Tankyrase inhibition blocks Wnt/β-catenin pathway and reverts resistance to PI3K and AKT inhibitors in the treatment of colorectal cancer

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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 CRC 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 CRC therapy.

ABSTRACT
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 hyper-activation as responsible for such resistance in colorectal cancer (CRC). 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 designs
CRC 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, clinico-pathological traits and gene expression patterns in 40 CRC 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.
13 out 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 clinico-pathological 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, reverts such resistance and represses tumor growth. FOXO3A content and activity predicts response to Wnt/β-catenin inhibition and together with β-catenin may be predictive biomarkers of drug-response providing a rational to stratify CRC patients to be treated with PI3K/AKT/mTOR and Wnt/β-catenin inhibitors.

INTRODUCTION
Colorectal cancer (CRC) 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 CRC (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, CRC 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 relocalization of FOXO proteins and consequent induction of 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 CRC 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 tumour 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).

Since 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 parsylation and degradation through the proteasome (17). Recently, a new family of tankyrase inhibitors was shown to stabilize 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 inhibitory drugs 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 clinicopathological traits implicated in CRC progression do not. We demonstrate that combining these drugs with NVP-TNK656, a new therapeutic small molecule inhibitor of Wnt/tankyrase pathway that reduces nuclear β-catenin (21), overcomes such resistance and represses tumor growth in CRC 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 indicates 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 CRC patients entering new clinical trials with PI3K/AKT and/or Wnt/β-catenin pathway inhibitors.

**MATERIAL 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; BKM120X2101,
Tumor response was assessed according to RECIST 1.0 or 1.1 (22, 23). We analyzed formalin-fixed paraffin-embedded (FFPE) tumor samples from CRC 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 VHIR - Vall d'Hebron Institute of Research (ID: 40/08 CEEA and 47/08/10 CEEA). Xenografts were obtained as described in (24). API2 (1 mg/kg in PBS-2% DMSO; Tocris Bioscience) was administered by intraperitoneal injection three times per week, NVP-TNKS656 (100 mg/kg) was injected subcutaneously bi-daily.

**Gene expression**

Gene expression of 292 selected genes was profiled in baseline tumors of patients treated with PI3K/ATK/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 (Affimmetrix). Data was acquired using the Affimmetrix 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 is deposited at ArrayExpress database (E-MTAB-2446). To perform RT-qPCR, RNA from endpoint tumor xenografts was used to synthesize cDNA using Superscript-III reverse transcriptase with oligo-dT and random hexamer primers (Life Technologies). A 7900HT qPCR System was used with Power SYBR-green (Applied Biosystems) and specific primer pairs. Relative gene expression was determined by the comparative \( C_T \) method (25) and significance was determined using an unpaired \( t \) test with Welch-correction. See details in Supplementary Methods.
Genotyping
Tumor samples from CRC 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 Methods. Frequent mutations in 57 oncogenes and tumor suppressor genes were interrogated (Supplementary Table S1). Microsatellite instability was analyzed using the MSI-Analysis System (Promega). Purified patient-derived cells were genotyped straight after surgical resection of patients’ tumors by Sequenom or Haloplex platforms. Genotyping by Sequenom (CLIA panel) was performed as previously described (10). Haloplex Target Enrichment System (Agilent Technologies) was used to capture the complete coding regions of 388 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). Non-tumoral 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 is available at SRA database at NCBI (BioProject ID: PRJNA242531).

Immunohistochemistry and immunofluorescence
Samples from paraffin-embedded tissues were stained as described in (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, Carpinteria, CA) 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 Methods.

DLD1F and HT29F cells were seeded on glass cover-slips and treated for six hours with 4OHT 100 nm (4-hydroxytamoxifen, 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 cell culture dishes. Cells were pre-
treated with NVP-TNKS656 (100 nM, Novartis) or DMSO for 48 h and then with API2 (20 μM, Tocris Bioscience) and/or NVP-BKM120 (2.4 μM, Selleck Chemicals) for another 48 h prior apoptosis analysis. Proportions of apoptotic cells were determined using the Annexin V-eGFP (BioVision) kit. Dead cells were detected as DAPI negative (1 mg/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 h in 4% PFA, permeabilized with PBS/1% Triton X-100 at room temperature for 3 h and blocked over night at 4ºC in PBS/1% Triton X-100/3% BSA. Samples were incubated for 24 h with primary antibodies (Supplementary Table S3). Secondary antibodies and Hoechst 33342 (5 μg/ml) were incubated over night at room temperature.

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

Western blot
The detailed protocol for protein extraction is described in Supplementary Methods. Western blot was performed as described in (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) (31). Cells were treated with NVP-TNKS656 100 nM (Novartis) for seven days and eGFP accumulation was measured by flow cytometry using a Navios Flow Cytometer (Beckman Coulter).

Statistics
We analyzed progression free survival of patients by the Kaplan-Meier method and compared the curves using a Log-rank (Mantel-Cox) test. We used Pearson’s 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’s 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, phosphoS6, Ki67 and cleaved-CASPASE3 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’s exact test served to analyze the differences in response among patients regarding nuclear β-catenin content, mutations affecting PIK3CA, KRAS, TP53, APC or tumor histological TNM status. P values lower than 0.05 were considered significant in all tests.

RESULTS
Effective pharmacological 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 CRC 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 CRC 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 and 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-CASPASE3 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 PIK3 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 indicates that nuclear β-catenin content conditions drug-response in patient-derived sphere cell cultures whereas frequent mutations in CRC do not (Fig. 1C and D, 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 (Fig. 1B, D and 2A). Treatment with NVP-TNKS656 caused a systemic reduction of nuclear β-catenin content and function in skin and intestine, tissues where 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-P5, with low basal nuclear β-catenin and FOXO3A content, probably representing a case of a CRC 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 phosphor-S6 or tumor growth upon API2 treatment in this model (Supplementary Fig. S5 and 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 phosphor-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 (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 end point of the in vivo experiments (Fig. 2B). The three models showed a distinct gene expression pattern that was modified by NVP-TNKS656 treatment (Fig. 3A, Supplementary Tables S7, S8 and 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 nine 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 suggests 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 Wnt/β-catenin pathway by NVP-TNKS656 alone did not promote apoptosis in DLD1 or HT29 colon cancer cells but enhanced the apoptosis induced by exogenous nuclear FOXO3A-ER (Fig. 4). This data confirms 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 patients’ tumors (Fig. 5A and B). Apoptosis was also proportional to FOXO3A content when sphere cultures were treated with a different tankyrase inhibitor, NVP-XAV939 (Supplementary Fig. S1). Similarly to β-catenin (10), patient-derived xenografts models and primary sphere cultures faithfully preserved the levels 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).

Lastly, by profiling 130 CRC 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 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 microsatellite instability and mutational status of KRAS, BRAF and PIK3CA, all molecular features relevant for CRC tumors. Any obvious correlation was observed between them and gene expression signatures distinctive of active FOXO3A/β-catenin transcription (Fig. 5C and 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 CRC tumors. Limited availability of samples from clinical trials only permitted the study of tumors at baseline from a cohort of 40 CRC patients treated with PI3K/AKT/mTOR pathway inhibitors in several phase I clinical trials (Fig. 6A, upper panel and 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 progression free survival (PFS) between patients treated with different PI3K, AKT or dual PI3K/mTOR drug subtypes (Fig. 6A, lower panel). 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 histological 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, nine out of 27 patients with tumors presenting low β-catenin continued treatment after CT scan evaluation, showing some degree of stabilized disease (Fig. 6C, upper panel). 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 nine patients were either more sensitive to anti-tumoral drugs in general or had slower tumor growth independently of treatment (Fig. 6C, lower panel 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, Supplementary Fig. S9A-F, Supplementary Tables S13 and S14). Only two out 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 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-I). 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 alpha, 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 outcome (PFS HR 3.96, CI95% 1.03-15.27; log rank p value 0.0158) (Supplementary Table S15).

Concerning gene expression patterns, we could analyze 31 out 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 cut-off selected to catalogue tumors histologically (Fig. 6B, Supplementary Figs. S8 and S10B).
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 CRC 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. Since 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 CRC 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 present study we observed that CRC patients with high nuclear β-catenin present a shorter progression free survival when treated with PI3K/AKT/mTOR pathway inhibitory drugs in the context of clinical trials. Such differential response contrasts with the fact that all CRC 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 CRC.

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 CRC patients beyond their combination with PI3K or AKT inhibitors. Indeed, we previously showed that cancer cells with high pro-apoptotic FOXO3A transcriptional 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 pharmacological 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 CRC 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 CRC. It is well described that oxidative stress promotes FOXO3A translocation from the cytoplasm to the nucleus and the consequent induction of genes involved in cells 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), since they may not recapitulate tumoral FOXO3A activity as consistently as PDX. Indeed, PDX are generally considered the best pre-clinical models to evaluate drug-response since they faithfully recapitulate patient’s disease preserving their molecular alterations and histopathological traits (24, 38, 39).

Other alterations frequent in CRC 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 CRC 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. This data suggests that the therapeutic potential of tankyrase inhibitors could be extended to the majority of CRC 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 parasylate and commit other proteins to degradation in addition to AXIN1 and 2 (40, 41), it could be of interest to evaluate to what extent the anti-tumoral 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 CRC tumors with high FOXO3A and β-catenin content and transcriptional activity. In particular, we observed that a particular set of genes was overexpressed in CRC 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 CRC 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 histological evaluation of nuclear FOXO3A and β-catenin content and the response to treatment, to finally define those gene sets associated to such histological 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 histological 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 CRC, 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 histopathological 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: 1) low nuclear β-catenin and FOXO3A activity, more suitable for the treatment with PI3K or AKT inhibitors alone, 2) high β-catenin and FOXO3A activity who could benefit from Wnt/β-catenin inhibitors alone and 3) 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 CRC therapy.

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AUTHOR CONTRIBUTIONS
O.A. performed most of the experiments and helped to interpret the results and write the manuscript. I.C., I.P. and S.P.T. helped to perform some experiments. G.A., R.D. and J.R. provided clinical follow up of all patients included in the study. S.L. selected the tissue specimens from which to derive cancer cells. A.P. Helped analyzing gene expression profiles by Nanostring. E.E. and R.C. performed surgery on colon cancer patients from whom PDX models were derived. P.N. performed histopathological evaluation and immunohistochemistry of β-catenin on tissue sections from CRC patients included in clinical trials with PI3K/AKT/mTOR inhibitors. A.V. performed mutational analysis of human colon carcinomas. W.S. provided the tankyrase inhibitor NVP-TNKS656 developed by Novartis and supervised the project. J.T. supervised the project. H.G.P designed all the experiments, interpreted all resulting data, supervised the whole project and wrote the manuscript.
ABBREVIATIONS
CRC, colorectal cancer; CT, computerized axial tomography; FFPE, formalin-fixed paraffin-embedded; NOD-SCID, nonobese diabetic/severe combined immunodeficiency; PDX, patient-derived xenograft; TNKS, tankyrase; TNM, tumor, Nodes and Metastasis.

REFERENCES

FIGURE LEGENDS

Figure. 1. CRC patient-derived cells with high amounts of nuclear β-catenin present high sensitivity to API-2 or NVP-BKM12 in combination with NVP-TNKS656. (A) Representative pictures of immunofluorescence and confocal microscopy of histological 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 standard deviation. (C) Column scatter plot showing the apoptosis induced in sphere cell cultures of these 10 patient-derived models treated as indicated. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. Horizontal lines indicate arithmetic mean values, and error bars show Standard Error of the Mean (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 API-2 (left upper panel) and NVP-BKM120 (right upper panel) or API-2 + NVP-TNKS656 (left lower panel) and NVP-BKM120 + NVP-TNKS656 (left lower panel) in sphere cell cultures of these 10 patient-derived models versus the histological amount of nuclear β-catenin in the original patient’s tumors. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. P values correspond to Pearson’s correlation test. (B, D) β-catenin relative units (r. u.) were calculated as described in Methods.

Figure. 2. NVP-TNKS656 stabilizes AXIN1 and reduces both, nuclear β-catenin and tumor growth alone or in combination with the AKT inhibitor API-2 in CRC PDX models. (A) Representative pictures of double immunofluorescent staining and confocal microscopy to detect β-catenin (red) and FOXO3A (green) in histological 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 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 +/- S.D. 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 (upper panels). β-tubulin was used as loading control. Blots were quantified using ImageJ software (lower panels). Protein expression levels are represented as relative units (r. u.) versus vehicle treated samples. Bars indicate standard deviation. \( P \) values correspond to unpaired t tests.

Figure. 3. Reduction of nuclear \( β \)-catenin content by NVP-TNKS656 regulates gene expression in CRC PDX models. (A) Three tumor xenografts from mice injected with PDX-P2, PDX-P30 or PDX-P5 treated with vehicle or NVP-TNKS656 were analyzed for gene expression at the end point of the experiments. Gene clustering diagrams shown were calculated using robust-multiarray-normalized expression values from genome-wide microarray analysis. Triplicates of each treatment are indicated as colored rectangles (R1, R2 and R3). Significance was calculated using two-tailed ANOVA with a significance cutoff of \( P \leq 0.05 \). Gene expression scaling was indicated from low (blue) to high (red). (B) Venn diagrams showing the number of genes down-regulated by NVP-TNKS656 in all PDX models and genes that belong to the TCF/\( β \)-catenin (44) or FOXO3A/\( β \)-catenin (10) transcriptional programs. Common genes are indicated on the right and enumerated in brackets. Asterisks indicate genes involved in metastasis (C) Relative mRNA expression levels of selected genes was confirmed by quantitative RT-PCR in the same xenograft tumors from the three PDX models treated with vehicle (blue bars) or NVP-TNKS656 (red bars). Expression values are shown as normalized \( ΔC_T \). Error bars \( ± S.D. \) of triplicate values obtained in three independent measurements are shown. \( P \) values correspond to unpaired t tests.

Figure. 4. NVP-TNKS656 sensitizes CRC cell lines to the apoptosis induced by FOXO3A. (A) Immunofluorescence and confocal microscopy to detect the translocation of exogenous FOXO3A(A3):ER\(^T\) from cytoplasm to nucleus of DLD1F and HT29F colon cancer cells upon tamoxifen (4OHT) treatment. Scale bar, 10 \( μm \). (B) Western blots showing the amount of exogenous FOXO3A(A3):ER\(^T\) in the nucleus and stabilization of AXIN1 in the cytoplasm of DLD1F and HT29F cells upon indicated treatments. The amounts of \( β \)-TUBULIN and LAMIN A/C were evaluated to confirm the purity of nuclear and cytoplasmic extracts and as loading control. (C) Left panel: Representative pictures of DLD1F cells expressing fluorescent green protein (eGFP) under the control of seven upstream TCF/\( β \)-catenin binding sites (7xTOP-eGFP) upon 5 days of NVP-TNKS656 treatment. Scale bar, 200 \( μm \). Right panel: 7xTOP-eGFP activity after treating DLD1F cells with vehicle or NVP-TNKS656 was assessed by flow cytometry. Bars indicate S.D. of...
experimental triplicates. (D) Apoptosis was measured in DLD1F and HT29F cells upon indicated treatments. Data are represented as fold change of apoptotic cells induced by the treatment compared to cells treated with vehicle. Bars show +/- S.D. of five replicates in six independent experiments. $P$ values correspond to unpaired t tests.

**Figure. 5. High FOXO3A activity in CRC patient-derived cells determines the apoptosis induced by NVP-TNKS656.** (A) Representative pictures of double immunofluorescence and confocal microscopy to detect $\beta$-catenin (red) and FOXO3A (green) in models PDX-P33 and PDX-P34. Arrowheads point cancer cells in PDX-P33 presenting high nuclear FOXO3A and $\beta$-catenin accumulation. PDX-P33 presented high and PDX-P34 low nuclear FOXO3A and $\beta$-catenin content. Lower panels show magnifications to better visualize $\beta$-catenin and FOXO3A subcellular localization. White dotted lines delineate nuclei stained with Hoechst 33342 (blue). Scale bars, 100 $\mu$m; magnifications, 50 $\mu$m. (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. FOXO3A relative units (r. u.) were calculated as described in methods. Apoptosis is represented as fold change of apoptotic cells induced by NVP-TNKS656 compared to the cells treated with vehicle. $P$ values correspond to Pearson’s correlation test. Nuclear $\beta$-catenin content for each model is also indicated. (C) Nanostring platform was used to analyze the expression of 31 FOXO3A/$\beta$-catenin and 21 TCF/$\beta$-catenin target genes in FFPE tumor samples from 130 colorectal cancer patients. Samples and genes were ordered by hierarchical clustering using uncentered Pearson correlation distance and complete linkage. Microsatellite stability and mutational status of $KRAS$, $PIK3CA$ and $BRAF$ were evaluated as indicated in methods. Patients showing highest expression of FOXO3A/$\beta$-catenin and TCF/$\beta$-catenin target genes are highlighted with a yellow box. Gene expression scaling is shown from low (blue) to high (red).

**Figure. 6. High nuclear $\beta$-catenin content is associated with resistance to PI3K/AKT/mTOR inhibitors in CRC patients.** (A) Table summarizing molecular targets, drugs and number of patients (40 in total) in each clinical trial whose baseline tumors were profiled. Kaplan-Meier analysis representing progression-free survival (PFS) of patients separated by drug subtype administered: dual PI3K/mTOR, PI3K or AKT inhibitors. Number of patients is show for each cohort (n). $P$ value was calculated by Log-rank (Mantel-Cox) Test. (B) Representative pictures of double immunofluorescence staining and confocal microscopy to detect $\beta$-catenin (red) and alpha-catenin (green) in CRC tumor sections of patients treated with PI3K or AKT inhibitors. Tumors present low
(upper image) or high (lower image) nuclear β-catenin content (Scale bar, 100 μm). Panels on the right show magnifications with fluorescent channels split to better visualize β-catenin subcellular localization (Scale bar, 20 μm). All sections were counterstained with Hoechst 33342 (blue) to detect nuclei. Dashed lines delineate nuclei. (C) Upper panel: Chart representing the time that each patient received a PI3K/mTOR, PI3K or AKT inhibitor and the time on the corresponding previous line of treatment. The 40 patients studied are split in those with tumors presenting high and those with low nuclear β-catenin accumulation. Pink vertical bar indicate the latest period of time when disease progression was evaluated by Computerized Axial Tomography (CAT) scan. Green horizontal bars correspond to patients who had progressed to treatments with PI3K/mTOR, PI3K or AKT inhibitors at the time of first CAT scan after initiating the clinical trial. Red bars show the time on treatment for those patients who had not progressed to treatment at the time of first CAT scan. Lower panel: Scatter plot representing the time on treatment with PI3K/mTOR, PI3K or AKT inhibitors versus the time on previous line of treatment for all 40 CRC patients. Their level of nuclear β-catenin is indicated. P value corresponds to Pearson’s correlation test. (D) Kaplan-Meier curves showing progression-free survival (PFS) of patients presenting tumors with high or low nuclear β-catenin content and treated with PI3K/mTOR, PI3K or AKT inhibitors (left) or the correspondent previous line of therapy (right). Number of patients (n) is show for each cohort. P value, Hazard Ratio (HR) and 95% confidence interval (CI) are shown and calculated by Log-rank (Mantel-Cox) test.
FIGURE 2

A

B

C

D

AXIN1 protein expression (f. U.)

VEHICLE

API2

TNKS656

API2 + TNKS656

P5

P2

P30

VEHICLE

TNKS656

API2

API2 + TNKS656

P5

P2

P30

Time (days)

Tumor growth (luminal area fold change)

Time (days)

Time (days)

AXIN1

p-TUB

TNKS656

API2

P5

P2

P30

p = 0.0067

p = 0.00041

p = 0.00025

p = 0.0482

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FIGURE 3

A

B

C

(1) P2/P30

S100A4

(4) P2/TCF

AXIN2

EPHB3

HOXA11

ID3

(2) P5/TCF

CIPH1A

CDKN3

(2) P2/FOXO3a

CLIC3

PHLD2

(9) P30/FOXO3a

SLC2A3

CAV1

CAV2

PLK2

MALT1

WWC2

CCDC68

WWTR1

ARHGAP29

(1) P5/FOXO3a

PYGL

VEHICLE

TNKS656

S100A4

CLIC3

AXIN2

SLC2A3

EPHB3

CAV2

ID3

CYR61

Normalized ΔCT

P = 0.0142

P = 0.0045

P = 0.0011

P = 0.033

P = 0.0012

P = 0.006

P = 0.003

P = 0.0006

P = 0.0283

P = 0.0025

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FIGURE 4

A

DLD1

HT29

VEHICLE

4OHT

FOXO3a β-catenin

B

DLD1

HT29

FOXO3a-HA

β-TUBULIN

LAMIN A/C

AXIN1

β-TUBULIN

LAMIN A/C

TNKS656 4OHT

- - + +

- - + +

C

VEHICLE

TNKS656

7xTOP-eGFP

TNKS656

- +

D

TNKS656 4OHT

- - + +

- - + +

APOTOPSIS

(% Annexin V positive cells)

7xTOP-eGFP activity (r. u.)

P = 0.0124

P < 0.0001

P = 0.0002

P = 0.0006

P = 0.0273

P = 0.0014
FIGURE 6

A

<table>
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<th>Target</th>
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<tr>
<td>AKT</td>
<td>LY278391</td>
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</tbody>
</table>

B

C

D

PREVIOUS LINE of TREATMENT

High Nuclear β-catenin
Low Nuclear β-catenin

HR = 0.612 (95% CI: 0.316-1.184)  
\( P = 0.1446 \)

Time on treatment with previous line (days)

0 60 120 180 240 300 600 1200 1800 2400 3000

HR = 2.208 (95% CI: 1.485-7.479)  
\( P = 0.008 \)

Time on treatment with PI3K/AKT/mTOR inhibitors (days)

0 60 120 180 240 300 600 1200 1800 2400 3000

Time on treatment with PI3K/mTOR inhi.

Previous line

Nuclear β-catenin

HR = 0.612 (95% CI: 0.316-1.184)  
\( P = 0.1446 \)

Time on treatment with previous line (days)

0 60 120 180 240 300 600 1200 1800 2400 3000

P = 0.4716
Clinical Cancer Research

Tankyrase inhibition blocks Wnt/β-catenin pathway and reverts resistance to PI3K and AKT inhibitors in the treatment of colorectal cancer

Oriol Arques, Irene Chicote, Isabel Puig, et al.

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