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Ras pathway activation in hepatocellular carcinoma and anti-tumoral effect of combined sorafenib and rapamycin *in vivo**

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Abstract

Background/Aims—The success of sorafenib in the treatment of advanced hepatocellular carcinoma (HCC) has focused interest on the role of Ras signaling in this malignancy. We investigated the molecular alterations of the Ras pathway in HCC and the antineoplastic effects of sorafenib in combination with rapamycin, an inhibitor of mTOR pathway, in experimental models.

Methods—Gene expression (qRT-PCR, oligonucleotide microarray), DNA copy number changes (SNP-array), methylation of tumor suppressor genes (methylation-specific PCR) and protein activation (immunohistochemistry) were analysed in 351 samples. Anti-tumoral effects of combined therapy targeting the Ras and mTOR pathways were evaluated in cell lines and HCC xenografts.

Results—Different mechanisms accounted for Ras pathway activation in HCC. *H-ras* was up-regulated during different steps of hepatocarcinogenesis. *B-raf* was overexpressed in advanced tumors and its expression was associated with genomic amplification. Partial methylation of RASSF1A and NORE1A was detected in 89% and 44% of tumors respectively, and complete methylation was found in 11 and 4% of HCCs. Activation of the pathway (pERK immunostaining) was identified in 10.3% of HCC. Blockade of Ras and mTOR pathways with sorafenib and rapamycin

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reduced cell proliferation and induced apoptosis in cell lines. *In vivo*, the combination of both compounds enhanced tumor necrosis and ulceration when compared with sorafenib alone.

Conclusions—Ras activation results from several molecular alterations, such as methylation of tumor suppressors and amplification of oncogenes (*B-raf*). Sorafenib blocks signaling and synergizes with rapamycin *in vivo*, preventing tumor progression. These data provide the rationale for testing this combination in clinical studies.

Keywords

Liver cancer; HCV; Signaling pathway; Molecular therapies; Ras; mTOR

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and primary cause of death in patients with liver cirrhosis [1]. In western countries, the increase in HCC is mainly due to the rising prevalence of hepatitis C infections [2]. Curative therapies can only be offered to approximately 30% of patients, complicated by high recurrence rates [3]. Therefore, new therapeutic strategies are urgently needed.

Similar to other solid tumors, genetic and epigenetic events are implicated in the development of HCC and result in aberrantly activated pathways [4,5].

The Ras pathway represents a dominant signaling network promoting cell proliferation and survival. The binding of different growth factors (e.g. EGF and IGF-1) to their receptors (e.g. EGFR, IGF-1R) induces activation of Ras, which in turn activates c-raf, MEK and ERK. In the nucleus, phosphorylated ERK activates transcription factors (e.g. Elk-1, c-Jun) that regulate the expression of genes involved in proliferation and survival. Aberrant activation of Ras pathway can occur at different levels [6]. Although point mutations in Ras gene seem to be common, their frequencies are rare in HCC [7,8]. Recently, a new family of tumor suppressor genes encoding Ras-binding proteins has been discovered [9,10]. Among them, RASSF1A and NORE1A are thought to act together to inhibit the mitogenic stimulation induced by Ras promoting apoptosis through activation of MST1 kinase [11,12]. Hypermethylation of these two genes has been reported in many cancers, including HCC [13–15].

Sorafenib is a multikinase inhibitor with potent activity against Raf-1 and B-Raf kinases, VEGFR-2 and -3, c-kit, and PDGFR- α , among others [16]. The recent Phase III SHARP trial demonstrated that sorafenib prolongs survival of patients with advanced HCC [17], resulting in FDA and EMEA approval in 2007 [18]. Sorafenib has emerged as standard of care in advanced cases, and new therapies should be combined with it, according to recommendations of an expert panel of the American Association for the Study of Liver Diseases [19]. We recently demonstrated that mTOR signaling plays an important role in HCC [20]. This effect was synergized through EGFR antagonism supporting the convenience of multi-target approaches in molecular therapy.

In this study, we characterized the molecular alterations underlying aberrant activation of Ras pathway in 351 human HCC samples and evaluated the anti-tumoral effects of sorafenib combined to rapamycin (mTOR inhibitor) in experimental models.

2. Materials and methods

2.1. Samples characteristics

Three hundred and fifty-one human samples were collected during liver resection or transplantation from three hospitals of the HCC Genomic Consortium: Mount Sinai School of

Medicine (NY), Hospital Clinic, Barcelona (Spain) and Istituto Nazionale Tumori, Milan (Italy). Tissue was collected with the required approvals from the Institutional Review Boards and patients' written consent.

An exploratory set of 77 samples was used to analyze expression levels of key genes of Ras pathway during stepwise hepatocarcinogenic process, as previously described [21] (Fig. 1). A replication set of 78 HCV-related HCC samples was used to more comprehensively investigate changes in mRNA expression in tumors. Clinical correlations were investigated in 82 patients who underwent liver resection selected from both cohorts (clinical training set). Clinically early tumors were divided into histologically early (single, well-moderately differentiated, no vascular invasion) and histologically advanced HCC (multinodular or >3 cm with vascular invasion or poorly differentiated). A tissue microarray with 196 HCC samples from different etiologies (HBV, HCV, alcohol-related) was constructed to validate clinical correlations (clinical validation set). Characteristics of patients of the clinical training and validation sets are reported elsewhere [20].

2.2. Quantitative Real-Time-PCR (qRT-PCR)

Total RNA was extracted from 50 mg fresh frozen tissue using *Trizol*[®] reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and purified with *RNeasy*[®] columns (Qiagen, Valencia, CA). RNA quality and integrity were measured with a bioanalyzer (Agilent, Palo Alto, CA). Complementary DNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Expression level of H-ras, B-raf, EGFR, NORE1A and RASSF1A mRNA was measured by Taqman Gene Expression Assays[®] (Applied Biosystems).

2.2.1. Single nucleotide polymorphism (SNP) array and oligonucleotide microarray—DNA was extracted using ChargeSwitch gDNA Mini Tissue Kit (Invitrogen) and quantified using PicoGreen (Invitrogen). A genome-wide scan of 238,000 tag SNP was conducted using the StyI chip of the 500K Human Mapping Array set (Affymetrix) at a median intermarker distance of approximately 10 kb. SNP array experiments were performed according to manufacturer's instructions and analyzed, as previously described [22].

Oligonucleotide microarray studies were conducted using Affymetrix U133 2.0[®] Array according to Affymetrix GeneChip Technical Manual.

2.3. Methylation-specific PCR

Genomic DNA was treated with sodium bisulfite using MethylDetector Kit[™] (Active Motif, Carlsbad, USA). RASSF1A and NORE1A primers [23,10] were purchased from Invitrogen (Suppl. Table 1). Fifty nanograms of bisulfite-treated DNA was amplified as follows: 95 °C 10 min, 35 cycles at 95 °C 40 s, 60 °C 50 s, 72 °C 40 s, 1 cycle 72 °C 10 min (RASSF1A); 95 °C 10 min, 40 cycles at 94 °C 1 min, 62 °C 1 min, 74 °C 1 min, 1 cycle 72 °C 10 min (NORE1A). DNA treated with M. SssI-methylase 4 U/μl (New England Biolabs, Beverly, MA) and water were used as positive and negative controls. PCR products were separated by 2% agarose gel electrophoresis.

2.4. Immunohistochemistry and tissue microarray

Formalin-fixed, paraffin-embedded tissues (4 μm) were baked for 30 min, de-paraffinized in xylene, and rehydrated in a graded series of ethanol solutions. Samples were incubated with anti-p-ERK antibody (phosphoThr202/Tyr204, Cell Signaling) at 4 °C overnight. Immunoreactivity was independently graded by three liver pathologists as follows: [1] Intensity, [2] cellular localization, [3] tumor or endothelial staining. Positivity was defined when the intensity of staining was +2 or higher. Immunostaining of paraffin sections of

xenograft tumors was performed using p-ERK and p-S6 (Ser240/244, Cell Signaling). Tumor microvessels were visualized by immunostaining with human Von Willebrand factor (vWF, Dako A0082). DeadEnd™ Colorimetric TUNEL (Promega) was performed for apoptosis quantification. Tissue microarray (TMA) blocks were constructed using the Advanced Tissue Arrayer ATA-100 (Chemicon International).

2.5. Cell lines and reagents

Cell assays were performed in DMEM supplemented with 10% FBS. Sorafenib (Bayer Corporation, West Haven, CT) was dissolved in DMSO for *in vitro* experiments and in Cremophor EL (Sigma)/95% ethanol (50:50) for *in vivo* experiments. Rapamycin (sirolimus, Wyeth) was purchased from our pharmacy and diluted in DMSO for *in vitro* assays. For *in vivo* experiments it was administered at 5 mg/kg/day.

2.6. Cell viability and proliferation assays

Cells were plated at 5000 cells/well in 24-well plates. Seventy-two hours after treatment, cells were incubated with tetrazoliumbromide (Sigma) for 1 h, solubilized in *N*-propanol, and the absorbance measured by spectrophotometer at 570 nm. For proliferation studies, 72 h after treatment cells were labeled with 1 μ Ci/mL (methyl-³H) Thymidine (Amersham) for 3 h, fixed in 1 N HCl, and lysed with 0.25% SDS/0.25M NaOH. Thymidine incorporation was measured by scintillation counting.

2.7. Western blot

Cells were plated at 150,000 into 6-well plates. One hour after addition of drugs, cells were incubated with 40 ng/mL rh-EGF (Invitrogen) for 10 min, lysed and pelleted. Blotting of membranes was performed with the following antibodies: ERK1–2 (Cell Signaling), phospho-ERK1–2 (Thr202/Tyr204, Cell Signaling), S6 (Cell Signaling), phospho-S6 (Ser240/244, Cell Signaling), and β -Tubulin (Santa Cruz).

2.8. Apoptosis assays

Flow cytometry was used to quantify the percent of cells with sub-diploid DNA content (sub-G0). Huh7 cells were plated at 50,000 cells/well (6-well plates) and treated after 24 and 72 h. Cells were collected at 96- and 120-h time points, stained with 50 μ g/mL propidium iodide (Sigma) and analyzed using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For demonstration of PARP cleavage, 150,000 cells/well were plated in 6-well plates, treated after 24 and 72 h and immunoblotted for PARP (Cell Signaling) as described above.

2.8.1. Xenograft model—Female *NU-NU* mice (Taconic Farms, NY) were maintained according to Mount Sinai School of Medicine institutional policies. Tumors were generated by injecting 5×10^6 Huh7 cells subcutaneously. Treatments started when tumors reached 100–300 mm³ in volume. Mice were randomized in 4 groups: placebo ($n = 6$, drug vehicle), sorafenib ($n = 9$, 30 mg/kg/day), rapamycin ($n = 9$, 5 mg/kg/day) and combination of sorafenib ($n = 9$, 30 mg/kg/day) plus rapamycin (5 mg/kg/day). Drugs were administered daily by gavage. Tumor dimensions were measured thrice/week, tumor weight was calculated using the following formula: length $\times$ (width)² $\times$ 0.4. Mice were euthanized when tumors reached 10% of their body weight or when skin overlying tumors became ulcerated. Mice were injected intraperitoneally with 5 μ g of rh-EGF (Invitrogen) 1–4 h after treatment and 5 min prior to euthanasia.

2.9. Statistical analysis

Comparisons between groups were made using the *t*-test or the non-parametric Mann–Whitney test for continuous variables, and the Fisher exact test for comparisons of proportions.

Correlations were calculated with the Pearson's coefficient or the non-parametric Spearman's coefficient. SNP-array data and gene expression microarray were analyzed as previously described [22]. The probability curves of recurrence were calculated according to Kaplan–Meier and compared by log-rank test. Early recurrence was defined as recurrence within the first two years following resection [19,24]. All calculations were done using the SPSS package (SPSS 15.0, Chicago, IL).

3. Results

3.1. Ras signaling in human HCV-related HCC

3.1.1. Gene expression analysis, copy number changes and promoter methylation of key genes involved in Ras signaling—Deregulated expression of genes involved in the Ras pathway was investigated in two independent cohorts of samples (exploratory and replication sets), as previously described (Fig. 1) [20,21]. H-ras was increasingly up-regulated during different stages of hepatocarcinogenesis (Fig. 2A). Although it was not overall up-regulated in the replication set, H-ras was expressed at high levels (fold change >3) in a subgroup of patients (8/78, 10.3%) (Fig. 2B). The expression levels of K-ras and N-ras were tested using microarray and did not show significant changes (data not shown). B-Raf was up-regulated in advanced tumors ($p = 0.041$) (Fig. 3A) and oligonucleotide microarray showed a similar trend ($p = 0.06$) (Fig. 3B). No significant changes in EGFR, RASSF1A and NRE1A expression were detected in either set by qRT-PCR (data not shown).

DNA copy number changes of key genes were analyzed using SNP array technology. Gains >3 of B-Raf were detected in 6 samples (6/82, 7.3%) with significant correlation with mRNA levels (Spearman coefficient = 0.54, $p < 0.0001$) (Fig. 3C and D). B-Raf is located on chromosome 7, in which polysomy defines a new class of HCC tumors, as we recently reported [22]. In the current study, 5 out of 6 tumors with B-Raf copy number >3 were classified in this molecular subgroup (Suppl. Fig. 1). The mean copy number of B-Raf within the Poly 7 class was 3.1, significantly higher than the mean of 2.16 in the rest of the molecular classes ($p < 0.05$).

Methylation status of RASSF1A and NRE1A promoters was analyzed using primers for both methylated and unmethylated DNA. Samples that did not show a product with either the PCR reaction were discarded. We observed the presence of both unmethylated and methylated alleles of RASSF1A in 89% of tumors and 2 normal livers (Suppl. Fig. 2 and Table 1). Interestingly, 11% of the samples (6/54) showed no signal from unmethylated primers and were defined as completely methylated. Partial methylation of NRE1A promoter was detected in 44% (24/54) of tumors and 2 normal livers. Complete methylation was found in 2/54 (4%) of HCC. We observed a non-significant decrease of NRE1A expression in tumors with promoter methylation compared to tumors without methylation (Suppl. Fig. 3).

3.1.2. Assessment of Ras pathway activation in human HCC—Phosphorylation of ERK, a downstream target of Ras, was analyzed in 78 samples belonging to the clinical training set. Eight out of 78 (10.3%) tumors were positive for p-ERK whereas 47/78 (60.3%) contained positive endothelial cells (Fig. 4). Among p-ERK positive tumors (8/78), 2 were characterized by RASSF1A and NRE1A methylation, 2 by high H-ras expression level (fold changes: 3.58 and 11.08) and one by B-Raf copy number gain. Staining of pERK was also analyzed by tissue microarray in an independent set of samples of different etiologies ($n = 196$), including HCV and HBV-related tumors. In this set, only 2.5% of tumors were p-ERK positive while 22% of the samples showed positive endothelial staining. A supervised analysis of microarray data was performed to identify a gene signature of p-ERK staining using the Significance Analysis of Microarrays Package. Five genes including the progenitor cell marker CD133 (PROM1) were included in the signature (q -value <0.01) (Suppl. Table 2).

3.1.3. Clinical implications of Ras pathway activation—Outcome analysis was predicted in a clinical training set of 82 patients with well-preserved liver function (>90% Child–Pugh’s A class) and early HCC (median tumor size:3.3 cm; BCLC 0-A > 80% of cases) treated by resection. Tumor size, BCLC class, macrovascular invasion, and multinodularity were independent predictors of recurrence in the multivariate analysis ($p < 0.05$). Up-regulation of H-ras >3-fold was associated with early recurrence (within 2 years after surgical treatment, $p = 0.007$, log-rank test) (Fig. 5). Median time to early recurrence in patients with high vs normal H-ras expression was 7 and 18 months, respectively. Interestingly, NORE1A methylation was more prevalent in advanced stages: 7/8 (87.5%) BCLC-C vs 19/45 (42%) BCLC-A/B, $p = 0.024$. Positive staining for pERK did not predict outcome, and was associated with small sized tumors in the clinical training and validation sets ($p = 0.03$ and $p = 0.018$, respectively).

3.2. Synergistic effects of sorafenib combined with rapamycin in vitro and in vivo

3.2.1. Blockade activity of sorafenib and rapamycin in Ras and mTOR signaling

—Before evaluating the anti-tumoral effect of sorafenib and rapamycin (mTOR inhibitor) combination in pre-clinical models, we assessed the expression levels of the downstream targets pERK and pS6 as surrogate markers of Ras and mTOR signaling activation in Huh7 cells. P-ERK in cells treated with sorafenib (1 μ M) was barely detectable, and p-S6 after treatment with rapamycin (5.5 nM) was markedly decreased (Fig. 6A). There was a clear synergistic effect in p-S6 decrease in the cells treated with both compounds.

3.2.2. Effects on proliferation, viability and apoptosis in HCC human cell lines

—Sorafenib at 1, 5 and 10 μ M concentrations decreased Huh7 viability by 19%, 55% and 74%, respectively ($p < 0.05$) (Suppl. Fig. 4A). Rapamycin at 5.5 nM and higher caused a 20–40% decrease in cell viability in Huh7, Hep3B and HepG2 cell lines (Suppl. Fig. 4B). Addition of rapamycin (5.5 nM) to sorafenib (1 μ M) further decreased cell viability from 81 to 47% in Huh7 ($p = 0.014$) (Suppl. Fig. 4C). Due to the remarkable sensitivity of Huh7 to sorafenib, we used low concentrations in subsequent experiments to capture potential synergistic effects with rapamycin. The combination led to significant decreased cell proliferation ($p = 0.008$) (Fig. 6B) and to increased percentage of cells in the subG0 phase compared to sorafenib alone (4.5–15% at 72 h) (Fig. 6D). Sorafenib 10 μ M induced apoptosis and this effect was enhanced when administered in combination with rapamycin as assessed by PARP cleavage immunoblotting (Fig. 6C).

3.2.3. Antitumoral effects in xenograft models—Tumor growth was inhibited in both monotherapy and combination groups ($p < 0.05$) (Fig. 7A). We observed significant tumor necrosis and concomitant ulceration of skin overlying the necrotic tumor in the combination arm (6/9 mice), whereas only small patches of skin ulceration were seen in 1/9 mice in the monotherapy groups ($p < 0.05$) (Suppl. Fig. 5A,B and 6A). Response to treatment was measured by tumor necrosis/ulceration and was superior in the combination-treated group ($p = 0.051$) (Fig. 7B). Survival analysis failed because animals were sacrificed due to presence of hemorrhagic skin ulcers.

Tumor necrosis was quantified by morphometry (12 images/tumor). Only the combination group had significantly greater necrotic area, a phenomenon not related to apoptotic cell measurement by TUNEL (Suppl. Fig. 6B and C). Tumor microvessel density was decreased in both sorafenib and sorafenib/rapamycin groups compared to controls ($p < 0.05$) (Suppl. Fig. 6D). Abrogation of downstream targets of sorafenib (p-ERK) and rapamycin (p-S6) was observed in tumors of each group (Suppl. Fig. 5C and D).

4. Discussion

Herein, we report a comprehensive and integrative genomic analysis of Ras pathway in a large cohort of human HCC samples. We found that different mechanisms account for Ras signaling in HCC, including over-expression (e.g. H-ras), DNA copy number gains (e.g. B-Raf), and aberrant methylation (e.g. RASSF1A) of key genes in this pathway. In addition, we demonstrated the synergistic effect of Ras and mTOR pathway blockade in experimental models of HCC, establishing the proof-of-principal to conduct clinical trials.

Up-regulation of principal mediators of the pathway, H-ras and B-Raf, was detected in HCC confirming their role in cancer. B-Raf overexpression was associated with copy number gains in B-Raf genomic locus (chromosome 7q34). We recently described a HCC molecular class defined by overexpression of genes located on chromosome 7, consistent with broad copy number gains on the same chromosome [22]. Previous studies have reported B-Raf number gains in follicular thyroid cancers, with clinical association with invasive phenotype [25,26].

Our data suggests that epigenetic mechanisms involving the tumor suppressor genes RASSF1A and NRE1A are implicated in HCC. All the samples analyzed showed RASSF1A methylation and 11% of them were completely methylated. The high frequency of RASSF1A promoter methylation could explain why we were unable to capture changes in its expression. NRE1A was both partially and completely methylated in 44 and 4% of tumors, respectively. A slight but not significant decreased expression was observed in patients with NRE1A methylation suggesting that multiple mechanisms could be implicated in its regulation. The frequencies of RASSF1A and NRE1A methylation in HCC reported in this study are consistent with other studies [27,28] and highlight the role of epigenetic mechanisms in Ras pathway activation. Surprisingly, we observed partial methylation of both genes in 2 out of 6 normal tissues, a phenomenon already observed for RASSF1A [29]. Recently, using a transposon-based unsupervised screening of potential new oncogenes and tumor suppressor genes in transgenic models of HCC, we identified MAP2K4, a negative regulator of Ras signaling, as a new candidate tumor suppressor gene in HCC [30]. These data were confirmed in our human samples (data not shown). MAP2K4 inactivation has been observed in pancreatic carcinomas, implicating deregulation of the stress-activated protein kinase pathway [31].

We next evaluated Ras/Raf/MAPK pathway activation at protein level using pERK as surrogate marker. Only 10.3% of 78 tumor samples stained for p-ERK, whereas 60% of tumor samples contained p-ERK-positive endothelial cells. This contrasts with previously reported universal activation of pERK in HCC [32], probably because we assessed signaling activation of tumors within early clinical stage (>80% BCLC 0-A class), whereas others tested pathway activation in advanced cases. Furthermore, we analyzed pERK by immunostaining, enabling an accurate discrimination between positive staining from endothelial and tumoral cells. Thus, blocking Ras signaling might prevent tumor proliferation and angiogenesis. Interestingly, immunostaining for p-ERK in endothelial cells within the tumors was more prevalent in smaller tumors ($p = 0.018$), a finding that needs further exploration.

The molecular pathogenesis of HCC is very complex, involving different pathways and molecular aberrations [33]. We herein provide comprehensive assessment of Ras signaling activation at early stages of HCC, providing the rationale for testing novel drugs blocking this cascade. Simultaneous abrogation of critical pathways involved in angiogenesis, proliferation and apoptosis will be required to yield major improvements in the management of HCC [34]. Sorafenib is currently established as the standard molecular therapy for HCC, and a consensus of the AASLD recommended exploring combinations with this drug as a first-line treatment option for advanced cases [19]. Recently, our group demonstrated mTOR pathway deregulation in a subset of HCC patients [20]. Thus, blocking both mTOR and Ras signaling emerges as an

appealing option to improve survival in HCC patients. In addition, a recent study identified MAPK activation as a consequence of mTOR inhibition, underscoring the potential of a combined therapeutic approach with mTOR and MAPK inhibitors [35]. In this study, we report a significant decrease in proliferation, induction of apoptosis *in vitro* and enhanced tumor necrosis in xenograft models. Nonetheless, the evaluation of survival and tumor growth endpoints was precluded by institutional guidelines requiring euthanasia in the presence of hemorrhagic skin ulcerations. No toxicity (assessed as weight loss) was detected. In conclusion, we demonstrate the efficacy of combining sorafenib with rapamycin, an inhibitor of mTOR signaling in pre-clinical models of disease, in accordance with the findings recently reported by Wang et al. [36]. These data, along with the evidence illustrating Ras activation in HCC as a result different mechanisms, provide clear justification for testing the combination therapy of sorafenib along with rapamycin or its analogs (everolimus, temsirolimus) in early phase clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HBV	Hepatitis B virus
LGDN	low-grade dysplastic nodules
HGDN	high-grade dysplastic nodules

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.jhep. 2009.03.028.

Fig. 1.

Flow chart of the samples analyzed in this study ($n = 351$). A training cohort of 155 samples including exploratory ($n = 77$) and replication sets ($n = 78$) was used to analyze molecular alterations of Ras signaling. Exploratory set: normal livers ($n = 10$), cirrhosis ($n = 10$), low ($n = 10$) and high ($n = 7$) grade dysplasia, very early ($n = 10$), early ($n = 10$), advanced ($n = 10$) and very advanced ($n = 10$) HCC. Replication set: 78 HCC samples. Clinical correlations of Ras pathway activation were investigated in clinical training ($n = 82$) and validation ($n = 196$) sets.

Fig. 2.

Box plot of the fold changes in H-ras expression by qRT-PCR in the exploratory (A) and replication sets (B) compared with normal livers. Results are expressed in logarithmic scale as fold changes normalized to 1 (mean expression in normal liver). c, control (normal liver); ci, cirrhosis; lg, low-grade and hg, high-grade dysplasia; ve, very early; e, early; a, advanced and va, very advanced HCC. °Significant outliers, * non-significant outliers.

Fig. 3.

(A) Box plot of the fold changes in B-Raf expression in the replication set compared with normal livers by qRT-PCR and (B) by oligonucleotide microarray. Results are expressed in logarithmic scale as fold changes normalized to 1 (mean expression in normal liver). c, control (normal liver); e, early; a, advanced HCC. °Significant outliers, * non-significant outliers. (C) Copy number gain of B-Raf. Each dot represents the copy number value for each HCC sample. Horizontal dashed lines indicate the copy number ranges found among non-tumoral counterparts. (D) Correlation between B-Raf mRNA expression levels (logarithmic scale) and B-Raf genomic copy number changes.

Fig. 4. (A) Immunostaining of phosphorylated ERK in tumor cells, (B) surrounding cirrhotic tissue, and (C) endothelial cells included in the tumor sections. (D) Vascular invasion within tumor was seen rarely.

Fig. 5. Kaplan–Meier curve showing correlation between H-ras expression levels (fold-change >3 compared with normal livers) and early recurrence in the replication set. Green line, H-ras >3; blue line: H-ras <3.

Fig. 6. (A) Immunoblotting for S6, ERK and phosphorylated forms after treatment with sorafenib (1 μ M) and rapamycin (5.5 nM) in Huh7 cells. (B) Proliferation assay in Huh-7 treated with sorafenib (1 μ M) and rapamycin (5.5 nM) and combination. Results are expressed as percentage of 3 H-thymidine incorporation (counts per million) compared with control. (C) Immunoblotting for PARP cleavage in Huh7 treated with both the compounds. (D) Histogram representing the percentage of cells in different phases of the cell cycle after treatment with sorafenib (1 and 5 μ M), rapamycin (5.5 nM) and combination in Huh7 by flow cytometry.

Fig. 7. (A) Tumor volume (mm³) in xenografts treated with sorafenib, rapamycin and combination compared to placebo * $p < 0.05$. (B) Kaplan–Meier curves showing tumor ulceration/necrosis as surrogate of response to sorafenib, rapamycin and combination. Tumor ulceration was not observed in the control (vehicle-treated) group.

Table 1
Frequencies of promoter methylation of RASSF1A and NORE1A in normal livers and HCV-related HCC samples.

Gene	Normal livers (n = 6)			HCC (n = 54)		
	Unmethylated	Partially methylated	Completely methylated	Unmethylated	Partially methylated	Completely methylated
RASSF1A	4 (67%)	2 (33%)	0 (0%)	0 (0%)	48 (89%)	6 (11%)
NORE1A	4 (67%)	2 (33%)	0 (0%)	28 (52%)	24 (44%)	2 (4%)