**ONLINE METHODS**

* **Study supervision**

The study was approved by the institutional Review Boards of Hospital Universitario HM Sanchinarro and conducted in agreement with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice guidelines. The patient gave written informed consent to participate in the study. Mice used in this research were treated humanely according to the regulations laid down by the Spanish National Cancer Research Centre (CNIO) Bioethics Committee.

* **DNA extraction**

DNA was extracted from leukocytes (gDNA), liver metastasis (tDNA), and basal and on-treatment plasma samples (cfDNA), using commercial kits according to the manufacturer’s instructions (Qiagen, Germany). The DNA amount was quantified with a Qubit™ Fluorometer (Thermofisher, USA) and reported in ng. cfDNA samples were also quantified using a modified version of human LINE-1-based quantitative real-time PCR and reported in genome equivalents (GE; GE being one haploid human genome weighing 3.3 pg). gDNA and tDNA was sheared to 300-bp fragments on a Covaris instrument (Covaris, Woburn, MA) according to standard procedures. The 2100 Bioanalyzer (Agilent, USA) was used to access the quality and size of the pre-processed and post processed samples and libraries.

* **Routine genetic analysis**

The FDA-approved Cobas mutation kit (Roche, Switzerland) was used to analyze the following mutations in the diagnostic biopsy tDNA: *KRAS* (G12S/R/C/V/A/D, G13D, Q61H, A146T), *NRAS* (Q61K/R/L/H), *BRAF* (V600E), and *PIK3CA* (E542K, E545K/G, Q546K, M1043I, H1047Y/R/L). The presence of the same mutations in the patient’s basal and on-treatment cfDNA samples was assessed by the highly sensitive BEAMing technique, as previously described1.

* **Whole-exome sequencing**

Sequencing libraries of cfDNA (15 ng), and gDNA and tDNA (70-110 ng) samples were prepared using the ThruPLEX Plasma- and DNAseq Kits (Rubicon Genomics Inc, USA), respectively. Barcode indices were added to samples during eight PCR cycles of template preparation, and 550 ng of each sample was processed through the SureSelectXT Target Enrichment System (Agilent SureSelect V5, ref. 5190-6208, protocol G7530-90000 version B1). xGen Blocking Oligos (IDT, Iowa, USA) were used as suggested by Rubicon Genomics. Captured targets were subsequently enriched by 11 cycles of PCR with KAPA HiFi HotStart (Kapa Biosystems), with a Tann of 60º and the following primers, which target generic ends of Illumina adapters: AATGATACGGCGACCACCGAGAT and CAAGCAGAAGACGGCATACGAGAT. For sequencing, magnetic bead-purified libraries with similar concentrations of cfDNAs and tDNA, and half the concentration of gDNA were pooled in order to increase coverage and favor the detection of non-inherited sub-clonal mutations. Sequencing was carried out in the Illumina HiSeq4000 platform. All sequencing data are going to be deposited in the European Nucleotide Archive (ENA) under the accession number ENA#202177, at the time of publication.

* **Somatic mutation call**

Bioinformatics analyses were performed using the NEXTGEN software (Softgenetics, USA), as previously described2. The detailed parameters used for the alignment and mutation call are provided as supplementary material. Briefly, FastaQ files were aligned using the BWA pipeline and the variants were processed by sequential stringent filters to exclude low-confidence variants. Only variants that passed the following filters were classified as high-quality and considered in the study: overall and allele scores ≥ 12; coverage ≥ 20; number of mutated reads ≥ 20; percentage of mutated reads ≥ 3% of cfDNA / tDNA and ≥ 35% of gDNA; F:R read balance ≥ 0.1; and F:R read percentage ≥ 0.45. The list of non-hereditary mutations detected by WES-cfDNA and WES-tumor was generated after disregarding germline variants (obtained by WES-gDNA). A detailed genomic annotation of the somatic mutations we identified, prediction of mutation pathogenicity based on predictor algorithms (SIFT, Polyphen2, LRT, Mutation Taster, Mutation Assessor, and other software packages included in the dbNSFP3), allele frequencies in population studies, such as 1000G and EXAC, and additional information are shown in Table S1.

* **TaqMan SNP genotyping assay**

A custom TaqMan® genotyping assay for the detection of the *KDR* c.2518C (L840L) and *KDR* c.2518C>T (L840F) alleles was designed using the Thermofisher online Design Tool (oligonucleotides and probes are shown in below). TaqMan® MGB (minor groove binder) probes incorporate a 5' reporter (VIC or FAM) and a 3' nonfluorescent quencher (NFQ).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay ID** | **Oligonucleotides** | **Sequence** | **5´ Reporter** | **3´ Reporter** |
| AHRSSQR  | primer forward | CTTGTTGCTGTTGTTGTTGACTCT | - | - |
| primer reverse | GCTTCAATCACTTGGCCAAAGG | - | - |
| probe wt | CGGCCAA**G**AGGCTTA | VIC | NFQ |
| probe L840F | CGGCCAA**A**AGGCTTA | FAM | NFQ |

* **Genetic/protein database and protein structure analyses**

Previously reported germline and somatic variants in *KDR* were retrieved from general population (EXACT4 and ESP5) and cancer (COSMIC6, GENIE7, PCAWGS8) sequencing public projects.The VEGFR2, EGFR, and ABL1 protein structures were obtained from the RCSB data bank; structurally analogous mutations in other cancer-relevant kinases were identified using MutationAligner9; kinase residues interacting with kinase inhibitors were mapped using the LigPlot10 software. Computational modeling of inhibitor binding to WT and L840F VEGFR2 was performed as previously described.

* **Generation and treatment of the Avatar patient-derived xenograft (PDX) model**

Liver metastasis biopsy was performed after tumor progression to capecitabine-bevacizumab rechallenge (Figures 1 and 1S). A fraction of the biopsy was used to generate the Avatar model as previously described by our group11,12. Expanded cohorts (five to six animals per arm) were treated with: anti-VEGF drugs (B20/murine and bevacizumab/human), VEGFR2 kinase inhibitors (axitinib, cabozantinib, cabozantinib:MEK inhibitor combo, lenvantinib, pazopanib, regorafenib, and sorafenib), and inhibitors of other kinases, such as afatinib (EGFR), crizotinib (MET), and MEK inhibitor (MAPK). Information on the treatment regimens is shown below.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Treatment** | **Company (catalog)** | **Administration** | **Dosis** | **Schedule** | **Period** |
| afatinib | MedChem Express (HY-10261) | oral | 20 mg/kg | 1X daily | 3 weeks\* |
| axitinib | Selleckchem (S1005) | oral | 30 mg/kg | 1X daily | 3 weeks\* |
| B20 | Merck | intraperitoneal | 5 mg/kg | 2X week | 3 weeks\* |
| bevacizumab | Genentech | intraperitoneal | 50 mg/kg | 2X week | 3 weeks\* |
| cabozantinib | MedChem Express (HY-13016) | oral | 10 mg/kg | 1X daily | 3 weeks\* |
| cabozantinib | MedChem Express (HY-13016) | oral | 30 mg/kg | 1X daily | 3 weeks\* |
| crizotinib | Selleckchem (S1068) | oral | 50 mg/kg | 1X daily | 3 weeks\* |
| MEKi | Selleckchem (S1036) | oral | 1 mg/kg | 1X daily | 3 weeks\* |
| MEKi + Cabozantinib | Selleckchem (S1036), MedChem Express (HY-13016) | oral | 1 mg/kg + 30 mg/kg | 1X daily | 3 weeks\* |
| pazopanib | MedChem Express (HY-10208) | oral | 50 mg/kg | 1X daily | 3 weeks\* |
| regorafenib | Selleckchem (S1178) | oral | 10 mg/kg | 1X daily | 3 weeks\* |
| sorafenib | Selleckchem (S7397) | oral | 60 mg/kg | 1X daily | 3 weeks\* |

\*or until the tumors reach the humane endpoint

* **Cell lines**

The human CRC cell lines used in the current study were selected based on their genotype in order to be as informative as possible for each experiment. Thus, we chose the Colo-320 cell line to interrogate the phenotypic changes caused by the overexpression of VEGFR2 mutants because it has the same genetic background as the patient´s tumor (mutated *TP53*/*APC* and WT *KRAS*/*BRAF*). The MDST8 CRC cell line was used for drug sensitivity studies because it naturally harbors the *KDR*/VEGFR2 R1032Q mutation, which we found to be a hot-spot VEGFR2 mutation in human cancers.

Colo320 and MDST8 colorectal cell lines were obtained from ATCC and cultured at 37°C in 5% CO2, in Roswelll Park Memorial Institute (RPMI) Medium 1640 + GlutaMAX (Gibco, USA) and Dulbeccos’ modified Eagle’s medium (DMEM) + 2 mM Glutamine (Gibco, USA), respectively, supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA). Porcine aortic endothelial (PAE) cell lines, kindly provided by Dr. Kurt Ballmer-Hofer, were grown in DMEM supplemented with 10% FBS.

* **Generation of stable colorectal and endothelial cell lines**

Colo-320 and PAE cell lines were used to generate cell lines stably expressing the VEGFR2 mutants using a previously described protocol12. Briefly, cells were seeded in 10-cm plates in the appropriate medium and were grown to 70% confluence. Transfection with constructs carrying either the empty vector or VEGFR2 (WT or mutant) was performed with polyethylenimine (PEI) as previously described13. Briefly, 30 µg of WT or mutant VEGFR2 plasmid (in the pBE vector containing the neomycine resistance gene, which confers resistance to the selection antibiotic G418) was mixed with 60 µl PEI (1 mg/ml in H2O) in 2 ml serum-free DMEM, incubated for 10 min at room temperature and added to the cells. Following a 3-h incubation at 37°C, the medium was changed, and the cells were allowed to grow to 100% confluence. Cells were re-seeded at a series of dilutions (1:1000 - 1:5000) in antibiotic selection medium (1 mg/ml G418) to allow for single colonies to grow, while non-transfected cells were dying. Individual colonies were consecutively transferred to 24-well and 6-well plates and screened by western blotting for VEGFR2 expression. To reduce polyclonality, colonies with the highest expression levels were subjected to 3 additional rounds of subcloning.

* **VEGF stimulation and western blotting**

Transiently transfected HEK293 cells or stable PAE cell lines expressing WT or L840F-KDR were starved in DMEM supplemented with 1% bovine serum albumin (BSA) for 4h at 37°C and were subsequently stimulated with 1.5 nM (60 ng/ml) VEGF165 for 10 min at 37°C. Following stimulation, the cells were scraped in lysis buffer (50 mM Tris pH = 8.0, 120 mM NaCl, 1% NP-40) supplemented with protease inhibitors (Roche, cat. Nr 04693159001) and phosphatase inhibitors (1 mM sodium orthovanadate and 20 uM phernylarsine oxide) and incubated for 30 min on ice. Cell lysates were collected as the supernanant of a centrifugation at 30,000 × *g* for 15 min and subjected to western blot analysis. The following antibodies were used to probe receptor activation: total KDR (Cell Signaling, cat. Nr 2479), phospho KDR at Y1175 (Cell Signaling, cat. Nr 2478). The secondary antibodies used were alkaline phosphatase (AP) conjugated (Southern Biotech). All antibodies were diluted at a 1:1000 ratio in 5% BSA in Tris-buffered saline, containing 0.05% Tween20 (TBST) buffer. The chemiluminescence signal was developed with the Novex AP Chemiluminescence substrate (Invitrogen, cat. Nr 100002906), recorded with an Amersham Imager 600 (Amersham), and quantified by ImageJ (NIH). Activation of KDR was assessed by the ratio of phospho-to-total signal.

* **Tissue immunofluorescence**

Immunofluorescence staining was performed to detect p-ERK and p-AKT. Formalin-fixed and paraffin-embedded tumors from Avatar models were cut into 3-mm-thick sections, deparaffinized, and preincubated with FBS to prevent nonspecific binding. The sections were incubated at room temperature for 30 min with a rabbit polyclonal antibody to p-ERK (1:300; Cell Signaling #9101) or a rabbit monoclonal antibody (D9E) to p-AKT (1:300, Cell Signaling #4060), followed by incubation with Alexa Fluor 555–conjugated donkey anti-rabbit IgG (1:400; Life Technologies#A27039) at 37ºC for 20 min. Nuclei were counterstained with DAPI (Molecular Probes) at 1:1,000 dilution, and the slides were mounted with Mowiol 4-88 (Calbiochem). Images were acquired with a confocal TCS-SP5 (AOBS-UV) (Leica Microsystems) confocal microscope, equipped with a 20xHCX PL APO 0.7 N.A. objective.

* **Proliferation assays**

Proliferation assays were performed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Briefly, cell lines were seeded in 96-well microtiter plates at a density of 10000 cells/well and were incubated for 24 h before adding the various drugs. A “mother plate” containing drugs at a concentration 200× higher than the final concentration to be used in the cell culture was prepared by serial dilutions of stock solutions of the drugs (10 mM) in DMSO. The appropriate volume from each drug (usually 2 μL) was added automatically (Beckman FX 96 tip) from this plate to the cell culture plate to reach the final concentration for each drug. Each concentration was assayed twice. The final concentration of DMSO in the tissue culture media did not exceed 1%. The cells were exposed to the drugs for 72 h and then analyzed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Cell proliferation values were plotted against drug concentrations and fitted to a sigmoid dose-response curve using the Activity base software from IDBS in order to calculate growth inhibition (GI50) values versus DMSO.

* **Cloning and mutagenesis**

The L840F and eight additional *KDR* mutations of interest identified on cancer databanks, as well as the K868M kinase-dead mutation, were generated by site-directed mutagenesis of WT *KDR*/VEGFR2 cloned on the pBE vector, using the QuikChange Kit (Agilent, USA) and the primers described in Table S5. Mutations were confirmed by Sanger sequencing of the entire open reading frame.

* **Transfection and xenograft models**

Colo-320 cell line-derived xenografts were generated from subcutaneous injections of 4 × 105 cells resuspended in phosphate-buffered saline (PBS) in four nude mice per genotype. Tumors were measured weekly and the animals were sacrificed within two months or when tumors reached the established humane endpoint. Mice injected with empty vector or the K868M kinase-dead mutant were kept alive and monitored weekly for four months.

* **Production of recombinant kinase domains of WT, L840F, and R1032Q VEGFR2**

The kinase domains (residues 806-1171) of WT, L840F, and R1032Q VEGFR2 without the kinase insert domain (aa 940-989) were cloned, tagged with 6×His at their C-terminus, and expressed in the baculovirus-infected insect cell system. Proteins were purified by affinity chromatography on HisTrap columns, followed by size-exclusion chromatography on a HiLoad 16/600 Superdex 200 prep grade column (GE Healthcare, USA), using an ÄKTA system (GE Healthcare, USA). Fractions containing kinase domains were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and concentrated by ultrafiltration up to 0.2 mg/ml. Protein mutations were confirmed by in-gel enzymatic digestion followed by liquid chromatography–mass spectrometry (LC-MS)/MS analysis.

* **Biochemical Assays**

The kinase activity of recombinant WT, L840F, and R1032Q VEGFR-2 (*KDR*) kinase domains, as well as that of a commercially available recombinant KDR cytoplasmic domain (residues 789-1356) (PV3660; ThermoFisher) that was used as a positive control were analyzed using the LANCE®Ultra time-resolved fluorescence resonance energy transfer (TR-FRET) assay from Perkin Elmer according to the manufactures’ instructions.. Briefly, the enzymes were titrated starting from an initial concentration of 5 μg/ml and proceeding with 1:4 serial dilutions, and were added to the reaction buffer (15 mM HEPES pH 7.4, 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 10 mM MgCl2, 0.1 mg/ml BGG, 2 mM DTT), containing 15 µM ATP and 200 nM UltralightTM-labeled Poly GT substrate in a total volume of 20 μl. The reaction was allowed to proceed in an Optiplate 384 from PerkinElmer for 60 min at room temperature. Reactions proceeded within the linear reaction time were then terminated by the addition of 20 mM EDTA and 4 nM Eu-W1024-labeled PY20 antibody. After an incubation of at least 60 min, the samples were excited with a Light Unit laser at 337 nm, and the emission of the LANCE Eu/APC (615/665 nm) was measured with an Envision reader (PerkinElmer). To test the effect of known VEGFR2 inhibitors on kinase activity, 0.3 ng WT VEGFR2 and 300 ng mutant VEGFR2 were used. The starting concentration of the inhibitors tested was 10 μM, followed by 1:5 serial dilutions. In order to calculate IC50 values of inhibition versus DMSO, the data were plotted against the inhibitor concentration and fitted to a sigmoid dose-response curve using the Activity base software from IDBS.

* **Immunohistochemistry**

Avatar tumor samples were fixed in 10% neutral buffered formalin (4% formaldehyde in solution) and paraffin-embedded. Subsequently, 3-µm-thick sections were cut from the samples, mounted in superfrost®plus slides, and dried overnight. Before staining, the sections were deparaffinized in xylene and re-hydrated through a series of decreasing ethanol concentration in water. Consecutive sections were stained with hematoxylin and eosin (H&E) and by immunohistochemistry, using an automated immunostaining platform (Ventana Discovery XT, Roche or Autostainer Plus Link 48). Antigen retrieval was first performed with high or low pH buffer (CC1m, Roche), endogenous peroxidase was blocked (3% hydrogen peroxide), and the slides were incubated with an anti p-ERK rabbit polyclonal primary antibody (1:300; Cell Signaling #9101) for 28 min. Subsequently, the slides were incubated with the corresponding visualization system (OmniRabbit, Ventana, Roche) with signal amplification conjugated with horseradish peroxidase. The signal was developed using 3,30-diaminobenzidine tetrahydrochloride (DAB) as a chromogen (Chromomap DAB, Ventana, Roche or DAB solution, Dako), while the nuclei were counterstained with Carazzi’s hematoxylin. Finally, the slides were dehydrated, cleared, and mounted with a permanent mounting medium for microscopic evaluation. The entire slide was scanned with a slide scanner (Axio Z1, Zeiss), and images were captured with the ZEN software (Zeiss) after evaluation by a trained veterinary pathologist. Image analysis and quantification were performed using the AxioVision software package (Zeiss).

* **Kaplan-Meier analysis of mCRC patients**

Kaplan-Meier survival data of 1,303 patients from four different cohorts of CRC patients with global gene expression and survival datasets available (GSE24551, GSE14333, GSE17538, GSE39582) were queried using the R2 microarray analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi). Concerning *KDR* gene expression in the combined datasets, 304 tumors had high levels of expression and 999 had low levels of expression.

**References (online methods)**

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