SUPPLEMENTARY INFORMATION

Materials and Methods of experiments on primary samples

TMA construction

Sufficient paraffin-embedded tissue for TMA construction was available in all cases. Formalin-fixed paraffin embedded tissues were first examined with H&E staining using whole sections to identify pathologically distinct areas of interest (tumour and adjacent normal). TMAs were constructed by punching 1.0mm discs and re-embedding them into a new paraffin block. From each sample, 4 cores (2 for neoplastic tissue and 2 for normal pancreas) of 1mm diameter were obtained from the donor block and transferred to receiver blocks using Quick-Ray® Tissue Micro Arrayer. Therefore, each receiver block was made of 60 cores belonging to 15 patients. Each TMA block was cut into 3μm sections and H&E staining was performed on every 25 section to assess retention of cancer tissue.

To ensure a reliable evaluation of immunostaining in each patient, TMAs were properly assembled (at the Campus Bio-Medico University of Rome) to allow the direct comparison of the neoplastic sample with its normal counterpart.

Immunohistochemistry

The sections were dewaxed and re-hydrated, endogenous peroxidases were blocked and antigen retrieval was performed with either Proteinase K (5μg/ml, for hERG1) or citrate buffer (pH 7.8 for Ki67 and pH 6.0 for EGF-R). Antibody dilutions were: 1:200 and 1:500 for the unpurified or purified α-hERG1-moAb, respectively, 1:100 for EGF-R (Santa Cruz Biotechnology) and 1:50 for Ki-67. Immunostaining was performed with a commercially available kit (PicTure Max kit and DAB, Invitrogen) according to manufacturer's instructions. To validate data obtained with anti-hERG1 antibodies we performed IHC on the same sets of samples also using the fully automated IHC and ISH Leica BOND-MAX system.

Scoring assessment

When evaluating IHC, no staining was scored as 0, 1–25% of stained cells was scored as 1, 26–50% as 2, 51– 75% as 3, and 76–100% as 4. Staining intensity was rated on a scale of 0–3, with 0 = negative; 1 = weak; 2= moderate, and 3= strong. The raw data were converted to the IHS score by multiplying the quantity and staining intensity scores. An IHS score ≥6 was considered as hERG1 positivity. Ki67 staining was evaluated by counting the number of positively stained nuclei and immunoreactivity was categorized by the percentage of nuclear staining. Samples were evaluated by two independent investigators (GP and EL). The study was conducted in a blinded fashion so that patients' outcomes were unknown to investigators performing IHC analyses.

Materials and Methods of molecular biology experiments

Real time Quantitative PCR (RQ-PCR)

Primers were as follows: hERG1 forward, 5' ACGTCTCTCCCAACACCAAC 3'; reverse, 5' GAGTACAGCCGCTGGATGAT 3'; and β-actin forward, 5' CAACGGCTCCGGCATGTG 3'; reverse, 5' CTTGCTCTGGGCCTCGTC 3'. The primers were used at a final concentration of 100nM.

Materials and Methods of *in vitro* experiments

Protein extraction, Immunoprecipitation (IP) and Western Blotting (WB)

Proteins (2 mg for cell line extracts and 1 mg for primary samples) were immunoprecipitated overnight at 4°C with anti-EGF-R (4µg/mg, sc-03 Santa Cruz Biotechnology) or with the purified α-hERG1-moAb antibody (5µg/mg) using protein A/G beads (Santa Cruz Biotechnology). WBs were performed using the following antibodies diluted in T-PBS BSA 5%: anti-pan-hERG1 (C54) (1:1000, DIVAL Toscana Srl), anti-EGF-R (1:200 sc-03, Santa Cruz Biotechnology), anti-tubulin (1:500, Sigma Aldrich).

Immunofluorescence (IF) laser confocal microscopy

IF experiments were performed employing α-hERG1-moAb directed against an extracellular epitope of the protein, without permeabilisation. Affinity purified mouse α-hERG1-moAb (DIVAL Toscana Srl) was diluted in blocking solution (PBS containing 10% BSA) to a final concentration of 1µg/ml and incubated overnight at 4°C. Alexa Fluor 488 labelled secondary anti-mouse antibody (Invitrogen) diluted in blocking solution (1:500) was used and incubated 1 hour at room temperature in the dark. Coverslips were mounted using fluorescence ProLong antifade mounting solution with DAPI (Invitrogen). Sequential images at 0.22µm intervals were obtained with C1 confocal microscope (Nikon) using laser excitation wavelength of 488 nm.

Cell migration assay

Cells were seeded on a "Basement membrane" consisting of RPMI 5X, HEPES (50 mmol/l), NaOH (1mol/l), laminin (0.1 mg/ml) fibronectin (1 mg/ml), Collagen IV (1 mg/ml). Migration was monitored for 5h for calculation of migration speed (µm/min) and distance covered within the experimental period (µm).

Cell viability assay

Cells viability was assessed using WST-1 assay (Roche, Mannheim, Germany). Cells were seeded in 96-well cell culture plates. After cell adhesion and spreading, cell medium containing EGF (20ng/ml) in the presence or not of E4031 (40µM) was added and cells were cultured for six hours in a humidified incubator at 37°C and 5% CO_2. Then medium was replaced adding 90µl of DMEM+10% FCS+ 20ng/ml EGF and WST-1 reagent (10 µl) was added into each well followed by incubation at 37°C for one hour. Then, the formazan dye formed after WST-1 cleavage carried out by living cells, was measured at 450 nm. The absorbance values were reported as ratio of mean absorbance values between treated and control primary cultures.

Materials and Methods of *in vivo* experiments

Cells

MIAPaCa-2 cell line, transfected with pGL4.51 [luc2/Neo] plasmid was used for tumour cell implantation. Cells were cultured in DMEM supplemented with Lglutamine (4 mM), 10% foetal bovine serum and geneticin (G418) (2.4 mg/ml) (Gibco) at 37°C in a humidified atmosphere of 5% CO2. Cells were harvested by a brief trypsinisation from semiconfluent culture dishes, washed several times, and collected in sterile PBS shortly before implantation. Cells were tested for microbial contamination (including mycoplasma) using a PCR-based analysis and were found to be negative.

Mouse Models

Mice were maintained in a sterile environment on daily twelve-hour light/dark cycles. Cages, bedding, and water were autoclaved, and the food was gamma irradiated. All manipulations were conducted under aseptic conditions using a laminar flow hood.

MIAPaCa-2-luc cells were injected into the pancreas of nu/nu mice and the animals were monitored by bioluminescence optical imaging at different time after injection. Seven days after injection, when no tumour mass was yet palpable, the counts per minute (cpm) were barely detectable. Afterwards, a progressive increase in the signal was observed, the maximum cpm value being reached 4 weeks after cell injection. At that time, local tumour growth occurred and complications such as stomach or intestinal obstruction occurred, accompanied by rapid weight loss and malnutrition. 4 weeks after cell injection mice were sacrificed and pancreatic tumour masses excised and examined. The mean value of the tumour masses was 378±32 mm3. Tumours were predominantly poorly differentiated, with a high degree of vascularisation.

We generated cohorts of *KPC* triple transgenic mice, interbreeding firstly *Pdx-1-Cre* mice with (Lox-STOP-Lox) *LSL-Kras*$^{G12D/+}$ mice (Hingorani et al, 2003) and then the resulting double transgenic *Pdx-1-Cre,LSL- Kras*$^{G12D/+}$ mice with *LSL-Trp53*$^{R172H/+}$ mice (Hingorani et al, 2005). Transgenic mice expressing Cre recombinase under the control of pancreas-specific *Pdx-1* promoter were crossed with *LSL-Kras*$^{G12D/+}$ mice. Among the progeny, *Pdx-1-Cre,LSL-Kras*$^{G12D/+}$ mice were crossed with *LSL-Trp53*$^{R175H/+}$ mice to obtain KPC mice. Based on these procedures, the progenitor cells of the developing pancreas of KPC transgenic mice expressed rearranged and activated *Kras*^{G12D} and *Trp53*^{R172H} alleles. Mice were maintained on a C57BL6 background, in plastic cages with a wire mesh providing isolation from the hygienic bed and were kept in temperature-, air-, and light-controlled conditions. They received food and water *ad libitum*.

Mice were genotyped by PCR analysis of genomic tail DNA, and they were monitored daily and sacrificed by cervical dislocation when they became moribund. Organs and tumors were explanted and fixed in PBS 4% formaldehyde for 24 h, then the samples were embedded in paraffin and cut into 7μm section used for IHC analysis (see below).

Tumour implantation

For orthotopic transplantation, general anaesthesia was performed by intraperitoneal application using Avertin (350 mg/kg) (Sigma). A median laparotomy was performed, approximately one cm in length; the peritoneum was opened, and the pancreas was carefully exposed by applying gentle traction at the stomach. Aliquot of 1×10^6 MIA PaCa-luc2 tumour cells in a volume of 10μl PBS was injected with an insulin syringe, 30 gauge, very slowly into the proximal part of the pancreas. Cells were implanted to visibly infiltrate the pancreatic tissue. The needle was slowly withdrawn after one-minute delay. The pancreas was then returned to the abdominal cavity, and the incision was closed in two layers using a continuous 4-0 Vicryl suture for the peritoneum and an interrupted suture for the skin. All animals tolerated the procedure well. After implantation, mice were inspected weekly for body weight loss, general condition, and tumour formation in the peritoneal cavity through bioluminescent optical imaging.

Tumour imaging

α-hERG1-moAb was conjugated with Alexa Fluor dye, according to protocol from the SAIVI Alexa Fluor 680 Antibody labelling kit (Invitrogen). Briefly, Alexa Fluor NHS esters were incubated with the protein in a basic medium (pH 9.3). Labelled α-hERG1-moAb was isolated and purified by gel filtration. The final dye-to-protein ratio (number of Alexa Fluor molecules coupled to each protein molecule) was between 2.5 and 3.5, according to manufacturer's instructions. All of the fluorescently labelled protein samples were aliquoted and stored in the dark at -80°C. All the fluorescent emission spectra were measured using Photon imager (Biospace Lab). The imager had a laser source for fluorescence excitation (λ=679nm), an emission filter (λ=702nm) for fluorescence detection, and a computer for data analysis. To analyse the specific target accumulation of the imaging probes, mice were injected with Alexa 680-labelled α-hERG1-moAb and 3 days after

injection, animal were anaesthetized, shaved and immunofluorescence was measured in whole animal and, after sacrifice, in the explanted organs.
For bioluminescent imaging anaesthetized mice bearing orthotopic tumours were injected intraperitoneally with 150mg/kg of XenoLight RediJect D-Luciferin (Caliper Life Sciences). Animals were imaged five minutes after Luciferin injection using the Photon imager (Biospace Lab). The stage is at a constant temperature of 37°C to maintain body temperature in the animals. An integration time of three minutes was used for bioluminescent image acquisition. Time profiles were generated for each of the signals bounded by circular region of interest (ROI) on the abdomen of each mouse.

Tumour harvesting and IHC

Animals were killed by cervical dislocation; organs and tumours were removed and fixed in 4% buffered formaldehyde for 24 h and embedded in paraffin. Tissue sections (7µm) were cut from blocks using a microtome (Leica RM2125/RM2125RT) mounted on positively charged slides for IHC analysis. After dewaxing and blocking endogenous peroxidases, sections were treated with proteinase K (Roche; 5 µg/mL in PBS) and UltraVBlock solution (LabVision) containing 0.1% Triton X-100, and then incubated with the anti-mERG1 monoclonal antibody (Lastraioli et al, 2012) (dilution 1:200 in PBS-UltraVBlock), overnight at 4°C. Immunostaining was carried out using a commercially available kit (PicTure Plus kit; Zymed). After washing with PBS, colour was developed by incubating the slides with the 3,3'-diamino-benzidine chromogen solution for 1-2 mins. Slides were then counterstained with Mayer's hematoxylin and mounted using Entellan mounting medium. Images were acquired on a Leica DM4000B microscope with a Leica DFC 320 camera using Leica Win software (Leica Microsystems; Milan, Italy).

Figures

Figure S1- EGF-R expression in MIAPaCa-2 and PANC-1 cell lines, evaluated by Western Blotting on total protein extracts. 30 µg of total protein were loaded. EGF-R detection was performed using an anti-EGF-R antibodies (Santa Cruz, SC- 03, dilution 1:200).

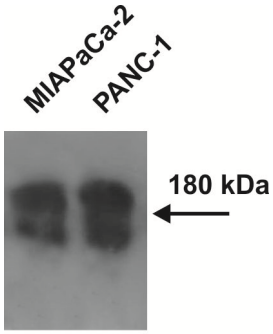
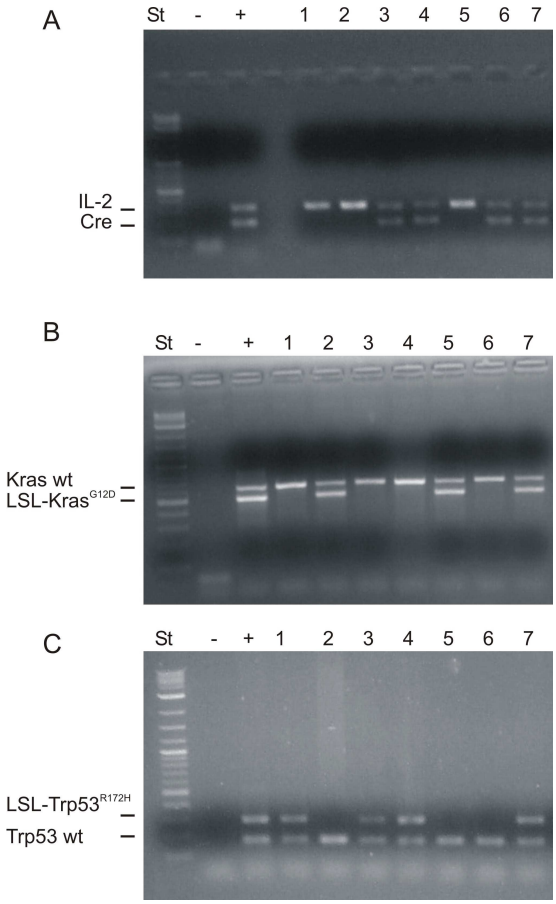


Figure S2. PCR for genotyping KPC transgenic mice. Genomic DNA was isolated using a Chelex protocol by incubation of a 0.2 cm of mice tails in 150 µl of 10% (w/v in water) Chelex 100 resin (BIO-RAD) for 4 h 25 at 56°C and 30 min at 98°C. PCR analysis were performed using Platinum® PCR SuperMix (Life Technologies), according to the manufacturer's instructions.
A) Amplification of *Cre* was performed with a specific primer pair: 5'-ACCAGCCAGCTATCAACTCG-3' and 5'-TTACATTGGTCCAGCCACC-3', which amplify a 200bp fragment, applying the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension cycle at 72°C for 3 min. Amplification of the *interleukin-2* (*IL-2*) gene by PCR was systematically performed on DNA as an internal PCR control, using the following primer pair: 5'-CTAGGCCACAGAATTGAAAGATCT-3' and 5'-GTAGGTGGAAATTCTAGCATCATCC-3', which amplify a 350bp fragment.
B) Amplification of *Kras* was performed with the following primers: forward 1: 5'-GTCTTTCCCCAGCACAGTGC-3'; reverse 1: 5'-CTCTTGCCTACGCCACCA GCTC-3'; reverse 2: 5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3', to detect the LSL-cassette (500bp) and the wt allele (620bp), applying the following PCR conditions: denaturation at 95°C for 2 min, 35 cycles at 95°C for 30 sec, 61°C for 30 sec, 72°C for 45 sec and a final extension cycle at 72°C for 10 min.
C) Amplification of *Trp53* was performed using the following primers (Jacks Lab): forward 1 (SD5'): 5'-AGCTAGCCACCATGGCTTGAGTAAGTCTGCA-3'; forward 2 (DT050500.2): 5'-TTACACATCCAGCCTCTGTGG-3'; reverse 1 (DT011200.3): 5'-CTTGGAGACATAGCCACACTG-3', to detect the LSL-cassette (270bp) and the wt allele (170bp), applying the following PCR conditions: denaturation at 94°C for 3 min, 31 cycles at 94°C for 1 min, 60°C for 1 min 30 sec, 72°C for 2 min and a final extension cycle at 72°C for 5 min.
-: water as negative control; +: positive PCR control.


A
St - + 1 2 3 4 5 6 7
IL-2
Cre
B
St - + 1 2 3 4 5 6 7
Kras wt
LSL-Kras^{G12D}
C
St - + 1 2 3 4 5 6 7
LSL-Trp53^{R172H}
Trp53 wt

1
2
3

Figure S3. Histological characterization of a representative tumour mass in a KPC mouse. Haematoxylin & Eosin staining. A) Low power picture in which tumour tissue architecture appears completely irregular and subverted when compared to healthy pancreatic tissue. The tissue is characterized by extensive necrosis (arrow). B) Higher power microphotograph of the same tumour mass as in A. Magnification: 10x (A) and 40x (B).

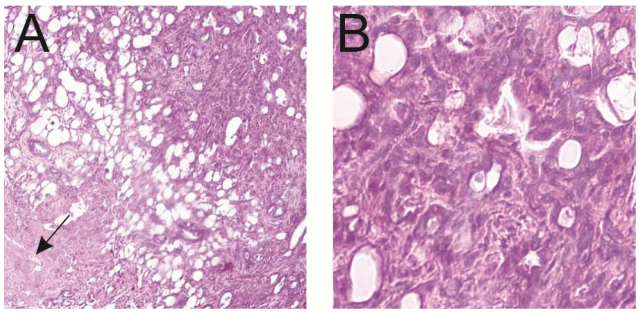


Figure S4- Relationship between hERG1 expression and proliferation in PDAC primary samples. A) Immunohistochemistry for hERG1. B) Immunohistochemistry for Ki67 in the same sample as in A). Magnification 20x.

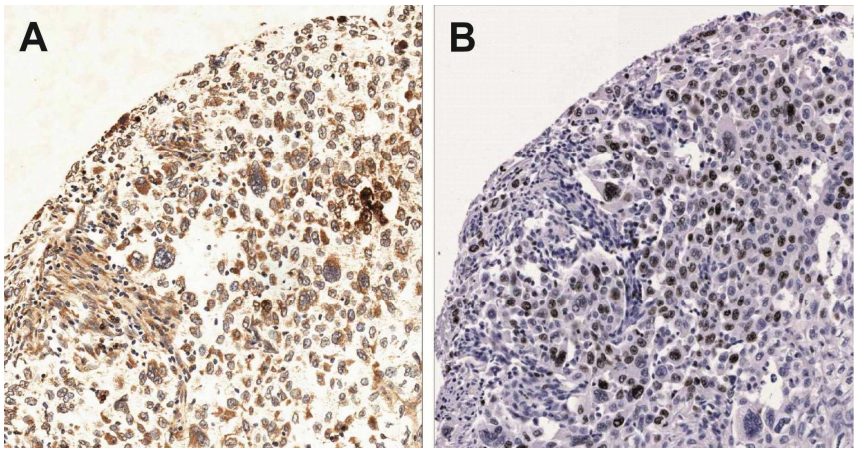


Figure S5. *In vivo* bioluminescent optical imaging of tumour growth. Panels represent sequential imaging of a single mouse taken on days 7, 14, 25 and 28 after surgical orthotopic implantation of MIAPaCa-luc2.

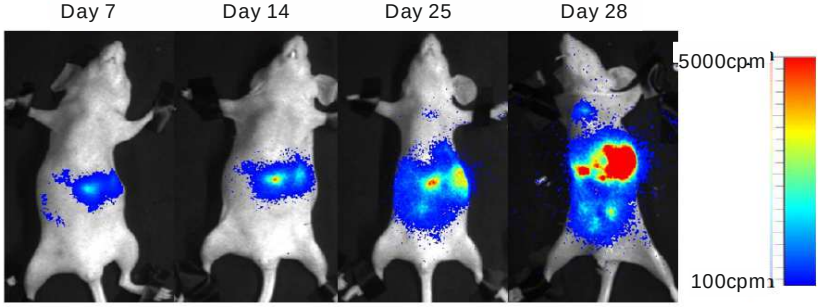


Figure S6. Quantification of tumour bioluminescence. Values represent the mean counts per minute (cpm) ± S.E. for a group of 16 mice. Progression of disease had a strong correlation with survival.

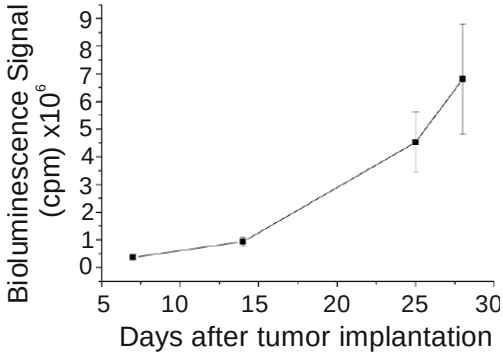
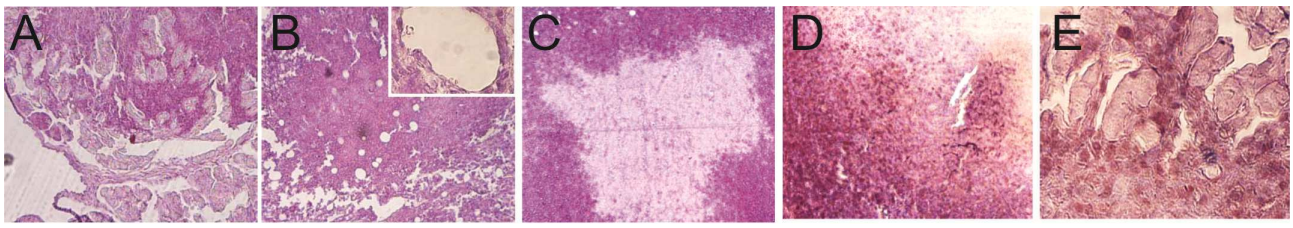


Figure S7. Histological characterization of primary MIA-PaCa-luc2 tumours. A-C) Haematoxylin & Eosin staining. Tumours were predominantly poorly differentiated, showed a high degree of vascularization (inset to panel B) and were composed of pleomorphic cells. Despite the presence of new blood vessels, tumours were associated with fairly extensive areas of degeneration and necrosis (panel C); D-E) hERG1-mAb staining. Immunohistochemical staining of tumour tissue demonstrated a strong positivity for hERG1, whereas hERG1-mAb did not stain healthy tissue (panel E). Magnification: 10x (A, B, C, D) and 40x (inset to B, E).



Tables

Table S1. Clinicopathological characteristics of the PDAC patients.

Variable Category	Number of Patients
Gender	
Female	21
Male	23
T stage	
T1	0
T2	7
T3	38
N stage	
N0	17
N1	27
M stage	
M0	41
M1	3
Staging	
I	18
II	23
III	0
IV	3
Grading	
Grade 1	3
Grade 2	25
Grade 3	16

Table S2. Raw data of hERG1 scoring and *Kras* mutational status in our TMA series.

Sample Id	*KRAS* status	Mutation type	hERG1 score	hERG1 expression
08 B 4818	Not determined	Not determined	9	+
08 B 3922	MUTATED	G12D (12Asp)	8	+
08 B 3894	MUTATED	G12D (12Asp)	0	-
08 B 3004	MUTATED	G12V (12Val)	0	-
08 B 2925	Not determined	Not determined	6	+
08 B 2876	MUTATED	G12V (12Val)	0	-
08 B 2113	MUTATED	G12V (12Val)	9	+
08 B 1373	MUTATED	G12D (12Asp)	2	-
08 B 1286	MUTATED	G12V (12Val)	0	-
07 B 4404	MUTATED	G12R (12Arg)	12	+
07 B 2017	Not determined	Not determined	8	+
07 B 1629	Not determined	Not determined	6	+
07 B 0631	Not determined	Not determined	12	+
07 B 0321	MUTATED	G12D (12Asp)	6	+
06 B 5473	MUTATED	G12D (12Asp)	12	+
03 B 0058	MUTATED	G12V (12Val)	6	+
01 B 2152	Not determined	Not determined	4	-
04 B 2023	MUTATED	G12D (12Asp)	8	+
02 B 4149	MUTATED	G12D (12Asp)	8	+
04 B 0587	Not determined	Not determined	4	-
04 B 1878	Not determined	Not determined	8	+
04 B 4172	Not determined	Not determined	0	-
05 B 0612	MUTATED	G12D (12Asp)	0	-
02 B 2581	MUTATED	G12V (12Val)	9	+
98 B 0252	Not determined	Not determined	6	+
06 B 4256	MUTATED	G12D (12Asp)	6	+
06 B 5392	Not determined	Not determined	4	-
05 B 1001	Not determined	Not determined	4	-
07 B 2305	Not determined	Not determined	1	-
05 B 4424	MUTATED	G12V (12Val)	6	+
00 B 0819	MUTATED	G12D (12Asp)	8	+
10 B 1732	MUTATED	G12R (12Arg)	8	+
05 B 3864	MUTATED	G12V (12Val)	3	-
04 B 1985	Not determined	Not determined	2	-
09 B 2856	Not determined	Not determined	4	-
05 B 3438	Not determined	Not determined	12	+
09 B 4797	Not determined	Not determined	2	-
02 B 3809	Not determined	Not determined	8	+
05 B 2893	Not determined	Not determined	8	+
09 B 7606	MUTATED	G12V (12Val)	12	+
09 B 3836	Not determined	Not determined	8	+
01 B 3563	MUTATED	G12D (12Asp)	4	-
09 B 1061	MUTATED	G12D (12Asp)	3	-
06 B 1757	MUTATED	G12R (12Arg)	12	+

Table S3. Distribution of hERG1 scoring and expression according to *Kras* mutational status in a small group of samples.

Sample Id	*KRAS* status	Mutation type	Mutation rate	hERG1 score	hERG1 expression
2012 B 11294	WILD TYPE	--	--	8	+
2013 B 902	WILD TYPE	--	--	8	+
2012 B 8973	WILD TYPE	--	--	6	+
2008 B 4818	WILD TYPE	--	--	8	+
2006 B 4256	WILD TYPE	--	--	9	+
2010 B 8744	MUTATED	G12C (12Cys)	24.5%	6	+
2012 B 8604	MUTATED	G12C (12Cys)	52.0%	12	+
2009 B 7606	MUTATED	G12D (12Asp)	42.3%	2	-
2008 B 3004	MUTATED	G12D (12Asp)	47.4%	6	+

References

Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA,Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA (2003) Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* **4**: 437-450.

Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA (2005) Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* **7**: 469-483.

Lastraioli E, Bencini L, Bianchini E, Romoli MR, Crociani O, Giommoni E Messerini L, Gasperoni S, Moretti R, Di Costanzo F, Boni L, Arcangeli A (2012) hERG1 Channels and Glut-1 as Independent Prognostic Indicators of Worse Outcome in Stage I and II Colorectal Cancer: A Pilot Study. *Transl Oncol* **5**: 105-112.