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

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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 HumanBeadchip27K 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 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-deoxycitidine.

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 post-translational 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 previously[12] (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

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, cm		
≤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, mo[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.
[a]Missing data for degree of differentiation (n = 69) and satellites (n = 72) of 241 cases.
[b]Follow-up period.

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 μM 5-aza-2′-deoxycitidine (DAC) (Sigma) for 48 hours.[14] For tetracycline-induced expression, cells were treated with 5 μg/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 HumanMethylation27 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