the previous line of treatment, ruling out the possibility that those 9 patients were either more sensitive to antitumoral drugs in general or had slower tumor growth independently of treatment (Figs. 6C, bottom) and D). Contrary to nuclear β-catenin content, relevant oncogenic mutations in PIK3CA, KRAS, APC, or TP53
genes, the site of tumor samples (primary tumor or metastasis) or TNM stage (T3 or T4) at the time of diagnosis did not correlate with any significant difference in response to PI3K/AKT/mTOR pathway inhibitors (Fig. 6C and D and Supplementary Fig. S9A–S9F; Supplementary Tables S13 and S14). Only 2 of 28 samples analyzed presented MSI, preventing the possibility to evaluate its impact on drug response (Supplementary Table S13).

Equivalent analyses were performed separately in patients treated with PKI-587 \((n = 11)\), BEZ235 \((n = 9)\), or NVP-BKM120 \((n = 6)\). We observed that cases with high nuclear β-catenin presented a shorter PFS upon PKI-587 or BEZ235 treatment (Supplementary Fig. S9G–S9I). However, results were not statistically significant as expected from the small number of cases available for the analyses.

To confirm the higher risk of progression in the β-catenin high–group, we performed a Cox Proportional Hazards analysis on a subset of our cohort \((n = 27)\) with complete annotation for variables potentially linked to outcome: age, gender, therapy (AKT, PI3K, pan-PI3K, PI3K-mTOR inhibitor), number of prior treatment lines, presence of liver metastasis, and molecular profile (PIK3CA, KRAS, TP53, APC mutations, PTEN loss). Even after multivariate adjustment, patients whose tumors had high nuclear β-catenin content still displayed a significantly worse...
Figure 5.
High FOXO3A activity in colorectal cancer patient-derived cells determines the apoptosis induced by NVP-TNKS656. A, representative pictures of double immunofluorescence and confocal microscopy to detect β-catenin (red) and FOXO3A (green) in models PDX-P33 and PDX-P34. Arrowheads point to cancer cells in PDX-P33 presenting high nuclear FOXO3A and β-catenin accumulation. PDX-P33 presented high and PDX-P34 low nuclear FOXO3A and β-catenin content. Bottom panels show magnifications to better visualize β-catenin and FOXO3A subcellular localization. White dotted lines delineate nuclei stained with Hoechst 33342 (blue). Scale bars, 100 μm; magnifications, 50 μm. B, Scatter plot comparing apoptosis induced by NVP-TNKS656 in xenograft-derived sphere cell cultures versus the histological amount of nuclear FOXO3A in the corresponding PDX models. (Continued on the following page.)
outcome (PFS HR 3.96, 95% confidence interval, 1.03–15.27; log-rank P 0.0158; Supplementary Table S15).

Concerning gene expression patterns, we could analyze 31 of the initial 40 baseline samples and observed that tumors responding to PI3K/AKT/mTOR pathway inhibitors clustered together (Supplementary Fig. S10A). The oncogenic mutations detected did not correlate with any of the gene expression clusters observed. We finally observed that the expression profile of a reduced set of genes could also help identifying tumors presenting high or low nuclear β-catenin content, cross-validating the initial
Discussion

The efficacy of several PI3K and AKT inhibitors is being tested in multiple clinical trials worldwide. Although initial results were promising in some tumor types, clinical responses are null or limited in most colorectal cancer patients (4–8). A reasonable hypothesis for such limited responses is the absence of a universal biomarker to select drug-sensitive patients or discard resistant cases. As activating mutations in PIK3CA gene confer sensitivity to PI3K/AKT pathway inhibitors in preclinical assays, they have been commonly used as inclusion criteria in clinical trials. However, PIK3CA mutations showed conflicting results in predicting response to single-agent PI3K or AKT inhibitors in early-phase clinical trials (7, 35–37). Similarly, we show here that mutations in genes frequently altered in colorectal cancer, including KRAS, TP53, or PIK3CA, do not predict response to PI3K/AKT pathway inhibitors. Interestingly, multivariate analysis indicates that APC mutations are a risk factor for patients treated with these inhibitors (Supplementary Table S15). This would suggest that oncogenic activation of the Wnt/β-catenin pathway could be a mechanism of resistance to PI3K and AKT inhibitors.

Indeed, we previously described that nuclear accumulation of β-catenin conferred resistance to PI3K and AKT inhibitors in colon cancer cells (10). In the current study, we observed that colorectal cancer patients with high nuclear β-catenin present a shorter PFS when treated with PI3K/AKT/mTOR pathway inhibitory drugs in clinical trials. Such differential response contrasts with the fact that all colorectal cancer patients in our cohort progressed to previous lines of treatment irrespective of their nuclear β-catenin content. These results on clinical samples indicate that nuclear β-catenin accumulation could be an independent predictive biomarker of resistance to PI3K and AKT inhibitors beyond other molecular alterations frequent in colorectal cancer.

In accordance, we explored here the therapeutic potential of overcoming such resistance by reducing nuclear β-catenin content with a new Wnt/tankyrase inhibitor, NVP-TNKS656. We show that it sensitizes patient-derived cells to PI3K or AKT inhibitors in vitro and in vivo, especially those with high accumulation of nuclear β-catenin and, thus, high oncogenic Wnt/β-catenin pathway activity.

Our results on Wnt/tankyrase inhibitors reveal a new therapeutic opportunity for the treatment of advanced colorectal cancer patients beyond their combination with PI3K or AKT inhibitors. Indeed, we previously showed that cancer cells with high proapoptotic FOXO3A transcriptionsal activity require high levels of nuclear β-catenin to preserve a viable balance between survival and apoptosis (10). Here, we observed that NVP-TNKS656 treatment was particularly effective in cells and tumors with high endogenous nuclear β-catenin and FOXO3A content, where the pharmacologic reduction of nuclear β-catenin promoted FOXO3A-dependent apoptosis. Therefore, it is expected that cancer cell survival in such endogenous FOXO3A-active scenario, more frequent in advanced metastatic colorectal cancer tumors (10), would rely on Wnt/β-catenin pathway activity. This could represent an exceptional opportunity to compromise cancer cell viability by treating patients with tankyrase inhibitors. Indeed, we identify by gene expression profiling a population of patients presenting tumors with high FOXO3A/β-catenin activity who could potentially respond best to the treatment with these Wnt/β-catenin pathway inhibitors.

However, very little is known about the determinants of nuclear FOXO3A activation in colorectal cancer. It is well described that oxidative stress promotes FOXO3A translocation from the cytoplasm to the nucleus and the consequent induction of genes involved in cell-cycle arrest, survival, apoptosis, and metastasis (9). Future investigations should reveal the precise contribution of oxidative stress and other stimuli originated in the surrounding tumor stroma on determining the final levels of activated FOXO3A. Our results suggest that describing FOXO3A-activating factors would be of particular interest to understand the response to Wnt/β-catenin inhibitors. The use of PDX models could be pivotal in these investigations since they preserve equivalent levels of FOXO3A than the original patients’ tumors. In fact, the efficacy of Wnt/tankyrase inhibitors observed in cancer cell lines or genetic mouse models has been modest (18–20), as they may not recapitulate tumoral FOXO3A activity as consistently as PDX. Indeed, PDX are generally considered the best preclinical models to evaluate drug response as they faithfully recapitulate patient’s disease preserving their molecular alterations and histopathologic traits (24, 38, 39).

Other alterations frequent in colorectal cancer could also be relevant determinants of response to Wnt/β-catenin inhibitors. For instance, truncating mutations in APC gene are present in more than 80% of colorectal cancer patients and constitutively activate the oncogenic Wnt/β-catenin pathway (3). Here we show that NVP-TNKS656 can reduce nuclear β-catenin content and repress tumor growth even in APC-mutant PDX models. These data suggest that the therapeutic potential of tankyrase inhibitors could be extended to the majority of colorectal cancer tumors. This wide spectrum would contrast with the lack of activity in APC-mutated tumors expected from the treatment with NVP-LGK974. This is the first Wnt/β-catenin pathway inhibitor tested in clinical trials (NCT01351103), which blocks porcupine activity and the maturation of Wnt ligands upstream in the oncogenic signaling. As tankyrases palmitoylate and commit other proteins to degradation in addition to AXIN1 and 2 (40, 41), it could be of interest to evaluate what extent the antitumoral capacity of tankyrase inhibitors rely on affecting any other cell processes beyond Wnt/β-catenin signaling.

Aiming to define clinically useful biomarkers to predict the response to PI3K/AKT and Wnt/β-catenin pathway inhibitors, we investigated the potential of gene expression signatures. Our initial results suggest that specific gene expression profiles could identify colorectal cancer tumors with high FOXO3A and β-catenin content and transcriptional activity. In particular, we observed that a particular set of genes was overexpressed in colorectal cancer tumors with high nuclear β-catenin content. Our drug treatment experiments indicate that such Wnt/β-catenin activated tumors could be resistant to PI3K and AKT inhibitors. Furthermore, we observed a significant correlation between nuclear FOXO3A accumulation and mRNA expression of one of its target genes, SLC2A3 (10). These results suggest that nuclear accumulation of FOXO3A and β-catenin observed by histology reflects their activation as transcription factors.
inducing the expression of their corresponding target genes. Finally, we could identify, using Nanostring nCounter platform, a population of colorectal cancer patients with tumors presenting high expression of FOXO3A/β-catenin and TCF/β-catenin target genes. Our preclinical data indicate that these patients could benefit from the treatment with Wnt/tankyrase inhibitors.

Together, these results suggest that gene expression profiling could help to build complex predictive biomarkers of response to PI3K/AKT and Wnt/β-catenin pathway inhibitors. However, selecting a precise set of genes to build robust signatures would require evaluating whole gene expression patterns by microarrays or RNAseq in prospective studies with fresh-frozen tumor samples. These expression profiles should be cross-compared with histologic evaluation of nuclear FOXO3A and β-catenin content and the response to treatment, to finally define those gene sets associated to such histologic and clinical traits. These biomarker exploratory studies should ideally focus on analyzing tumor biopsies taken from progressive lesions at the time of inclusion in clinical trials and not from archival samples. In the particular case of nuclear FOXO3A, we have observed that advanced metastatic tumors present the highest proportion of cases activated for this transcription factor (10). Therefore, validating the use of nuclear FOXO3A accumulation and its associated gene expression signatures as potential biomarker of sensitivity to Wnt/β-catenin pathway inhibitors would require taking biopsies preferentially from metastatic lesions that will actually be progressing at the time of patients’ inclusion in clinical trials.

Hence, we suggest combining histologic evaluation of FOXO3A and β-catenin with functional gene expression signatures to build complex predictive biomarkers of response to PI3K/AKT and Wnt/β-catenin pathway inhibitors in colorectal cancer, which may help the design of future clinical trials with this family of drugs. Similarly, current studies are analyzing the expression of 50 selected genes by Nanostring nCounter platform to facilitate a more precise definition of breast cancer subtypes (42, 43), whose differential response to treatment is currently under validation in several clinical trials. In the same line as our proposal, such gene expression signatures are being combined with clinical evaluation of hormone receptors and HER2 protein levels by histopathologic techniques.

In summary, we propose combining gene expression profiling and histology to define nuclear β-catenin and FOXO3A content and activity as predictive biomarkers of drug response (Supplementary Fig. S11). We hypothesize that this molecular prescreening could establish three groups of patients presenting tumors with: (i) low nuclear β-catenin and FOXO3A activity, more suitable for the treatment with PI3K or AKT inhibitors alone, (ii) high β-catenin and FOXO3A activity who could benefit from Wnt/β-catenin inhibitors alone and (iii) high nuclear β-catenin but low FOXO3A activity who may benefit from combined treatments. Such molecular stratification of patients could represent a significant improvement in response to therapy and an important step forward towards reverting the long-stalled scenario of colorectal cancer therapy.

**Disclosure of Potential Conflicts of Interest**

J. Tabernero is a consultant/advisory board member for Novartis. No potential conflicts of interest were disclosed by the other authors.

**Disclaimer**

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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