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RESEARCH ARTICLE

Genome-wide analysis of DNA methylation identifies novel cancer-related genes in hepatocellular carcinoma

Masahiro Shitani · Shigeru Sasaki · Noriyuki Akutsu · Hideyasu Takagi · Hiromu Suzuki · Masanori Nojima · Hiroyuki Yamamoto · Takashi Tokino · Koichi Hirata · Kohzoh Imai · Minoru Toyota · Yasuhisa Shinomura

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Abstract Aberrant DNA methylation has been implicated in the development of hepatocellular carcinoma (HCC). Our aim was to clarify its molecular mechanism and to identify useful biomarkers by screening for DNA methylation in HCC. Methylated CpG island amplification coupled with CpG island microarray (MCAM) analysis was carried out to screen for methylated genes in primary HCC specimens [hepatitis B virus (HBV)-positive, n=4; hepatitis C virus (HCV)-positive, n=5; HBV/HCV-negative, n=7]. Bisulfite pyrosequencing was used to analyze the methylation of selected genes and long interspersed nuclear element (LINE)-1 in HCC tissue (n=57) and noncancerous liver tissue (n=50) from HCC patients and in HCC cell lines (n=10). MCAM analysis identified 332, 342, and 259 genes that were methylated in HBV-positive, HCV-positive, and HBV/HCV-negative HCC tissues, respectively. Among these genes, methylation of *KLHL35*, *PAX5*, *PENK*, and *SPDYA* was significantly higher in HCC tissue than in noncancerous liver tissue, irrespective of the hepatitis virus status. LINE-1 hypomethylation was also prevalent in HCC and correlated positively with *KLHL35* and *SPDYA* methylation. Receiver operating characteristic curve analysis revealed that methylation of the four genes and LINE-1 strongly discriminated between HCC tissue and noncancerous liver tissue. Our data suggest that aberrant hyper- and hypomethylation may contribute to a common pathogenesis mechanism in HCC. Hypermethylation of *KLHL35*, *PAX*, *PENK*, and *SDPYA* and hypomethylation of LINE-1 could be useful biomarkers for the detection of HCC.

Keywords Hepatocellular carcinoma · DNA methylation · CpG island · LINE-1 · Biomarker

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M. Shitani · S. Sasaki (✉) · N. Akutsu · H. Takagi · H. Suzuki · H. Yamamoto · Y. Shinomura (✉)
First Department of Internal Medicine,
Sapporo Medical University,
S1, W16, Chuo-Ku,
Sapporo 060-8543, Japan
e-mail: ssasaki@sapmed.ac.jp
e-mail: shinomura@sapmed.ac.jp

H. Suzuki · M. Toyota
Department of Molecular Biology, Sapporo Medical University, Sapporo, Japan

M. Nojima
Department of Public Health, Sapporo Medical University, Sapporo, Japan

T. Tokino
Medical Genome Science, Research Institute for Frontier Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

K. Hirata
First Department of Surgery, Sapporo Medical University, Sapporo, Japan

K. Imai
Division of Novel Therapy for Cancer, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Introduction

Hepatocellular carcinoma (HCC) is one of the most common human malignancies, worldwide [ ]. Chronic infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) are well-documented risk factors for the development of HCC, while chronic alcoholism and various environmental factors, including aflatoxin B1, are also believed to be important risk

factors [2, 3]. The development and progression of HCC is often a complex, multistep process entailing the evolution of normal liver through chronic hepatitis and cirrhosis to HCC, but HCC can also arise in a noncirrhotic liver. In either case, the process is influenced by multiple genetic changes, including allelic deletions, chromosomal losses and gains, DNA rearrangements, and gene mutations [4]. In addition, a growing body of evidence suggests that epigenetic changes such as DNA methylation and histone modification also play crucial roles in hepatocarcinogenesis.

Two seemingly contradictory epigenetic events coexist in cancer: global hypomethylation, which is mainly observed in repetitive sequences throughout the genome, and regional hypermethylation, which is frequently associated with CpG islands within gene promoters [5]. Hypermethylation of CpG islands is a common feature of cancer and is associated with gene silencing. Although the classical two-hit theory posits that tumor suppressor genes are inactivated by gene mutation or deletion, it is now recognized that DNA hypermethylation is a third mechanism by which inactivation of tumor suppressor genes occurs, and that it plays a significant role in tumorigenesis. In contrast to the CpG islands, repetitive DNA elements are normally heavily methylated in somatic tissues. About 45 % of the human genome is composed of repetitive sequences, including long interspersed nuclear elements (LINEs) and short interspersed nuclear element [6], and studies have shown that methylation of such repetitive elements can serve as a surrogate for the global methylcytosine content [7]. In that regard, LINE-1 hypomethylation is known to occur during the development of various human malignancies, including HCC [8, 9].

HCC is generally diagnosed at an advanced stage of tumor progression, and a large fraction of HCC cases are fatal. Thus, a better understanding of the underlying molecular mechanisms and identification of genes critical for early detection of HCC and therapeutic intervention would be highly desirable. Although a number of hyper- or hypomethylated loci have been identified in HCC [10–12], only a few studies have been conducted to unravel the genome-wide methylation status [13–15]. In the present study, we carried out genome-wide CpG island methylation analysis in a set of primary HCC specimens, with and without hepatitis virus infection. We also evaluated the hypomethylation of LINE-1 and assessed its association with aberrant CpG island hypermethylation in HCC.

Materials and methods

Tissue samples and cell lines

A total of 57 primary HCC specimens (HBV-positive, n=21; HCV-positive, n=21; HBV/HCV-negative, n=15) were obtained through surgical resection or needle biopsy at Sapporo Medical University Hospital. Corresponding samples of noncancerous liver tissue were also obtained from 50 patients. HBV surface (HBs) antigen and anti-HCV antibody were measured serologically. An informed consent was obtained from all patients before collection of the specimens. The ten liver cancer cell lines (HT17, PLC/PRF/5, Li-7, huH-1, HuH-7, HepG2, Hep3B, HLE, HLF, and JHH-4) used have been described previously [11]. To analyze restoration of gene expression, cells were treated with 2.0 μM 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma, St Louis, MO, USA) for 72 h, replacing the drug and medium every 24 h. Genomic DNA was extracted using the standard phenol–chloroform procedure. Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and then treated with a DNA-free kit (Ambion, Austin, TX, USA). Genomic DNA and total RNA from normal liver tissue from a healthy individual were purchased from BioChain (Hayward, CA, USA).

Methylated CpG island amplification coupled with CpG island microarray

Methylated CpG island amplification (MCA) was performed as described previously [13]. Briefly, 500 ng of genomic DNA was digested with the methylation-sensitive restriction endonuclease *Sma*I (New England Biolabs, Ipswich, MA, USA), after which it was digested with the methylation-insensitive restriction endonuclease *Xma*I. The adaptors were prepared by addition of the oligonucleotides RMCA12 (5′-CCGGGCAGAAAG-3′) and RMCA24 (5′-CCACCGCCATCCGAGCCTTTCTGC-3′). After ligation of the digested DNA to the adaptors, PCR amplification was carried out. Using a BioPrime Plus Array CGH Genomic Labeling System (Invitrogen), MCA amplicons from the HCC samples were labeled with Alexa Fluor 647, while amplicons from a normal liver sample was labeled with Alexa Fluor 555. The labeled MCA amplicons were then hybridized to a custom human CpG island microarray containing 15,134 probes covering 6,157 unique genes (G4497A; Agilent Technologies, Santa Clara, CA, USA) [16]. After washing, the array was scanned using an Agilent DNA Microarray Scanner (Agilent technologies), and the data were processed using Feature Extraction software ver. 10.7 (Agilent Technologies). The data were then analyzed using GeneSpring GX ver. 11 (Agilent Technologies).

Methylation-specific PCR

Genomic DNA (1 μg) was modified with sodium bisulfite using an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), and methylation-specific PCR (MSP) was performed as described previously [17]. Briefly, PCR was run in a 25-μl

volume containing 50 ng of bisulfite-treated DNA, 1× MSP buffer [67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH4)_2SO_4$, 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol], 1.25 mM dNTP, 0.4 μM each primer, and 0.5 U of JumpStart REDTaq DNA Polymerase (Sigma). The PCR protocol for MSP entailed 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; and a 7 min final extension at 72°C. Primer sequences and PCR product sizes are shown in Supplementary Table 1.

Bisulfite pyrosequencing analysis

Bisulfite pyrosequencing analysis was performed as described previously [17]. The PCR protocol entailed 5 min at 95°C; 45 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; and a 7-min final extension at 72°C. PCR products were then bound to Streptavidin Sepharose beads HP (Amersham Biosciences, Piscataway, NJ); after which, the beads containing the immobilized PCR product were purified, washed, and denatured using a 0.2 M NaOH solution. After addition of 0.3 μM sequencing primer to the purified PCR product, pyrosequencing was carried out using a PSQ96MA system (Qiagen, Hilden, Germany) and Pyro Q-CpG software (Qiagen). Primer sequences and PCR product sizes are shown in Supplementary Table 1.

Quantitative RT-PCR

Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was carried out using TaqMan Gene Expression Assays (*KLHL35*, Hs00400533_m1; *PAX5*, Hs00172003_m1; *PENK*, Hs00175049_m1; *SPDYA*, Hs00736925_m1; *GAPDH*, Hs99999905_m1; Applied Biosystems, Foster City, CA, USA) and a 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. SDS1.4 software (Applied Biosystems) was used for comparative delta Ct analysis, and *GAPDH* served as an endogenous control.

Statistical analysis

To compare differences in continuous variables between groups, *t* tests or ANOVA with post hoc Tukey's tests were performed. Fisher's exact test or chi-squared test was used for analysis of categorical data. Receiver operator characteristic (ROC) curves were constructed based on the levels of methylation. Values of $P<0.05$ (two-sided) were considered statistically significant. Statistical analyses were carried out using SPSS statistics 18 (IBM Corporation, Somers, NY, USA) and GraphPad Prism ver. 5.0.2 (GraphPad Software, La Jolla, CA, USA).

Results

Genome-wide CpG island methylation analysis in HCC

To screen for CpG island hypermethylation in HCC, we carried out methylated CpG island amplification coupled with CpG island microarray (MCAM) analysis using a set of HCC tissue specimens (HBV-positive, $n=4$; HCV-positive, $n=5$; HBV/HCV-negative, $n=7$). As in an earlier study in which the same array system was used, we utilized a signal ratio (Cy5/Cy3) of >2.0 as the criterion for a methylation-positive probe [13]. The average number of methylated probe sets in the HCC specimens was 566 (range 159–846). To assess the association between hepatitis virus infection and methylation status, we categorized the HCC specimens according to their viral status. The average numbers of methylated probe sets in HBV-positive, HCV-positive, and the HBV/HCV-negative HCC specimens were 574, 598, and 539, respectively, which did not significantly differ ($P=0.840$). Interestingly, however, the numbers of methylated probe sets were more varied among HBV/HCV-negative HCCs, which is indicative of their varied pathological backgrounds (Fig. 1a).

To identify commonly methylated genes in HCC, we selected genes that were methylated in at least two tumors in each group. Among the HBV-positive HCCs, 443 probe sets (corresponding to 332 unique genes) satisfied this criterion. Among the HCV-positive HCCs, 476 probe sets (342 unique genes) satisfied the criterion, and among the HBV/HCV-negative HCCs, 348 probe sets (259 unique genes) satisfied the criterion. Collectively, 714 probes (514 unique genes) were selected as commonly methylated genes. Of those, 137, 146, and 47 probe sets were methylated in only HBV-positive, HCV-positive, or HBV/HCV-negative HCC tissues, respectively (Fig. 1b). By contrast, a large number of genes were methylated in multiple categories, and 169 probe sets were methylated in all three groups (Fig. 1b). Consistent with the above results, unsupervised hierarchical clustering analysis demonstrated that some genes were methylated irrespective of the hepatitis virus status, and that HCV-positive HCCs exhibited the largest number of methylated genes (Fig. 1c, Supplementary Fig. 1). Gene ontology analysis of the commonly methylated genes revealed that genes related to "multicellular organismal process," "developmental process," and "system development" are significantly enriched among the methylated genes (Supplementary Table 2). In addition, pathway analysis suggested that some of the methylated genes are involved in differentiation and development (Supplementary Fig. 2).

Identification of novel genes methylated in HCC

Our MCAM analysis suggested that some genes were methylated in a hepatitis virus-specific manner, but a larger

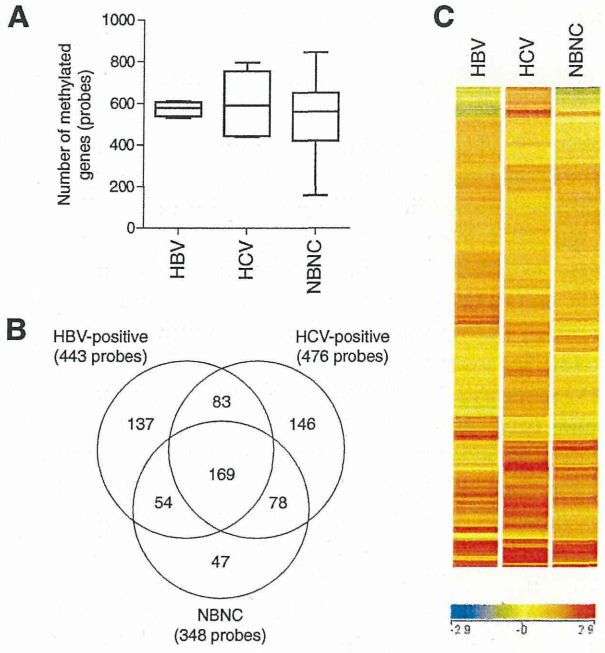


Fig. 1 Genome-wide analysis of CpG island methylation. **a** MCAM analysis was carried out using a series of HCC tissue specimens (HBV-positive, n=4; HCV-positive, n=5; HBV/HCV-negative, NBNC, n=7). MCAM data were categorized into three groups based on the hepatitis virus status, and the numbers of methylated genes in the respective categories are shown. **b** Venn diagram analysis of the methylated genes in the indicated categories. **c** Gene tree view of the MCAM analysis results. A set of 714 probes (514 unique genes) were selected as commonly methylated genes, after which, hierarchical clustering was performed. Each row represents a single probe

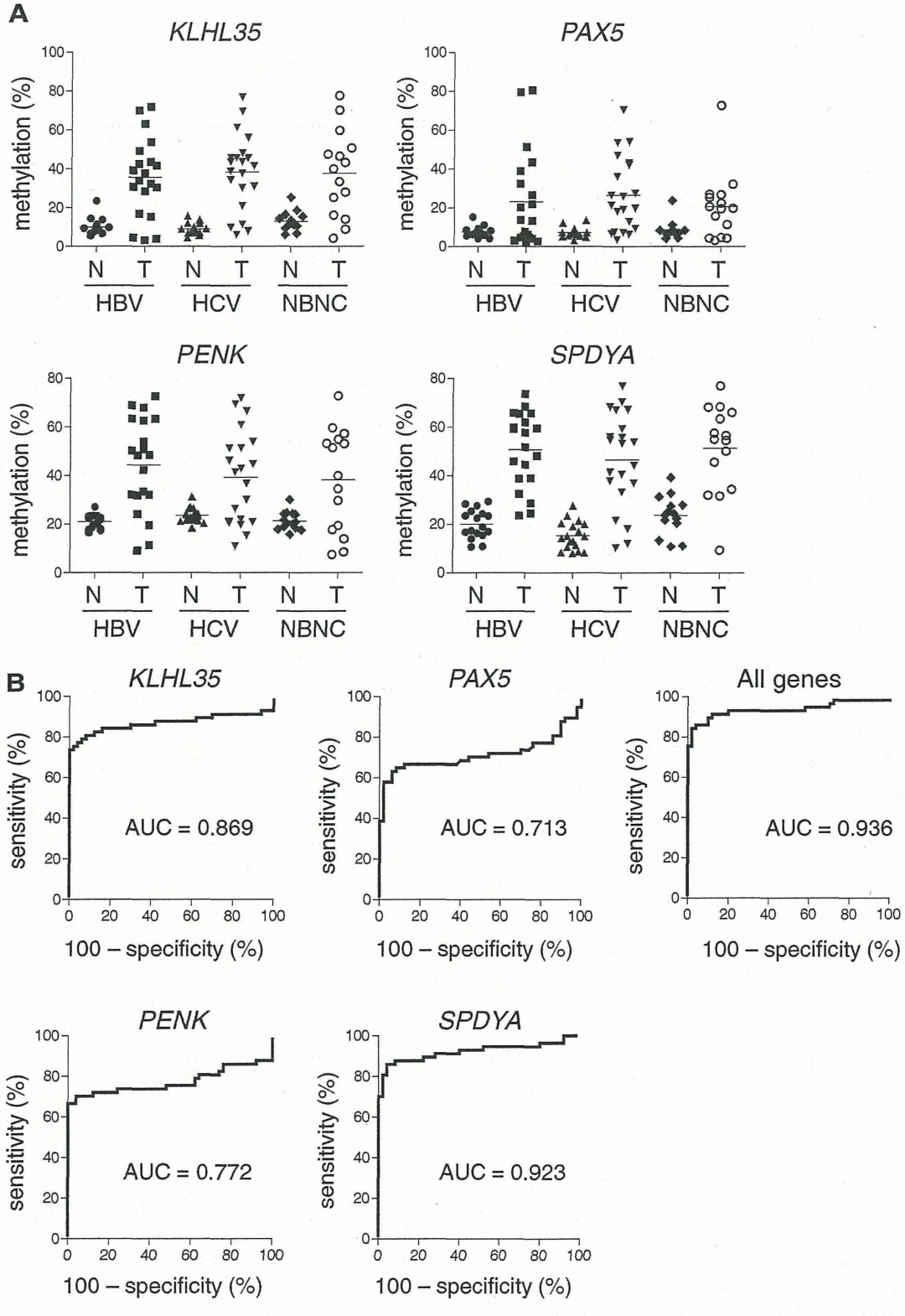
number were commonly methylated in HCC. Because recent studies have suggested that aberrant DNA methylation could be a useful diagnostic marker for HCC, we next aimed to identify novel genes frequently methylated in HCC. Among the genes commonly methylated irrespective of hepatitis virus status, we selected 14 (*KLHL35*, *PAX5*, *PENK*, *SPDYA*, *LTBP2*, *DLX1*, *PGBD1*, *WNT9A*, *ADRA1A*, *RHOBTB1*, *GDNF*, *WNT11*, *MLL*, and *PLEC1*) and carried out MSP to assess their methylation status in a series of HCC cell lines (Supplementary Fig. 3). We found that four (*KLHL35*, *PAX5*, *PENK*, and *SPDYA*) of the genes were frequently methylated in HCC cell lines, but showed only little or no methylation in normal liver tissue from a healthy individual (Supplementary Fig. 3). We therefore used quantitative bisulfite pyrosequencing to further analyze the methylation levels of these four genes (Supplementary Figs. 4 and 5).

To determine the extent to which these genes are aberrantly methylated in primary tumors, we analyzed a set of primary HCC specimens (HBV-positive, n=21; HCV-positive, n=21; HBV/HCV-negative, n=15) and corresponding noncancerous liver tissues from the same patients (HBV-positive, n=18; HCV-positive, n=18; HBV/HCV-negative, n=14). Bisulfite pyrosequencing analysis revealed the methylation levels of the four genes to be significantly higher in tumor tissues than in their noncancerous counterparts (*KLHL35*, 37.9 vs. 10.4 %, P<0.001; *PAX5*, 23.4 vs. 7.7 %, P<0.001; *PENK* 41.1 vs. 22.0 %, P<0.001; *SPDYA*, 49.7 vs. 19.3 %, P<0.001) (Supplementary Fig. 6). Moreover, these genes were frequently methylated in HCCs, irrespective of the hepatitis virus infection (*KLHL35*, HBV-positive, 37.5 vs. 9.9 %, P<0.001; HCV-positive, 38.3 vs. 9.0 %, P<0.001; HBV/HCV-negative, 37.7 vs. 13.0 %, P<0.001; *PAX5*, HBV-positive, 22.2 vs. 7.6 %, P=0.014; HCV-positive, 26.5 vs. 7.2 %, P<0.001; HBV/HCV-negative, 20.7 vs. 8.5 %, P=0.017; *PENK*, HBV-positive, 45.1 vs. 20.9 %, P<0.001; HCV-positive, 39.2 vs. 23.5 %, P=0.001; HBV/HCV-negative, 38.2 vs. 21.3 %, P=0.006; *SPDYA*, HBV-positive, 51.5 vs. 19.8 %, P<0.001; HCV-positive, 46.6 vs. 15.3 %, P<0.001; HBV/HCV-negative, 51.3 vs. 23.7 %, P<0.001) (Fig. 2a). The association between the methylation of each gene and the clinicopathological features are shown in Table 1. Methylation of *KLHL35* and *PAX5* was correlated with greater age, and *SPDYA* methylation was moderately correlated with higher PIVKA-II levels, but we found no other significant correlations (Table 1). We also generated an ROC curve and observed that methylation of the four genes discriminated strongly between tumor tissues and noncancerous liver tissue, suggesting that methylation of these genes could be a useful tumor marker (Fig. 2b). The most discriminating cutoffs for *KLHL35*, *PAX5*, *PENK*, and *SPDYA* were 14.8 % (sensitivity, 82.5 %; specificity, 88.0 %), 12.5 % (sensitivity, 63.2 %; specificity, 94.0 %), 28.4 % (sensitivity, 70.2 %; specificity, 96.0 %), and 30.3 % (sensitivity, 86.0 %; specificity, 94.0 %), respectively.

Analysis of *KLHL35*, *PAX5*, *PENK*, and *SPYDA* methylation and expression

We next tested whether methylation of *KLHL35*, *PAX5*, *PENK*, and *SPYDA* was associated with their silencing in HCC. Bisulfite pyrosequencing analysis revealed that the degree to which these genes were methylated varied among the HCC cell lines, but it was always much higher than in normal liver tissue from a healthy individual (Fig. 3a). Quantitative RT-PCR analysis confirmed an inverse relationship between methylation and expression of *KLHL35*

Fig. 2 Quantitative methylation analysis of the genes identified by MCAM. **a** Summary of the bisulfite pyrosequencing analysis of *KLHL35*, *PAX5*, *PENK*, and *SPDYA* in tumor tissue (*T*) and noncancerous liver tissue (*N*) from HBV-positive, HCV-positive, and HBV/HCB-negative (NBNC) HCC patients. **b** ROC curve analysis of the methylation of the indicated genes. The area under the ROC curve (*AUC*) for each site conveys its utility (in terms of sensitivity and specificity) for distinguishing between HCC tissue and corresponding noncancerous liver tissue from the same HCC patients



and *PAX5* in the cell lines and normal liver tissue (Fig. 3b), whereas methylation of *PENK* and *SPDYA* did not correlate significantly with their expression levels. The expression of *PENK* was undetectable in seven HCC cell lines and in

Table 1 Association between clinicopathological features and DNA methylation in HCC

	N	*KLHL35* methylation			*PAX5* methylation			*PENK* methylation			*SPDYA* methylation			LINE-1 methylation		
		Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value	Mean	SD	*P* value
Age																
≤63	24	30.3	17.7	0.003	18.3	17.1	0.026	41.7	19.3	0.583	51.2	17.9	0.945	49.4	14.8	0.571
>64	23	47.2	18.5		32.3	24.1		44.8	19.6		50.9	15.0		47.2	10.6	
Sex																
M	39	37.5	20.3		22.6	20.9		40.2	19.6		50.1	18.1		47.8	13.3	
F	18	37.2	22.0	0.953	25.2	19.9	0.652	43.1	19.6	0.602	48.7	16.7	0.771	51.5	12.0	0.318
Virus																
HBV	21	35.6	20.3		23.1	24.5		44.3	19.4		50.7	15.4		50.4	13.9	
HCV	21	38.3	19.5		26.5	19.0		39.2	18.8		46.6	19.6		50.2	12.2	
NBNC	15	36.1	22.2	0.900	20.7	17.2	0.698	38.2	21.0	0.603	51.3	18.0	0.668	44.7	12.9	0.359
Child-Pugh																
A	44	39.2	20.0		25.5	22.3		43.4	19.6		51.4	15.6		48.6	12.4	
B	3	29.7	18.2	0.426	19.9	11.8	0.672	41.0	17.1	0.842	45.3	29.5	0.536	44.6	20.6	0.609
PIVKA-II (mAU/ml)																
≤21	16	40.0	19.5		24.0	25.7		42.1	19.1		53.5	14.1		48.0	11.5	
22–66	16	35.8	11.8		23.4	14.2		44.6	14.0		42.9	15.9		52.9	10.7	
>67	15	40.1	26.9	0.795	28.3	24.8	0.802	42.8	24.9	0.933	57.1	16.6	0.039	43.8	15.1	0.136
AFP (ng/ml)																
≤7.4	16	39.3	19.3		25.8	24.1		41.4	19.0		49.4	17.0		47.4	9.2	
7.5–55.0	16	44.9	20.2		31.1	23.2		51.5	15.6		55.0	16.7		50.6	13.4	
>55.1	15	31.1	18.7	0.150	18.1	16.3	0.256	36.3	21.0	0.078	48.7	15.8	0.509	46.9	15.7	0.695
Cirrhosis																
0	27	35.3	23.4		22.2	22.2		40.0	22.1		51.8	18.2		47.7	14.7	
1	24	40.7	16.5	0.353	25.7	20.3	0.559	44.2	17.8	0.467	50.5	15.8	0.795	49.2	11.3	0.687
Vascular invasion																
0	42	38.3	18.5		24.0	21.0		43.9	19.5		52.3	15.1		48.2	12.8	
1	9	35.7	28.9	0.353	23.1	23.3	0.559	33.1	21.5	0.467	46.1	24.2	0.795	49.3	15.1	0.687
TNM stage																
1	6	29.5	15.4		15.0	9.8		50.6	12.1		43.3	20.8		58.4	11.8	
2	20	37.7	20.4		24.6	20.3		44.7	19.3		53.7	11.8		47.5	13.2	
3	13	45.4	14.3		24.4	23.4		43.0	19.3		55.5	13.8		44.8	10.6	
4	6	32.4	28.6	0.335	30.9	28.4	0.639	29.4	23.3	0.262	41.6	25.5	0.181	47.5	16.1	0.200
Multiple cancer																
0	33	38.3	22.1		26.2	23.9		43.9	20.8		51.1	18.5		48.8	14.2	
1	13	38.6	14.3	0.964	22.4	16.9	0.609	41.7	16.4	0.732	50.5	10.7	0.911	48.3	8.5	0.916

NBNC HBV/HCV-negative

normal liver tissue, irrespective of the methylation status (Fig. 3b). Conversely, although *SPDYA* was highly methylated in a majority of HCC cell lines, its expression was detectable in all cells, and most of the HCC lines exhibited greater *SPDYA* expression than did normal liver tissue (Fig. 3b). The above results suggest that *KLHL35* and *PAX5* are epigenetically silenced in HCC cells. Consistent with that idea, treating methylated cell lines with a DNA methyltransferase inhibitor, 5-aza-dC, restored the expression of *KLHL35* and *PAX5* (Fig. 3c). On the other hand, the expression of *PENK* and *SPDYA* does not appear to be affected by methylation.

Analysis of LINE-1 methylation and its association with gene hypermethylation

It was previously reported that LINE-1 is frequently hypomethylated in HCC, though most of those studies focused on HBV-positive tumors. Similarly, by using the bisulfite