Neuroblastoma RAS Viral Oncogene Homolog (NRAS) Is a Novel Prognostic Marker and Contributes to Sorafenib Resistance in Hepatocellular Carcinoma

Abstract
Inhibition of the RAS-RAF-ERK-pathway using sorafenib as a first-line and regorafenib as a second-line treatment approach is the only effective therapeutic strategy for advanced hepatocellular carcinoma (HCC). Recent studies suggest that wild-type KRAS and HRAS isoforms could majorly contribute to HCC progression and sorafenib resistance. In contrast, the role of neuroblastoma RAS viral oncogene homolog (NRAS) in HCC remained elusive. In this study, wild-type NRAS was found to be overexpressed in HCC cell lines, preclinical HCC models, and human HCC tissues. Moreover, NRAS overexpression correlated with poor survival and proliferation in vivo. However, siRNA-pool–mediated NRAS knockdown showed only slight effects on HCC proliferation, clonogenicity, and AKT activity. We determined that KRAS upregulation served as a functional compensatory mechanism in the absence of NRAS, which was overcome by combined inhibition of NRAS and KRAS in HCC cells. Furthermore, NRAS expression was elevated in sorafenib-resistant compared to nonresistant HCC cells, and NRAS knockdown enhanced sorafenib efficacy in resistant cells. In summary, NRAS appears to be a prognostic marker in HCC and contributes to sorafenib resistance. Regarding potential therapeutic strategies, NRAS inhibition in HCC should be combined with KRAS inhibition to prevent KRAS-mediated rescue effects.

Introduction
Hepatocellular carcinoma (HCC) is a major cause of cancer-related mortality [1,2]. Sorafenib as a first-line [3–5] and regorafenib as a second-line [6] approach are the only effective therapeutic strategies for advanced HCC. Both sorafenib and regorafenib target multiple kinase-related pathways including the RAS-RAF-ERK-pathway in HCC cells, underlining the crucial role of RAS signaling in HCC [3,7,8]. In most recent studies, our group showed that the wild-type RAS isoform Kirsten rat sarcoma (KRAS) is a promising candidate diagnostic and therapeutic target majorly contributing to acquired resistance to RAF inhibitors in HCC and other types of cancer [9–11]. Moreover, we found that the HRas proto-oncogene (HRAS) isoform is upregulated in HCC and affects patient outcome [12].

Unlike KRAS and HRAS, the precise function of neuroblastoma RAS viral oncogene homolog (NRAS) was unclear in HCC. Mouse models of primary liver cancer driven by oncogenic NRAS have been established previously [13]; however, several studies suggested that NRAS mutations only rarely occur in human HCC [14,15]. In contrast to mutated NRAS, the role of wild-type NRAS in HCC progression and therapy resistance remained completely unknown and was addressed in this study.

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Materials and Methods

Cells and Cell Culture
The human HCC cell lines PLC (ATCC CRL-8024), HepG2 (ATCC HB-8065), and Hep3B (ATCC HB-8064) were described previously [11]. Murine Hepa129 cells originate from a C3H/HeN mouse and were obtained from the NCI-Frederick Cancer Research and Development Center (DCT Tumour Repository). Sorafenib-resistant HCC cells (Hep3B) were generated by long-term (3-4 months) exposure of cells to sorafenib with stepwise dose escalation (0.5 μM per week) up to 10 μM [11]. In parallel, nonresistant, untreated Hep3B cells were cultured and used as controls. As soon as the resistant cells were able to tolerate 8 μM of sorafenib without signs of toxicity, proliferation and anchorage-dependent growth assays were performed. Sorafenib (“ Nexavar”) was purchased from Selleckchem (Munich, Germany). Primary human hepatocytes were isolated as described [16].

Human Material
Paired human HCC tissues and corresponding nontumorous liver tissues originated from patients that underwent partial hepatectomy. The tissue microarray comprising paraffin-embedded human HCC tissue samples was analyzed as described [11,17,18]. All experimental procedures were performed according to the guidelines of the nonprofit state-controlled Human Tissue and Cell Research (HTCR) foundation with informed patients’ consent [18]. Sampling and handling of patient material were performed in accordance with the ethical principles of the Declaration of Helsinki.

Immunohistochemistry
Immunohistological analysis was performed as previously described [11]. In brief, after deparaffinizing/dewaxing in xylene and rehydration in a graded series of isopropanol, antigen retrieval was achieved by microwave in Tris-EDTA buffer. After peroxidase block (Dako, Hamburg, Germany), the sections were incubated with anti–phospho-ERK antibody (1 in 100 dilution; Cell Signaling, Frankfurt am Main, Germany), anti–Ki-67/MIB-1 (1 in 50 dilution, Dako GmbH, Hamburg, Germany) (Abcam, Cambridge, UK; 1 in 2,000 dilution), anti–KRAS antibody (1 in 50 dilution; Abcam), or a validated and specific NRAS antibody (1 in 200 dilution, Abcam). As a next step, the slides were washed three times with PBS and then incubated with HRP-labeled polymer (conjugated with anti-rabbit secondary antibody) before again washing three times with PBS. Staining was performed with DAB (Dako) followed by counterstaining with hematoxylin (Merck, Darmstadt Germany). NRAS staining was described qualitatively using “0” (low/negative), “1” (moderate), or “2” (high). KRAS membrane localization was described qualitatively using “0” (negative): cytoplasmic/endomembranous staining, “1” (positive): <50% of cells show positive plasma membrane staining, or “2” (strong positive): >50% of cells show positive plasma membrane staining. Quantification of pERK staining (“0”: <5%; “1”: 5%-20%; “2” more than 20% positive cells) was also performed in HCC tissues.

Protein Analysis
Protein extraction and Western blotting analysis were performed as described elsewhere [11]. The following antibodies were used: anti–phospho-ERK (1 in 4000 dilution; Cell Signaling, Frankfurt am Main, Germany), anti–ERK (1 in 1000 dilution; Cell Signaling), anti–KRAS antibody (1 in 1000 dilution; Abcam), anti–phospho-AKT (1 in 2000 dilution; Cell Signaling), anti–AKT (1 in 2000 dilution; Cell Signaling), and anti–NRAS (1 in 1000 dilution, Abcam). For visualization of immunoreactions, the NBT/BCIP (Sigma-Aldrich) staining technique was used. Computational densitometry of the scanned Western blot images was performed using the “ImageJ” program (National Institutes of Health, USA).

Cell Proliferation, Clonogenicity and Migration Analysis
The xCELLigence System (Roche) was used to analyze real-time cell proliferation as described previously [11]. Stem cell properties of cancer cells (clonogenicity) were analyzed using clonogenic assays as described [12]. Cell migration was analyzed using the Boyden chamber system as described [9].

RNA Expression Analysis
Total RNA isolation and reverse transcription were performed as described previously [11]. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using a Lightcycler (Roche, Mannheim, Germany) as described [9]. The following primer pairs were used: 18S (5′-GCA ATT TTC CCC CAT GAA CG-3′ and 5′-GGG ACT TAA TCA AGG CAA GC-3′), BAX (5′-TGC AGA GGA TTA TGG CCG CTC CGG-3′ and 5′-CAC CCA ACC ACC CTT GTC TTG GA-3′), BCL-2 (5′-AGG CAC CCA GGG TGA TGC AA-3′ and 5′-GTT GAG GAG CTC TTC AGG GA-3′), BCL-3 (5′-TGA CAG CAG CCT CCA GA AC-3′ and 5′-CGG AGA GAA GAC CAT TG-3′), and NRAS (5′-TGG TGG GGA GCA AGT GTG AC-3′ and 5′-TTG TGG TGC GTC AGG AGA G-3′), KRAS (5′-TTG AGC TGG TGG CTT CAG CA-3′ and 5′-AGC CCT CCC CAG TCC TCA TG-3′), HRAS (5′-TGG TGG GGA AAC AGT GTG AC-3′ and 5′-TTG TGG TGC GTC AGG AGA G-3′), MAPK14 (5′-GGG AAG TCC ATC TCG GCT CG-3′ and 5′-CTG CCT TCC CAG GT-3′), LIN28 (5′-CGG TGG GGG CAT CTG TAA GTT GG-3′ and 5′-TGG CGG CCT CTC ACT CCC AAT AC-3′), MDR2 (5′-GGT AAA TCA TAC TGC GAC GG-3′ and 5′-CGG GGT CCG CGC TCA AG-3′), and NRAS (5′-ATG AGG ACA GCC GAA GG CT-3′ and 5′-TGA GTC CCA TCA TCA CTG-3′).

Quantification of Apoptosis
Fluorescence-activated cell sorting and the “ApoDETECT ANNEXIN V-FITC KIT” (Invitrogen distributed by Life Technologies, Darmstadt, Germany) were used to quantify apoptotic cells as described earlier [19].

Transfecting Cells with Si-RNA-Pools and MicroRNAs
A total of 2 × 10⁴ cells were seeded per well in six-well plates. The Lipofectamine RNAmax transfection reagent was used (Life Technologies, Darmstadt, Germany). Si-RNA-pools against the human HRAS, KRAS, and NRAS mRNAs were used (functionally verified by siTOOLs Biotech GmbH, Planegg, Germany). Si-RNA-Pools consist of 30 single si-RNAs and are considered to reduce off-target effects [9]. For transfection of microRNAs, 5 μl (20 mM per microRNA) of commercially available pre-miR-622 (Ambion) and the corresponding pre-miR negative Control #1 (Ambion) were transfected per six-well plate. Total RNA and protein were isolated for 48 hours after transfection as described [9].

In Silico Analysis
In silico analysis of RNA expression of NRAS was performed using Gene Expression Omnibus (GEO) datasets (GEO profiles). A murine Mdr2-knockout HCC model-derived dataset was used. The Mdr2-KO mouse represents a model for a beta-catenin–negative subgroup of human HCCs characterized by downregulation of multiple tumor suppressor genes [20]. Additionally, the Trim24-KO murine HCC
A model was used to determine gene expressions in wild-type as compared to Trim24-deficient mice. Trim24 knockout mice also spontaneously develop HCCs [21]. Immunostainings of NRAS in human tissues were explored using the human proteinatlas (https://www.proteinatlas.org/) database. Oncomine cancer microarray database analysis for gene expressions was performed using the website https://www.oncomine.org/. Kaplan-Meier survival curve analysis was performed using the SynTarget/BioProfiling database [22,23].

Figure 1. Expression of NRAS in HCC. (A) Nras mRNA levels in liver tissues derived from homo- (N=6) as compared to heterozygous (N=6) Mdr2-knockout (KO) mice (*: P<.05). (B) Nras mRNA levels in HCC (N=6) as compared to nontumor liver tissues (N=6) derived from a Trim24-knockout (KO) mouse model (*: P<.05). (C) Nras protein levels (Western blot analysis) in murine HCC cells (Hepa129) as compared to primary murine hepatocytes (*: P<.05 vs. hepatocytes). (D) NRAS mRNA levels in nontumorous liver tissues ('Liver') as compared to HCC patient tissues. Data were obtained from the Oncomine cancer microarray database using the datasets 'Roessler Liver 2,' 'Roessler Liver,' and 'Wurmbach Liver' (OE: overexpression) (*: P<.05 vs. 'Liver'). (E) GEO dataset analysis comparing NRAS expression in human metastatic HCC tissues ('MET') as well as primary tumor tissues that had metastasized ('PT metastatic') and nonmetastatic HCC tissues ('nonmetastatic') (*: P<.05 vs. nonmetastatic). (F) NRAS mRNA expression levels (qRT-PCR analysis) in human HCC cell lines (PLC, Hep3B, HepG2) as compared to primary human hepatocytes (*: P<.05 vs. hepatocytes). (G) Summarized NRAS protein expression (Western blot analysis) in human HCC cell lines (PLC, Hep3B, HepG2) as compared to primary human hepatocytes (*: P<.05 vs. hepatocytes) The exemplary Western blot image shows NRAS expression in hepatocytes compared with the HepG2 HCC cell line.
Additionally, the "SurvExpress-Biomarker validation for cancer gene expression" database (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp) was used as described [24].

**Statistical Analysis**

All results are expressed as mean ± SEM. The Student’s t test or one-way analysis of variance, if appropriate, was used for statistical
comparisons between groups. The level of significance was $P<0.05$ ("ns": nonsignificant; "*": $P<0.05$). The number of independent experiments was $n \geq 3$ (if not depicted otherwise). Calculations were performed using the GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA) and SPSS (SPSS Statistics 23, IBM Corp., Armonk, NY).

## Results

### Expression of Wild-Type NRAS in HCC

First, we aimed at investigating the expression of NRAS in murine and human HCC models, cell lines, and patient tissues. The Mdr2-knockout (KO) mouse represents an established model of human HCC development [20]. GEO dataset analysis revealed that Nras was significantly overexpressed in homozygous as compared to heterozygous Mdr2-KO mice (Figure 1A), pointing to a potential role of Nras in HCC development in this model system. Trim24-deficient (KO) mice (Figure 1B) represent another experimental model of hepatocarcinogenesis [21]. A Trim24-KO mouse derived GEO dataset showed significant upregulation of NRAS expression by both "best separation" ($P=0.0045$) was confirmed using additional TCGA data

### Effect of NRAS Expression on HCC Patient Survival

The strong overexpression of NRAS prompted us to ask whether NRAS expression could affect survival of HCC patients. Kaplan-Meier (overall) survival analysis was performed using the "SynTarget/BioProfiling" database and a The Cancer Genome Atlas (TCGA) HCC dataset [22,23]. High NRAS expression was a predictor for poor patient outcome (i.e., overall survival) ($N=370$, $P=0.0211$) (Figure 3A). Poorer outcome of patients with high as compared to low NRAS expression by both "best separation" ($P=0.0001$) and "median separation" ($P=0.0045$) was confirmed using additional TCGA data
available from the human proteinatlas database (Suppl. Figure 4). Moreover, survival analysis using the "SurvExpress" Biomarker validation for cancer gene expression database [24] was performed. Computational stratification into "low-risk" and "high-risk" patient groups (based on prognostic index) revealed marked overexpression of NRAS as well as reduced overall survival of high- compared to low-risk groups in three available datasets (Figure 3B). In summary, these results indicated high NRAS expression as a predictor of poor outcome in HCC patients.

**Function of Wild-Type NRAS in HCC**

To further explore the role of NRAS in HCC, we performed functional analysis after NRAS suppression in HCC cells in vitro. A si-RNA-pool ("si-NRAS": functionally verified pool of 30 single si-RNAs against the human NRAS mRNA) and an corresponding si-RNA-control-pool ("Control") were used for specific gene knockdown and concomitant reduction of off-target effects. NRAS knockdown was established in two HCC cell lines (Hep3B, PLC) (Figure 4, A and B). Si-NRAS-treated HCC cells showed slight, nonsignificant reduction of proliferation (Figure 4C). Moreover, NRAS knockdown significantly reduced clonogenicity in Hep3B cells (Figure 4D). However, only slight, nonsignificant inhibition of colony formation was observed in PLC cells (P=.07) (Figure 3D). HCC cell migration was also not affected by NRAS knockdown as determined using Boyden chamber assays (Figure 3E). Western blot analysis revealed that, only in Hep3B cells, NRAS knockdown was sufficient to significantly impair AKT activation (Figure 3F). AKT activity was not affected in PLC cells, and ERK activity was not altered in both HCC cell lines after NRAS knockdown (Figure 3, F and G). In summary, NRAS suppression did not affect migration and ERK activation and had only moderate and partially nonsignificant effects on proliferation, clonogenicity, and AKT activation in HCC.

**Loss of NRAS in HCC Cells Is Rescued by KRAS Upregulation**

In the light of the strong overexpression of NRAS (Figure 1 and 2) and the marked effects on HCC patient survival (Figure 3), we had expected stronger functional effects (Figure 4) after NRAS knockdown or in all HCC cell lines, respectively. Interestingly, NRAS immunoreactivity was found to be significantly correlated with KRAS membrane staining in patient-derived tissue microarray samples (Table 1). Moreover, in contrast to NRAS expression "alone" [which did not correlate with ERK activation in patient-derived HCC tissues (N=45, Fisher’s exact P=.134, Spearman R=0.194, P=.214)], co-positivity for both NRAS and KRAS staining was significantly correlated with ERK-activation (N=37, Fisher’s exact P=.042, Spearman R=.374, P=.042). Therefore, we hypothesized that NRAS might co-function with other RAS isoforms and that loss of NRAS in HCC cells could potentially be compensated by other RAS proteins. To adress this hypothesis, the canonical "non-NRAS" RAS isoforms (i.e., KRAS and HRAS) were knocked down in HCC cells (Hep3B, PLC) using si-RNA-pool–mediated mRNA suppression. Here, combined si-HRAS and si-KRAS treatment served as "control"
and was compared to cells that were additionally treated with si-NRAS. In the absence of KRAS and HRAS, knockdown of NRAS significantly impaired proliferation in both HCC cell lines (Figure 5A, Suppl. Figure 5). qRT-PCR analysis revealed that, after NRAS knockdown, KRAS was significantly upregulated (by ~20%) in HCC cells, while HRAS expression was unaffected (Figure 5B).

We have previously demonstrated that KRAS inhibition strongly impairs proliferation in HCC [11]. Therefore, the current observations together with our previous study pointed to KRAS (and not HRAS) as a major functional rescue gene in the absence of NRAS. Accordingly, knockdown of HRAS alone did not affect proliferation in both HCC cell lines (Figure 5C). To confirm the hypothesis that specifically KRAS (and not HRAS) is sufficient to rescue NRAS knockdown in HCC, we performed additional proliferation analysis. Here, si-NRAS effects were analyzed in 1) the absence of HRAS (si-HRAS versus si-HRAS+si-NRAS) and 2) the absence of KRAS (si-KRAS versus si-KRAS+si-NRAS). In the absence of HRAS [which had shown no effect on proliferation (Figure 5C)], additional NRAS knockdown did not significantly reduce the proliferation capacity of HCC cells (Figure 5D). In contrast and in line with our previous
study [11], KRAS inhibition alone was sufficient to strongly reduce proliferation. Notably, NRAS knockdown further enhanced inhibition of proliferation in the absence of KRAS (Figure 5D). In contrast, migration was not affected by combined NRAS and KRAS knockdown (Suppl. Figure 6). This resembled our previous study showing that KRAS alone also did not regulate migration [11] as well
as the nonsignificant effects of single NRAS knockdown on migration seen in this study (Figure 4). Together, these data indicated that KRAS (but not HRAS) upregulation serves as a functional compensatory mechanism for HCC proliferation after loss of NRAS in HCC (Figure 5E). Based on our finding that NRAS affected survival in HCC (Figure 3; Suppl. Figure 4), we explored whether NRAS and KRAS might also co-function on patient survival.

"SyntTarget/BioProfiling" database analysis revealed a patient dataset with statistically significant and significant effects on poor patient outcome of co-upregulated NRAS and KRAS levels ($P=0.0269$, $\chi^2=4.9$) compared to less/nonsignificant effects of upregulated "NRAS-only" ($P=0.0462$, $\chi^2=4.0$) or "KRAS-only" ($P=0.0686$, $\chi^2=3.3$) (Figure 5F). Moreover, "SurvExpress" Biomarker validation for cancer gene expression database analysis revealed significant co-upregulation of NRAS and KRAS and poorer overall (Figure 5G) and recurrence-free (Figure 5H) survival in high-risk as compared with low-risk patient groups. In summary, NRAS co-functioned with KRAS in HCC, and loss of NRAS was functionally rescued by KRAS upregulation.

Expression and Function of NRAS in Aquired Sorafenib Resistance

Since our previous study had revealed strong impact of wild-type KRAS on RAF inhibitor resistance in HCC [11], we next asked if also NRAS might affect sorafenib resistance. First, long-time exposure to slowly increasing doses of sorafenib was performed to establish sorafenib-resistant Hep3B and PLC cell clones. Functionally, these cells revealed marked resistance to sorafenib exposure compared to nonresistant cells (Figure 6, A and B). Moreover, the resistant cells showed enhanced expression of the chemoresistance-associated stem cell marker Lin-28 homolog A (LIN28A) [30] and sorafenib-resistance–induced mitogen-activated protein kinase 14 (MAPK14) [31] (Figure 6C). NRAS but not HRAS mRNA expression was significantly upregulated in sorafenib-resistant as compared to nonresistant HCC cells (Figure 6D). Western blot analysis confirmed that also NRAS protein levels were upregulated in sorafenib-resistant Hep3B cell clones as compared to nonresistant Hep3B cells (Figure 6E). Since resistant cells were cultured in sorafenib-containing medium to ensure constant selection pressure, we excluded that sorafenib treatment could directly induce NRAS expression in HCC cells (Suppl. Figure 7). Functionally, real-time cell proliferation analysis showed that si-RNA–mediated NRAS knockdown (alone) was sufficient to inhibit cell proliferation in sorafenib-resistant HCC cells (Figure 6F). Resembling nonresistant cells, NRAS knockdown partially but nonsignificantly upregulated KRAS but not HRAS in resistant cells (Suppl. Figure 8). Furthermore, NRAS knockdown did not affect apoptosis in these cells (Suppl. Figure 9) but restored sorafenib sensitivity in resistant cells (Figure 6G). In summary, these data revealed that in aquired resistance to sorafenib, HCC cells upregulate NRAS expression and NRAS inhibition could be sufficient to enhance sorafenib sensitivity. Regarding these data together with the previously described function of KRAS in sorafenib resistance [11], our findings suggest that co-targeting of NRAS and KRAS might be an effective therapeutic approach to overcome sorafenib resistance in HCC.

Discussion

Although RAS proteins are among the most desirable therapeutic targets in cancer, they were considered to be "undruggable" for a many years [4]. Meanwhile, technical improvements have resurrected the concept of effective RAS inhibition and promoted the so-called "RAS renaissance" [4, 32–35]. In HCC, RAS proteins have commonly no mutations in the known hotspot regions [2, 36]. NRAS and KRAS are mutated in <5% of HCCs [14]. Therefore, until recently, RAS proteins were only poorly investigated in HCC, and their potential diagnostic and therapeutic functions remained unclear.

Novel studies by our group demonstrated that wild-type KRAS can function as a potent therapeutic target in "non–KRAS-mutated" cancer types including malignant melanoma [9, 10] and HCC [11]. At a first glance, melanoma and HCC are cancer types without remarkable similarities. However, apart from their primary sites, on a molecular level, both melanoma and HCC strongly depend on the RAS-RAF-ERK-pathway. In advanced melanoma, specific BRAF inhibition represented the only effective therapeutic option for many years and is still considered as a first-line therapeutic strategy for patients with BRAFV600E mutations [37]. Likewise, the unspecific RAS inhibitors sorafenib and regorafenib are the only approved systemic therapy options for intermediate and advanced HCC patients [3, 7, 8]. We found recently that inhibition of wild-type KRAS inhibits proliferation, clonogenicity and RAS downstream signaling in melanoma and in HCC in vitro and in vivo. Moreover, both cancer types showed strong upregulation of wild-type KRAS, which was released by the downregulated tumor-suppressive microRNA-622 [10, 11]. KRAS expression also correlated with tumor stages and patient survival [9, 11]. Furthermore, we found that HRAS is a prognostic marker in HCC and revealed that the novel RAS inhibitor rigorosertib exerted strong functional effects on HCC cells [12]. In summary, these previous studies highlighted the importance of wild-type RAS proteins in HCC.

In contrast to KRAS and HCR, the role of NRAS in HCC was elusive. A recent transcriptome profiling study revealed that NRAS was dysregulated in fibrolamellar HCC, but potential clinical implications
or the function of NRAS have not been investigated [38]. Another recent study found that NRAS and c-MYC are co-upregulated by insulin-like growth factor II in HCC, but the specific function of NRAS was not explored [39]. Therefore, until now, there was no mechanistic evidence for the potential function of wild-type NRAS in HCC. Here, we newly demonstrated marked overexpression of wild-type NRAS in HCC cell lines, murine HCC models, and patient tissues, and NRAS expression correlated with poor patient survival. In vitro analysis using specific si-RNA-pool–mediated NRAS knockdown showed only slight effects on HCC cell proliferation, clonogenicity, and AKT activity, and...
nonsignificant effects on migration and ERK activation. Significant reduction of clonogenicity and AKT-signaling after NRAS knockdown was found only in the Hepa3B cell line which had revealed the highest levels of NRAS mRNA as compared with other HCC cell lines used in this study. Strikingly, RAS-isofrom–specific antibodies revealed that NRAS immunoreactivity correlated with KRAS membrane staining in patient-derived tissue samples. Further, in contrast to NRAS expression alone, co-positivity for both NRAS and KRAS staining significantly correlated with ERK activation. After NRAS knockdown, KRAS but not HRAS was upregulated in HCC cells, thereby rescuing pro-proliferation effects of NRAS. These data suggest that, apart from inhibition of KRAS[11], a combinatorial approach targeting both KRAS and NRAS could be even more effective in HCC. HRAS, however, was indeed shown to serve as a prognostic marker in HCC[12] but did not affect proliferation or was sufficient to compensate for loss of NRAS in this study. These results are in accordance with novel findings that confirm specific functions of different RAS isoforms in other cancer types like pancreatic cancer[40]. Accordingly, in contrast to KRAS, NRAS revealed no binding sites for the KRAS-targeting microRNA-622 and was not regulated by this microRNA in HCC cells. Together, different RAS isoforms display nonexchangeable, specific functions in HCC and potentially also in other types of cancer.

The efficacy of RAS inhibition underlines the importance of RAS downstream signaling pathways such as the MAPK and the PI3K pathway. Moreover, it highlights the major clinical issue of acquired resistance to RAF inhibitors. Escape pathway activation of RAS-RAF-ERK is considered to be a crucial mediator of chemoresistance in HCC[5,41]. In our previous studies, wild-type KRAS was upregulated in (B)RAF-inhibitor–resistant cancer cells, and inhibition of KRAS could almost completely break resistance to vemurafenib[9] and sorafenib[11]. Also, other studies suggested wild-type KRAS as an emerging therapeutic target in cancer therapy resistance[42,43]. Accordingly, novel phase II/III clinical studies reveal that upstream inhibition of MAPK and PI3K pathways by, e.g., EGFR inhibition is sufficient in wild-type KRAS/NRAS colorectal cancer[44]. Another study highlighted the importance of wild-type RAS proteins by revealing that wild-type HRAS and NRAS promote mutant KRAS-driven tumorigenesis[45]. In the current study, NRAS expression was significantly overexpressed in sorafenib-resistant HCC cells compared to nonresistant cells. Moreover, NRAS knockdown partly restored sorafenib efficacy in resistant HCC cells. In contrast to nonresistant cells, NRAS inhibition alone was sufficient to significantly impair proliferation in resistant cells, and NRAS knockdown only slightly and nonsignificantly induced KRAS upregulation. These findings suggest that sorafenib-resistant cells more strongly depend on NRAS as compared to nonresistant cells.

In summary, this study indicates wild-type NRAS as a prognostic marker in HCC. Furthermore, combined NRAS and KRAS inhibition might represent a novel therapeutic approach which could be achieved by pharmacologic "pan-RAS" or "dual-RAS" (i.e., KRAS and NRAS) inhibition.

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Author Contributions

P. D., A. K. B., and C. H. conceived the project, analyzed the data, and wrote the manuscript. P. D., A. G., L. W., and V. F. designed the experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.11.011.

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Figure 6. Expression and function of NRAS in acquired sorafenib resistance. (A) Representative images of crystal violet–stained nonsorafenib and sorafenib-resistant ("Sora-resistant") Hep3B cells. A total of 100,000 cells were seeded in 6-well plates and allowed to attach for 6 hours. Subsequently, cells were treated with different doses of sorafenib (0, 2, 4, 6, 8, 10 μM) for 72 hours. (B) Real-time cell proliferation analysis (xCELLigence) of sorafenib-resistant and nonresistant HCC cells treated with different doses of sorafenib (0, 2, 4, 6 μM) for 128 hours (ns: nonsignificant vs. Control). (C) LIN28 and MAPK14 mRNA expression levels (qRT-PCR analysis) in nonresistant as compared to sorafenib-resistant ("Sora-resistant") Hep3B cells (*: P<0.05 vs. nonresistant). (D) NRAS (left panel) and HRAS (right panel) mRNA expression levels (qRT-PCR analysis) in nonresistant as compared to sorafenib-resistant ("Sora-resistant") Hep3B cells (*: P<0.05 vs. non-resistant). (E) Exemplary images (left panel) and densitometry (right panel, representing N=3 pairs) of Western blot analysis of NRAS protein levels in nonresistant compared to sorafenib-resistant ("Sora-resistant") Hep3B cells (∗: P<0.05 vs. nonresistant). (F) Proliferation (relative cell number after 72 hours of cultivation under normal conditions in serum-containing media) of si-NRAS as compared to control-transfected sorafenib-resistant and nonresistant HCC cells, respectively. (G) Real-time cell proliferation analysis (xCELLigence). Sorafenib-resistant Hep3B cells were transfected with a control-si-RNA-pool (Control) or a si-RNA-pool against the NRAS mRNA (si-NRAS). After seeding, cells were allowed to attach for 24 hours. Afterwards, cells were treated with different doses of sorafenib (0, 2, 4, 6 μM) for 96 hours. The graph depicts summarized quantifications (∗: P<0.05 vs. untreated cells (i.e., 0 μM sorafenib)).
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