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Genome-Wide Methylation Analysis and Epigenetic Unmasking Identify Tumor Suppressor Genes in Hepatocellular Carcinoma

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Abstract

BACKGROUND & AIMS—Epigenetic silencing of tumor suppressor genes contributes to the pathogenesis of hepatocellular carcinoma (HCC). To identify clinically relevant tumor suppressor genes silenced by DNA methylation in HCC, we integrated DNA methylation data from human primary HCC samples with data on up-regulation of gene expression after epigenetic unmasking.

METHODS—We performed genome-wide methylation analysis of 71 human HCC samples using the Illumina Human Beadchip 27K array; data were combined with those from microarray analysis of gene re-expression in 4 liver cancer cell lines after their exposure to reagents that reverse DNA methylation (epigenetic unmasking).

RESULTS—Based on DNA methylation in primary HCC and gene re-expression in cell lines after epigenetic unmasking, we identified 13 candidate tumor suppressor genes. Subsequent validation led us to focus on functionally characterizing 2 candidates, sphingomyelin phosphodiesterase 3 (*SMPD3*) and neurofilament, heavy polypeptide (*NEFH*), which we found to behave as tumor suppressor genes in HCC. Overexpression of *SMPD3* and *NEFH* by stable transfection of inducible constructs into an HCC cell line reduced cell proliferation by 50% and

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Supplementary Material

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Author names in bold designate shared co-first authorship.

Conflicts of interest

The authors disclose no conflicts.

20%, respectively (SMPD3, $P = .003$ and NEFH, $P = .003$). Conversely, knocking down expression of these genes with small hairpin RNA promoted cell invasion and migration in vitro (SMPD3, $P = .0001$ and NEFH, $P = .022$), and increased their ability to form tumors after subcutaneous injection or orthotopic transplantation into mice, confirming their role as tumor suppressor genes in HCC. Low levels of SMPD3 were associated with early recurrence of HCC after curative surgery in an independent patient cohort ($P = .001$; hazard ratio = 3.22; 95% confidence interval: 1.6–6.5 in multivariate analysis).

CONCLUSIONS—Integrative genomic analysis identified *SMPD3* and *NEFH* as tumor suppressor genes in HCC. We provide evidence that *SMPD3* is a potent tumor suppressor gene that could affect tumor aggressiveness; a reduced level of SMPD3 is an independent prognostic factor for early recurrence of HCC.

Keywords

nSMase2; NEFH; Sphingomyelin Phosphodiesterase; 5-aza-2-deoxycytidine

Age-adjusted incidence rates of hepatocellular carcinoma (HCC) have tripled in the United States from 1975 through 2005,¹ and worldwide this cancer is the third leading cause of cancer death.² Currently, the most effective systemic therapy for patients with HCC is treatment with the tyrosine kinase inhibitor sorafenib, which increases median survival and time to radiologic progression by nearly 3 months.^{3,4} The mechanisms contributing to hepatocarcinogenesis are unclear, however, it is widely accepted that HCC exhibits numerous genetic abnormalities, such as chromosomal alterations, gene amplifications, and mutations, as well as epigenetic alterations.^{5,6} A number of locus-specific studies in HCC report promoter DNA methylation–associated silencing of tumor suppressor genes,^{7–9} and increased DNA methylation levels of tumor suppressor genes have also been reported to correlate positively with HCC development and progression.^{5,10} Aberrant DNA methylation and other posttranslational histone modifications are frequently found in surrounding tissues^{5,6} and in patients with chronic hepatitis or cirrhosis, conditions that commonly precede development of this disease.¹¹

In this study, we have performed a systematic and combinatorial approach to identify hypermethylated tumor suppressor genes in HCC. First, we performed array-based analysis on 27,578 CpG sites in 71 primary HCC samples and 8 nondiseased (ND) normal tissues to identify genes differentially methylated between primary HCC and ND normal liver. Second, we performed microarray analysis of 4 liver cancer cell lines from an epigenetic unmasking experiment to identify genes up-regulated after DNA de-methylation and inhibition of histone de-acetylation. Finally, we combined both datasets to provide a list of biologically relevant hypermethylated genes for further validation and characterization as tumor suppressor genes and prognostic markers in HCC.

Materials and Methods

Patients

We analyzed a previously reported genomic dataset of hepatitis C–related HCC patients with prognostic information ($n = 77$) as our training cohort, as described previously¹² (NCBI Gene Expression Omnibus, accession number GSE9829). For prognostic validation, we analyzed a previously reported genomic dataset of mixed etiology HCC patients from the HCC Genomic Consortium with prognostic information ($n = 164$), GEO accession GSE19977.¹³ Clinical demographics are summarized in Table 1. All patients were treated with curative resection. Curativity of resection was confirmed by postsurgical histological

and imaging assessment every 3 to 6 months. Tumor recurrence was determined based on typical imaging features or histological confirmation.

Cell Culture and Treatment

For epigenetic unmasking, cells were treated with 500 nm trichostatin A (TSA) (Sigma, St Louis, MO) for 24 hours and 1 μ M 5-aza-2'-deoxycytidine (DAC) (Sigma) for 48 hours.¹⁴ For tetracycline-induced expression, cells were treated with 5 μ g/mL tetracycline (Sigma) for 48 hours and cultured in tetracycline-reduced fetal bovine serum (Invitrogen, Life Sciences Technologies, Santa Clara, CA) containing media.

Methylation Profiling

Whole genome methylation analysis was performed using the Human Methylation 27 BeadChip (Illumina, San Diego, CA) on 71 samples from the HCC Genomic Consortium, 24 samples comprising 12 HCC samples with 12 paired adjacent normal from cooperative tissue network (CHTN) and 8 ND normal adult liver samples (Figure 1).

Genes were considered to be hypermethylated if the β value for one or more of their corresponding probes showed a fold increase of ≥ 1.5 in the mean β of HCC samples above the mean β of normal ND liver, and if this change was statistically significant ($P \leq .05$). In addition, we only considered probes passing a minimum cut-off value of 0.3 mean β value of HCC samples (Figure 1).

For detailed methods and statistical analysis see Supplementary Material.

Results

Patient Characteristics

Table 1 summarizes clinical characteristics of the patients analyzed in this study. The HCC Genomic Consortium is split into 2 groups, 1 that is hepatitis C-related only and 1 with mixed etiology. Patients from the HCC Genomic Consortium were predominantly Barcelona Clinic Liver Cancer stage A and Child-Pugh class A with tumor sizes of >2 cm. Clinical information for samples collected from the CHTN tissue bank were not available and are therefore not listed; however, in contrast to patients within the HCC Genomic Consortium, these patients do not have a background of viral infection. This situation reflects the differences in obtaining samples from these different sites. Although the HCC Genomic Consortium collects samples from clinical sites, Cold Spring Harbor Laboratory utilizes a commercial tissue bank. The differing etiologies and method of collection between both groups allowed us to evaluate how these variables affect the application of our approach to identify novel tumor suppressor genes in a cancer phenotype.

Candidate Tumor Suppressor Gene Discovery

A total of 678 genes were identified as being significantly hypermethylated in HCC samples compared with ND normal liver tissues (Figure 1, Supplementary Table 1). Among the list of significantly hypermethylated genes in primary HCC were 37 known tumor suppressor genes, including adenomatous polyposis coli, *p16*, glutathione S-transferase 1 (*GSTP1*), retinoblastoma 1, and *SPINT2*, which are known to be silenced by DNA methylation in HCC,^{15,16} giving us confidence in our methodology.

In parallel, we performed an epigenetic unmasking experiment¹⁴ on the liver cancer cell lines, SNU-449, HepG2, Hep3B, and JHH2 with DAC treatment, which blocks DNA methylation, and TSA to inhibit histone deacetylation (Figure 1). The optimal treatment conditions were determined by the robust re-expression of *GSTP1* and retention of the

unmethylated *GSTP1* band by combined bisulfite restriction analysis upon treatment with 500 nM DAC for 48 hours and 1 μ M TSA for 24 hours, indicating de-methylation of *GSTP1* (Supplementary Figure 1).

RNA microarray was used to select for genes that were re-expressed after DAC/TSA treatment. We identified genes that were transcriptionally silent during the mock treatment (<1.5 times the median background probe intensity), but were re-expressed after the DAC/TSA combination treatment. A total of 253 genes passed this cut-off (Figure 1, Supplementary Table 2).

As a means to identify biologically relevant candidate tumor suppressor genes silenced by DNA methylation, we filtered the list of genes identified by array analysis as being hypermethylated in primary HCC with genes up-regulated from the epigenetic unmasking procedure. Using this approach, we arrived at a list of the following 13 candidate genes: actin-like 6B (*ACTL6B*); C19orf30; diacylglycerol kinase (*DGKI*); *DLX1*; *ELOVL* fatty acid elongase 4 (*ELOVL4*); lactate dehydrogenase B (*LDHB*); lecithin retinol acyltransferase (phosphatidylcholine–retinol O-acyltransferase) (*LRAT*); *MLF1*; neurofilament, heavy polypeptide (*NEFH*); *PPM1N*; peripherin (*PRPH*); *SLC8A2*; and sphingomyelin phosphodiesterase 3 (*SMPD3*) (Figure 1, Supplementary Table 3). These genes share some ontology by DAVID pathway analysis—*SLC8A2*, *PPM1N*, *DGKI*, and *SMPD3* are involved in ion binding; *DLX1*, *MLF1*, and *SMPD3* are involved in development; and *LDHB*, *LRAT*, and *SMPD3* are involved in metabolic processes. Enrichr analysis¹⁷ reveals that several of these candidates, *LRAT*, *DLX1*, *NEFH*, *PRPH*, *DGKI*, *ELOVL4*, *SMPD3*, and *MLF1* are targets of at least 2 members of the PRC2 polycomb repressive complex and *DLX1*, *NEFH*, *DGKI*, *ELOVL4*, *SMPD3*, *MLF1* are marked by the active histone mark H3K4me1 in the adult liver (Supplementary Table 4).

We also wanted to assess the suitability of using a much smaller group of samples for candidate gene selection, as we acknowledge that, in some instances, analysis of large sample numbers is not an option. By performing the same analysis described here on methylation data from samples obtained from CHTN (comprising just 12 HCC samples with 12 paired adjacent liver), we identified 403 significantly hypermethylated genes. When we filter this list using the list of 253 genes up-regulated after epigenetic unmasking, we arrive at the following 10 candidate genes: *ACTL6B*, *DGKI*, *ELOVL4*, *PPM1N*, *G-protein-coupled receptor 63* (*GPR63*), *LDHB*, *LRAT*, *NEFH*, *PRPH*, and *SMPD3*. Nine of which were also identified using the larger dataset described here, validating our approach.

Gene-Specific Validation of Candidate Tumor Suppressor Genes in Primary HCC

Using HCC paired with adjacent liver tissue from CHTN, we confirmed the methylation status of 6 of 9 candidates that were identified in both independent analyses (ie, *SMPD3*, *NEFH*, *LDHB*, *ACTL6B*, *PRPH*, and *DGKI*) using the fully quantitative method of methylation-specific pyrosequencing (Supplementary Figure 2). We observed significant hypermethylation in tumor samples compared with their adjacent counterparts for *DGKI* ($P = .002$), *NEFH* ($P = .016$), *LDHB* ($P = .004$), *ACTL6B* ($P = .003$), and *SMPD3* ($P = .002$), but not *PRPH* ($P = .686$) (Figure 2A).

For those genes with significant hypermethylation in tumor samples, *DGKI*, *NEFH*, *LDHB*, *ACTL6B*, and *SMPD3*, we analyzed the expression status in 12 samples from paired HCC using quantitative real-time polymerase chain reaction. *SMPD3* exhibited significantly reduced expression in all 12 tumors when calculating fold change from paired adjacent ($P = .001$). Nine of 11 tumors that were assessed for methylation were also hypermethylated, correlating significantly with loss of expression ($P = 5.95 \times 10^{-6}$, Fisher's). *NEFH* exhibited reduced expression in 7 of 12 tumor samples, which was not significant ($P = .601$);

however, 4 of the 7 tumors with reduced expression were also hypermethylated, and none of the samples expressing NEFH were methylated ($P = .029$, Fisher's). *LDHB* exhibited reduced expression in 6 of 12 samples (only 2 of 6 samples with reduced expression were hypermethylated) and *ACTL6B* in 6 of 12 samples (only 4 of 6 samples with reduced expression were hypermethylated), neither of these were significant for loss of expression ($P = .642$, $P = .282$, respectively). By contrast, the expression levels of *DGKI* were significantly increased in 11 of 12 HCC samples ($P = .0002$) (Figure 2B).

Microarray analysis of samples obtained from the HCC Genomic Consortium revealed significantly reduced expression in tumors compared with ND normal liver for *ACTL6B* ($P = .01$), *C19orf30* ($P = .015$), *PPMIN* ($P = .03$), *LRAT* ($P = 2.51 \times 10^{-14}$), *SLC8A2* ($P = .027$), and *SMPD3* ($P = 5.03 \times 10^{-6}$) (Supplementary Figure 3). We did not observe significant correlation between gene silencing and hypermethylation obtained from array data for these samples.

Methylation is not the only mechanism of gene silencing, and DNA methylation is often observed without accompanying gene silencing (reviewed extensively¹⁸ and elsewhere). In addition, other mechanisms to silence expression of a tumor suppressor gene should be expected, such as deletion and inactivating mutations.¹⁸ On samples obtained from the HCC Genomic Consortium, we performed copy number analysis within a 0.5-Mb window for those genes where promoter methylation was confirmed in our paired samples (Figure 1). We observed copy number loss of *SMPD3* and *NEFH* in 21% and 13% of samples, respectively (Supplementary Figure 4A). The level of copy number loss of *SMPD3* and *NEFH* in HCC vs adjacent cirrhotic tissues when using the same cut-offs is comparable with *cyclin-dependent kinase inhibitor 2A*, *PTEN*, and *RBI*, which are tumor suppressor genes known to be deleted in HCC (Supplementary Figure 4B). Samples exhibiting copy number loss of *SMPD3* or *NEFH* tended to also be hypermethylated (Supplementary Figure 4C).

In samples from the HCC Genomic Consortium, we observed copy number loss of *ELOVL4* in 26% of patients and significant loss of expression of *LRAT* ($P = 2.51 \times 10^{-14}$), and copy number loss of *LRAT* in 19% of patients (Supplementary Figure 4D). Due to assay limitations, we were unable to confirm methylation status of *ELOVL4* and *LRAT* by pyrosequencing.

Overexpression of SMPD3 and NEFH Reduces Cell Proliferation in a Human HCC Cell Line

Due to the presence of methylation and loss of expression in at least half of the tumors surveyed, as well as evidence of copy number loss, we next wanted to test whether overexpression of *SMPD3* or *NEFH* leads to an inhibitory effect on cell proliferation. To achieve this, we created constructs, pDEST *SMPD3* or pDEST *NEFH*, using a tetracycline operator plasmid (TetO) that allows for expression of either *SMPD3* or *NEFH* under the influence of an inducible promoter (Supplementary Figure 5A and B). To correct for treatment with the inducible agent tetracycline and the effects of gene overexpression, we also created an inducible construct pDEST β -galactosidase, to express the gene for bacterial β -galactosidase, which is neutral (Supplementary Figure 5C).

The HCC cell line JHH-7 has low or absent *SMPD3* protein expression and *NEFH* messenger RNA expression (Supplementary Figure 5), and was successfully cloned with a tetracycline repressor plasmid (TetR) necessary for repression in the absence of tetracycline to produce TetR JHH-7 cells (Supplementary Figure 5A and B). This TetR cell line was used to introduce pDEST *SMPD3* and pDEST *NEFH* TetO plasmids, to give JHH-7 pDEST *SMPD3* and JHH-7 pDEST *NEFH*.

By inducing *SMPD3* expression with tetracycline in JHH-7 pDEST *SMPD3* cells, we observed that proliferation is significantly reduced by at least 50% at 96 hours post induction in these cells compared with noninduced cells ($P = .003$) (Figure 3A).

We observe a less striking, but significant, effect on cell proliferation when expression of *NEFH* is induced in JHH-7 pDEST *NEFH* cells. Cell proliferation is significantly reduced by 20% ($P = .034$) in cells induced to express *NEFH* at 96 hours post induction (Figure 3B).

We see no significant effect on cell proliferation in cells induced to overexpress the control gene β -galactosidase, arguing against cell toxicity upon tetracycline treatment and any aberrant effects of gene overexpression in these cells (Figure 3C).

Loss of *SMPD3* and *NEFH* Promotes Tumorigenicity

We knocked down expression of *Smpd3* and *Nefh* in an immortalized line of mouse embryonic hepatoblasts lacking tumor protein p53 and overexpressing *v-myc myelocytomatosis viral oncogene homolog (avian)* (PHMI cells) that are not tumorigenic in vivo,¹⁹ these cells provide an appropriate sensitized background where a single lesion can trigger tumorigenesis.^{20,21} Using these cells, we first assessed the tumorigenic effects of short hairpin RNAs (shRNAs) on migration and invasion in vitro and then assessed their ability to promote tumorigenesis after subcutaneous injection and orthotopic liver transplantation via intrasplenic injection into recipient mice.

Successful knockdown of *Smpd3* by 3 independent shRNAs was confirmed by Western blotting (Supplementary Figure 6A). shRNA 4 provided the highest level of *Smpd3* knockdown as evidenced by a barely detectable protein band. As *Nefh* encodes a protein product of 220 kD and was difficult to detect by Western blotting, successful knockdown of *Nefh* by 2 independent shRNAs—shRNA 2 and shRNA 5—was confirmed by quantitative real-time polymerase chain reaction (Supplementary Figure 6B). Both short hairpins provided >90% knockdown of *Nefh*.

Because enforced expression of *SMPD3* or *NEFH* in an HCC cell line reduces cell proliferation, we assessed whether knockdown of these proposed tumor suppressor genes can affect other aspects of tumor biology, namely cell migration and invasion, in PHMI cells in vitro before performing in vivo experiments. Compared with cells transfected with the nontargeting shRNA to Renilla luciferase, cell migration was significantly increased in PHMI cells with shRNA knockdown of *Nefh* ($P = .022$) or *Smpd3* ($P = .001$) (Figure 4A and B). Cell invasion was dramatically enhanced in cells with knockdown of *Smpd3* ($P = .005$), but not *Nefh* (Figure 4C and D). This result indicates that loss of *Nefh* can promote cell migration but not cell invasion. Loss of *Smpd3*, on the other hand, can promote both cell migration and invasion, suggesting that the loss of *Smpd3* might have more pronounced effects on tumor growth and aggressiveness than the loss of *Nefh*.

We then tested if reduced levels of either of these genes could play a tumorigenic role in vivo. A significant increase in tumor volume was observed in nude mice subcutaneously transplanted with PHMI cells with shRNA-mediated knockdown of either *Smpd3* or *Nefh* vs PHMI cells with shRNA directed to the nontarget control (Figure 5). A significant increase in tumor volume was observed at 5 weeks post injection in animals with shRNA-mediated *Smpd3* knockdown compared with background from control animals (shRNA 1, $P = .035$; shRNA 4, $P = .01$; shRNA 6, $P = .007$) (Figure 5A).

By week 7, the knockdown of *Smpd3* by shRNA 4 resulted in a mean tumor volume of around 400 mm³, significantly higher than background ($P = .002$) and the partial knockdown of *Smpd3* by shRNAs 1 and 6 in tumors of approximately 200 mm³ were again

significantly higher than background ($P = .001$ and $P = .05$, respectively) (Figure 5A). The level of knockdown achieved by each hairpin was proportional to the tumorigenic effect observed in the subcutaneous tumorigenicity assay, indicating specific knockdown of *Smpd3* and arguing against off-target effects (Figure 5A).

A significant but less pronounced increase in tumor volume was observed at 5 weeks post injection in animals with shRNA-mediated *Nefh* knockdown (shRNA 2, $P = .0006$; shRNA 5, $P = .0009$) and by week 8, the mean tumor volumes of cells with knockdown of *Nefh* by shRNA 2 and shRNA 5 are approximately 300 mm³ and 200 mm³, respectively, and significantly higher than background (shRNA 2, $P = .002$; shRNA 5, $P = .005$) (Figure 5B). Again, off-target effects of these shRNAs are unlikely because shRNA 2 and shRNA 5 knock down *Nefh* to similar levels and the resulting tumors are within 100 mm³ of each other (Figure 5B).

These results strongly suggest that *Smpd3* and *Nefh* have tumor-suppressive functions in the liver and that *Smpd3* is a more potent tumor suppressor gene than *Nefh*.

When PHMI cells depleted for *Smpd3* or *Nefh* protein expression using the previously described most potently acting shRNAs were transplanted into the livers of recipient mice, tumors developed within 6 weeks (Figure 5C). Microscopic examination of the resultant in situ liver tumors classified them as aggressive, solid hepatocellular carcinomas. The tumors appeared to be composed of a population of undifferentiated cells growing as a sheet without any histological evidence of gland formation or any other structures. The cells were large with a more basophilic staining cytoplasm than normal liver. We established that the liver tumors arose from the *Smpd3*- and *Nefh*-depleted PHMI cells, as the tumors were green fluorescent protein–positive. These tumors were highly proliferative, indicated by strongly positive immunohistochemical staining for proliferating cell nuclear antigen (Figure 5C).

SMPD3 Expression Is an Independent Prognostic Factor for Early Recurrence

To evaluate the potential association of *SMPD3* expression and recurrence of HCC, patients from the HCC Genomic Consortium HCV HCC ($n = 77$) were divided into 2 groups according to levels of high and low *SMPD3* expression of microarray data by performing log-rank testing in the interquartile range to determine the optimal cut-off (Supplementary Figure 7 and Supplementary Table 5). Clinical variables of high and low *SMPD3* expression groups are listed in Supplementary Table 6. The clinical variables between both groups are similar and differences are not statistically significant. Kaplan-Meier plots (Figure 5D and E) were used to assess association between recurrence and *SMPD3* expression. The “*SMPD3*-low” group showed significantly higher early recurrence compared with “*SMPD3*-high” group ($P = .018$, Figure 5D). We were able to validate this finding in an independent cohort from the HCC Genomic Consortium ($n = 164$; $P = .001$; Figure 5E). The association of low *SMPD3* expression level with early recurrence remained significant in multivariate analysis performed on the validation cohort (hazard ratio = 7.1; 95% confidence interval: 1.6–6.5; $P = .001$), even after adjusting with other significant predictors of early recurrence, Barcelona Clinic Liver Cancer stage B (hazard ratio = 3.31; 95% confidence interval: 2.12–23.92; $P = .002$) and presence of satellites (hazard ratio = 3.17; 95% confidence interval: 1.78–5.64; $P = .0001$), indicating an independent prognostic implication of *SMPD3* expression (Table 2).

Early recurrence is generally due to dissemination of primary tumor cells, within the first 2 years of resection, and is associated with tumor aggressiveness.^{18,22}

Discussion

Some success has been achieved in treating solid malignancies using a combination of histone deacetylase and DNA methyltransferase inhibitors.²³ Recent advances in HCC treatment strategies, such as those proposed by Lachenmayer et al, in which the preclinical efficacy of histone deacetylase inhibition combined with sorafenib therapy is assessed, holds great promise in the treatment and management of this disease.²⁴ Defining hypermethylated biomarkers to identify patients who would most benefit from this type of combined treatment is an important area of research.

Recently, the vertical integration of methylation data with both transcriptomic and genomic data was described as a method to identify tumor suppressor gene candidates in HCC. This study by Neumann et al identified 3 candidates (ie, *PER3*, *PROZ*, and *IGFLAS*) in a single cohort of HCC, 2 of which, *PER3* and *IGFALS*, exert tumor-suppressive activities in vitro.²⁵

In this study, we combined whole genome methylation data using the Human Beadchip 27K array from primary HCC samples with re-expression data after epigenetic unmasking in a panel of liver cancer cell lines. Although similar methods have previously been used individually¹⁴ or combined with other methods²⁵ to identify candidate tumor suppressor genes, to our knowledge, we are the first to use this particular combined approach to successfully identify tumor suppressor genes that can be functionally validated in vivo. With this approach, we identified 13 candidate tumor suppressor genes and, when functionally characterized, 2 candidates (*NEFH* and *SMPD3*) behave as tumor suppressor genes in vivo. Although it is feasible that more candidates could be revealed by relaxing our strict criteria, an important factor in this study was to obtain a manageable number of genes for downstream validation and functional characterization efforts, both in vitro and in vivo. In addition, by applying the same criteria of candidate gene selection to an independent set of just 12 HCC samples, we identify several candidates that were selected from the HCC Genomic Consortium cohort (comprising 71 HCC samples), indicating that this method of candidate tumor gene discovery can be applied to smaller sample sizes without greatly compromising the identification of a number of potentially relevant candidate genes. That we observe no overlap with candidates reported by Neumann et al²⁵ most probably reflects our differing approaches to candidate gene discovery. A comparison of *SMPD3* and *NEFH* antiproliferative activity with the growth suppressive activity of *PER3* and *IGFLAS* as identified by Neumann et al can be found in Supplementary Table 7.

Within the candidate tumor suppressor genes from our combined analysis, several share biological features, as assessed by DAVID or Enrichr analysis. Interestingly, 8 genes, including *SMPD* and *NEFH* are targets of at least 2 members of the PRC2 polycomb repressive complex, indicating that these genes are targeted for epigenetic repression during development (reviewed in Simon and Kingston²⁶). Six genes, including *SMPD3* and *NEFH*, are marked by the active histone mark H3K4me1 in adult liver, indicating that these genes are maintained in the active state in the normal liver, suggesting a functional impairment when these genes undergo a silencing event.

Several of our candidates have been reported to be silenced in cancer previously. *ELOVL4* is methylated with associated loss of expression in pancreatic cancer²⁷ and *LRAT* expression is reduced in prostate, renal, and breast cancer.²⁸ We observed striking copy number loss for these genes in a subset of HCC samples. In addition, *LRAT* exhibited a significant reduction of expression, suggesting that either of these genes may play a role in the pathogenesis of HCC and warrant further study.

LDHB is silenced by methylation in gastric²⁹ and prostate cancer³⁰ and decreased expression of *LDHB* enhances claudin-1-mediated hepatoma cell invasiveness, suggesting that the loss of this gene may play an important role in HCC.³¹ In this study, HCC samples exhibit significant DNA methylation of the CpG island associated with the promoter region of *LDHB*; however, *LDHB* expression was not significantly down-regulated in either of the independent sets of samples analyzed in this study.

In this report, we present novel evidence that both *SMPD3* and *NEFH* function as tumor suppressor genes. The hypermethylation and silencing of *SMPD3* and *NEFH* have previously been linked to various malignancies,^{32,33} but not yet HCC, and have not, as yet, been functionally characterized as tumor suppressor genes in any cancer.

Overexpression of *NEFH* disrupts normal cell structure and function³⁴ and its loss is proposed to activate the Akt/ β -catenin pathway and increase glycolysis.³³ We demonstrate that *NEFH* is significantly hypermethylated in a subset of HCC; we also show evidence of copy number loss and reduced expression of *NEFH*. Overexpression of *NEFH* reduces cell proliferation and knockdown promotes cell migration in vitro and tumor growth in vivo, implicating *NEFH* as a bona fide tumor suppressor gene, however, we observe a weaker tumor suppressive role for *NEFH* than we do for *SMPD3*.

SMPD3 is located at 16q22, an area that is frequently deleted in HCC.³⁵ We also observe copy number loss of *SMPD3* in 21% of samples from the HCC Genomic Consortium, suggesting that *SMPD3* is a tumor suppressor gene that is inactivated by both deletion and/or promoter hypermethylation in HCC. *SMPD3* may also behave as a tumor suppressor gene in other cancers because deletion of *Smpd3* has been reported in a mouse *Trp53*-driven osteosarcoma and missense mutations of *SMPD3* have been identified in human leukemia.³⁶ *SMPD3* encodes a sphingomyelinase that cleaves sphingomyelin to produce ceramide, which, via the phosphoinositide-3-kinase/AKT pathway, is involved in growth arrest, differentiation, and apoptosis.^{37,38} We confirmed that loss of *SMPD3* impairs apoptosis and increases phosphorylation of Akt at Ser473 (data not shown). We show that *SMPD3* is significantly hypermethylated and exhibits down-regulated expression in the majority of primary HCCs. Overexpression of *SMPD3* reduces cell proliferation and knockdown promotes both tumor migration and invasion in vitro, indicating that loss of this gene enhances cell migration and, more importantly, promotes cell invasion. In addition, we demonstrate that knockdown of *Smpd3* promotes dramatic tumor growth in vivo. Prognostic analysis indicates that *SMPD3* appears to be an independent prognostic factor of early recurrence of HCC, the loss of which can influence the aggressiveness of this cancer. Detecting *SMPD3* loss might offer a novel and effective therapeutic strategy for HCC patients who could perhaps be treated with inhibitors of the phosphoinositide-3-kinase/AKT pathway.

In summary, we believe that our approach to identify biologically meaningful tumor suppressor genes epigenetically silenced in HCC is a powerful and cost-effective method that can be applied to small sample sizes to yield meaningful results. This work has led to the discovery and characterization of 2 genes in HCC that behave as tumor suppressor genes in vivo and provided multiple candidates for future analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ACTL6B	actin-like 6B
CHTN	cooperative tissue network
CpG	cytosine preceding guanine
DAC	5-aza-2' deoxycytidine
DGKI	diacylglycerol kinase, iota
ELOVL4	ELOVL fatty acid elongase 4
GSTP1	glutathione S-transferase 1
HCC	hepatocellular carcinoma
LDHB	lactate dehydrogenase B
LRAT	lecithin retinol acyltransferase (phosphatidylcholine–retinol O-acyltransferase)
PRPH	peripherin
ND	nondiseased
NEFH	neurofilament, heavy polypeptide
shRNA	short hairpin RNA
SMPD3	sphingomyelin phosphodiesterase 3
TSA	trichostatin A

References

1. Altekruse SF, McGlynn KA, Reichman ME. Hepatocellular carcinoma incidence, mortality, and survival trends in the United States from 1975 to 2005. *J Clin Oncol.* 2009; 27:1485–1491. [PubMed: 19224838]
2. Ferlay J, Shin HR, Bray F, et al. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer.* 2010; 127:2893–2917. [PubMed: 21351269]
3. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med.* 2008; 359:378–390. [PubMed: 18650514]
4. Villanueva A, Hernandez-Gea V, Llovet JM. Medical therapies for hepatocellular carcinoma: a critical view of the evidence. *Nat Rev Gastroenterol Hepatol.* 2013; 10:34–42. [PubMed: 23147664]

5. Calvisi DF, Ladu S, Gorden A, et al. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J Clin Invest.* 2007; 117:2713–2722. [PubMed: 17717605]
6. Nishida N, Goel A. Genetic and epigenetic signatures in human hepatocellular carcinoma: a systematic review. *Curr Genom.* 2011; 12:130–137.
7. Matsuda Y, Ichida T, Matsuzawa J, et al. p16(INK4) is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology.* 1999; 116:394–400. [PubMed: 9922321]
8. Lim SO, Gu JM, Kim MS, et al. Epigenetic changes induced by reactive oxygen species in hepatocellular carcinoma: methylation of the E-cadherin promoter. *Gastroenterology.* 2008; 135:2128–2140. 2140 e1–8. [PubMed: 18801366]
9. Harder J, Opitz OG, Brabender J, et al. Quantitative promoter methylation analysis of hepatocellular carcinoma, cirrhotic and normal liver. *Int J Cancer.* 2008; 122:2800–2804. [PubMed: 18351580]
10. Nishida N, Kudo M, Nagasaka T, et al. Characteristic patterns of altered DNA methylation predict emergence of human hepatocellular carcinoma. *Hepatology.* 2012; 56:994–1003. [PubMed: 22407776]
11. Ammerpohl O, Pratschke J, Schafmayer C, et al. Distinct DNA methylation patterns in cirrhotic liver and hepatocellular carcinoma. *Int J Cancer.* 2012; 130:1319–1328. [PubMed: 21500188]
12. Chiang DY, Villanueva A, Hoshida Y, et al. Focal gains of VEGFA and molecular classification of hepatocellular carcinoma. *Cancer Res.* 2008; 68:6779–6788. [PubMed: 18701503]
13. Villanueva A, Hoshida Y, Battiston C, et al. Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. *Gastroenterology.* 2011; 140:1501–1512. e2. [PubMed: 21320499]
14. Cameron EE, Bachman KE, Myohanen S, et al. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet.* 1999; 21:103–107. [PubMed: 9916800]
15. Nishida N, Nagasaka T, Nishimura T, et al. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. *Hepatology.* 2008; 47:908–918. [PubMed: 18161048]
16. Edamoto Y, Hara A, Biernat W, et al. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int J Cancer.* 2003; 106:334–341. [PubMed: 12845670]
17. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinform.* 2013; 14:128.
18. Gronbaek K, Hother C, Jones PA. Epigenetic changes in cancer. *APMIS.* 2007; 115:1039–1059. [PubMed: 18042143]
19. Zender L, Xue W, Cordon-Cardo C, et al. Generation and analysis of genetically defined liver carcinomas derived from bipotential liver progenitors. *Cold Spring Harb Symp Quant Biol.* 2005; 70:251–261. [PubMed: 16869761]
20. Zender L, Xue W, Zuber J, et al. An oncogenomics-based in vivo RNAi screen identifies tumor suppressors in liver cancer. *Cell.* 2008; 135:852–864. [PubMed: 19012953]
21. Xue W, Kitzing T, Roessler S, et al. A cluster of cooperating tumor-suppressor gene candidates in chromosomal deletions. *Proc Natl Acad Sci U S A.* 2012; 109:8212–8217. [PubMed: 22566646]
22. Hoshida Y, Toffanin S, Lachenmayer A, et al. Molecular classification and novel targets in hepatocellular carcinoma: recent advancements. *Semin Liver Dis.* 2010; 30:35–51. [PubMed: 20175032]
23. Juergens RA, Wrangle J, Vendetti FP, et al. Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov.* 2011; 1:598–607. [PubMed: 22586682]
24. Lachenmayer A, Toffanin S, Cabellos L, et al. Combination therapy for hepatocellular carcinoma: additive preclinical efficacy of the HDAC inhibitor panobinostat with sorafenib. *J Hepatol.* 2012; 56:1343–1350. [PubMed: 22322234]
25. Neumann O, Kesselmeier M, Geffers R, et al. Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors. *Hepatology.* 2012; 56:1817–1827. [PubMed: 22689435]

26. Simon JA, Kingston RE. Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol.* 2009; 10:697–708. [PubMed: 19738629]
27. Omura N, Li CP, Li A, et al. Genome-wide profiling of methylated promoters in pancreatic adenocarcinoma. *Cancer Biol Ther.* 2008; 7:1146–1156. [PubMed: 18535405]
28. Simmons DP, Peach ML, Friedman JR, et al. Evidence that sequence homologous region in LRAT-like proteins possesses anti-proliferative activity and DNA binding properties: translational implications and mechanism of action. *Carcinogenesis.* 2006; 27:693–707. [PubMed: 16234259]
29. Maekawa M, Taniguchi T, Ishikawa J, et al. Promoter hypermethylation in cancer silences LDHB, eliminating lactate dehydrogenase isoenzymes 1–4. *Clin Chem.* 2003; 49:1518–1520. [PubMed: 12928234]
30. Leiblich A, Cross SS, Catto JW, et al. Lactate dehydrogenase-B is silenced by promoter hypermethylation in human prostate cancer. *Oncogene.* 2006; 25:2953–2960. [PubMed: 16547507]
31. Kim JH, Kim EL, Lee YK, et al. Decreased lactate dehydrogenase B expression enhances claudin 1-mediated hepatoma cell invasiveness via mitochondrial defects. *Experimental cell research.* 2011; 317:1108–1118. [PubMed: 21356207]
32. Corcoran CA, He Q, Ponnusamy S, et al. Neutral sphingomyelinase-3 is a DNA damage and nongenotoxic stress-regulated gene that is deregulated in human malignancies. *Mol Cancer Res.* 2008; 6:795–807. [PubMed: 18505924]
33. Kim MS, Chang X, LeBron C, et al. Neurofilament heavy polypeptide regulates the Akt-beta-catenin pathway in human esophageal squamous cell carcinoma. *PloS One.* 2010; 5:e9003. [PubMed: 20140245]
34. Szebenyi G, Smith GM, Li P, et al. Overexpression of neurofilament H disrupts normal cell structure and function. *J Neurosci Res.* 2002; 68:185–198. [PubMed: 11948664]
35. Zhang WD, Hirohashi S, Tsuda H, et al. Frequent loss of heterozygosity on chromosomes 16 and 4 in human hepatocellular carcinoma. *Jpn J Cancer Res.* 1990; 81:108–111. [PubMed: 1970554]
36. Kim WJ, Okimoto RA, Purton LE, et al. Mutations in the neutral sphingomyelinase gene SMPD3 implicate the ceramide pathway in human leukemias. *Blood.* 2008; 111:4716–4722. [PubMed: 18299447]
37. Hannun YA. Functions of ceramide in coordinating cellular responses to stress. *Science.* 1996; 274:1855–1859. [PubMed: 8943189]
38. Oskouian B, Saba JD. Cancer treatment strategies targeting sphingolipid metabolism. *Adv Exp Med Biol.* 2010; 688:185–205. [PubMed: 20919655]

Figure 1.

Study overview. Seventy-one patients from the HCC Genomic Consortium were profiled for methylation status and 4 liver cancer cell lines analyzed for gene re-expression after epigenetic unmasking to identify candidate tumor suppressor genes (TSGs). Candidates were validated for methylation and gene expression in 12 paired HCC samples from CHTN and copy number variation (CNV) in the HCC Genomic Consortium. Functional validation was performed in vitro and in vivo for the strongest candidates and prognostic implications explored.

Figure 2.

Candidate gene methylation validation. (A) Methylation-specific pyrosequencing analysis of 6 candidate genes—SMPD3, NEFH, DGKI, LDHB, PRPH, ACTL6B—in 12 paired normal HCC samples. Normal tissue is indicated by a *black box* and *whisker*, tumor tissue is indicated by a *red box* and *whisker*. Values are displayed as the percentage of methylation at each CpG site. (B) Transcript expression analysis of 12 primary HCC samples relative to matched normal for SMPD3, NEFH, DGKI, and ACTL6B exhibiting hypermethylation in tumor vs normal by pyrosequencing analysis. Data from quantified quantitative real-time polymerase chain reaction values were normalized to matched normal tissue and log-2 transformed ($\pm$ SD, * P .05, ** P .01, *** P .001).

Figure 3.

Overexpression of SMPD3 and NEFH significantly reduces cell proliferation. JHH-7 pDEST SMPD3 (A), JHH-7 pDEST NEFH (B), and JHH-7 pDEST β -galactosidase (LACZ), and (C) human hepatocellular carcinoma cells were induced to express their respective transgenes with 5 μ g/mL of tetracycline. Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 48, 72, and 96 hours post induction. Assays were performed in triplicate. Cell viability measurements of JHH7 pDEST SMPD3 and JHH7 pDEST NEFH were normalized by those obtained for the control cells JHH7 pDEST LACZ.

Figure 4.

Depletion of SMPD3 and NEFH significantly increases cell migration and/or invasion. (A) Effect on cell migration of tumor protein p53 ($p53^{-/-}$; Myc mouse hepatoblasts (PHMI cells) with shRNA directed to either Nefh or Smpd3. Panels are, from left to right: migration of cells with nontargeting knockdown (REN shRNA), migration of cells with knockdown of Nefh, and migration of cells with knockdown of Smpd3. (B) Quantification of cell migration for each cell line. Cell number is averaged over 5 fields of view. (C) Effect on cell invasion of $p53^{-/-}$; Myc mouse hepatoblasts (PHMI cells) with shRNA directed to either Nefh or Smpd3. Panels are, from left to right: invasion of cells with non-targeting knockdown, invasion of cells with knockdown of Nefh, and invasion of cells with knockdown of Smpd3.

(D) Quantification of cell invasion for each cell line. Cell number is averaged over 5 fields of view.

Figure 5. Depletion of Smpd3 and Nefh promotes liver carcinoma formation. (A) Subcutaneous growth of tumor protein p53 (p53)^{-/-}; Myc hepatoblasts infected with either nontargeting shRNA, shRNA 4, shRNA 6, or shRNA 1 to Smpd3 (n = 6 injections, asterisks indicate that the indicated tumor group is significantly different than controls, error bars denote ±SD, *P < .05; **P < .01; ***P < .001). Tumor volumes were determined from 2 to 7 weeks post injection. (B) Subcutaneous growth of p53^{-/-}; Myc hepatoblasts infected with either nontargeting shRNA or shRNA2 or shRNA 5 to Nefh (n = 6 injections, asterisks indicate that the indicated tumor group is significantly different than controls, error bars denote ±SD, *P < .05; **P < .01; ***P < .001). Tumor volumes were determined from 5 to 8 weeks post injection. (C) Images of mouse livers and sections taken 6 weeks after transplantation of

p53^{-/-}; Myc mouse hepatoblasts with knockdown of either Smpd3 or Nefh. *Panel* columns are, from *left to right*: intact livers; fluorescent imaging of intact liver for green fluorescent protein (GFP)-positive transplanted cells; H&E staining of liver tissue sections showing the border between normal liver and carcinoma (*arrows*); immunohistochemical detection of GFP; and immunohistochemical detection of proliferating cell nuclear antigen (PCNA). The last 3 are from the same tissue block. Scale bars = 100 μ m. (D) Association of SMPD3 expression with time to early and late recurrence after surgery early recurrence in patients from HCC Genomic Consortium. SMPD3 high-expressing patients are indicated in *blue* and SMPD3 low-expressing patients are indicated in *red*. (E) Early recurrence in patients from validation cohort. SMPD3 high-expressing patients are indicated in *blue* and SMPD3 low-expressing patients are indicated in *red*.

Table 1

Clinical Data of Patients Included in Study

Variable	HCC Genomic Consortium	
	HCV HCC (n = 77, training)	Mixed etiologies (n = 164, validation)
Age older than 60 y	56 (71.8)	110 (67.1)
Male sex	52 (66.7)	113 (68.9)
Origin		
Barcelona	23 (29.9)	36 (22)
Milan	25 (32.1)	58 (35.4)
New York	29 (37.2)	70 (42.7)
Child-Pugh class A	69 (88.5)	101 (61.6)
Etiology		
Hepatitis C virus	77 (100)	37 (22.6)
Hepatitis B virus		39 (23.8)
Alcohol		12 (7.3)
Other		21 (12.8)
Tumor size, <i>cm</i>		
2	10 (12.8)	32 (19.5)
>2	67 (87.2)	119 (72.6)
Degree of differentiation ^a		
Well	19 (24.4)	25 (15.2)
Moderate	41 (52.6)	55 (33.5)
Poor	16 (20.1)	16 (10)
Satellites ^a		
Absent	25 (29.5)	110 (52.4)
Present	4 (6.4)	30 (11.0)
Vascular invasion		
Absent	33 (42.3)	96 (58.5)
Present	44 (57.1)	49 (29.9)
BCLC stage		
0	6 (7.7)	17 (10)
A	59 (75.6)	122 (74.4)
B	5 (6.4)	9 (35.5)
C	7 (9)	1 (0.6)
Albumin level <3.5 mg/dL	12 (15.4)	7 (4.3)
Bilirubin level >1 mg/dL	17 (21.8)	38 (23.2)
AFP level >100 mg/dL	20 (25.6)	23 (14.0)
Events		
Follow-up period, <i>mo</i> ^b	52.9 (24–108)	85 (60.9–117.2)
Deaths	20 (25.6)	28 (17.1)
Late recurrence	10 (12.8)	24 (14.6)
Early recurrence <2 y	28 (35.9)	39 (23.8)

NOTE. Data are expressed as median (range, quartile 25–quartile 75).

AFP, α -fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HCV, hepatitis C virus.

^aMissing data for degree of differentiation (n = 69) and satellites (n = 72) of 241 cases.

^bFollow-up period.

Table 2
Univariate and Multivariate Analysis of Clinical Variables and SMPD3 Expression for Early Recurrence

Clinical variables	Univariate analysis			Multivariate analysis		
	n	P value	HR	95% CI	n	P value
Origin	157	.76	1.06	0.73–1.53		
Male sex	151	.228	0.71	0.41–1.24		
Age older than 60 y	151	.785	1.84	0.61–1.93		
Etiology, hepatitis C	146	.143	2.93	0.69–12.31		
Size >2 cm	151	.295	1.63	0.65–4.09		
Albumin level <3.5 g/L	140	.157	1.72	0.81–3.64		
Bilirubin level >1 mg/dL	146	.28	1.35	0.78–2.35		
AFP level >100 mg/dL	142	.246	1.42	0.79–2.55		
Vascular invasion	145	.216	1.4	0.83–2.39		
Satellites	140	.0000012	3.92	2.26–6.79	138	.0000876
BCLC stage A	149	.632	1.25	0.5–3.16		3.17
BCLC stage B	149	.003	5.67	1.79–18	138	.00156
Child Pugh A	140	.122	0.21	0.03–1.53		7.103
Degree of differentiation, moderate or poor	95	.2	1.4	0.84–2.35		
SMPD3 low expression	163	.002	2.7	1.44–5.09	138	.0011
						3.222
						1.6–6.5

NOTE. Bold type indicates independent significance.

AFP, α -fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; HR, hazard ratio.