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Short Communication

Hypomethylation of Chromosome 1 Heterochromatin DNA Correlates with q-Arm Copy Gain in Human Hepatocellular Carcinoma

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Using comparative genomic hybridization (CGH) analysis, we, and others, have shown that there is a high and consistent incidence of chromosome 1q copy gain in human hepatocellular carcinoma (HCC). Chromosome 1 rearrangements, that involved pericentromeric breakpoints, have also been frequently reported in karyotypic studies of HCC. Satellite DNA hypomethylation has been postulated as the mechanism underlying the induction of chromosome 1 pericentromeric instability in many human cancers and in individuals with the rare recessive disorder ICF (immunodeficiency, centromeric heterochromatin instability, facial anomalies). In this study, we have investigated the role of DNA hypomethylation in 1q copy gain in HCC by examining the methylation status of chromosome 1 heterochromatin DNA (band 1q12). Thirty-six histologically confirmed samples of HCC were studied (24 paired tumor and adjacent nontumorous liver tissues, and 12 tumor only). Hypomethylation of satellite 2 (Sat2) DNA in 1q12 was analyzed by Southern blotting using methyl-sensitive enzyme digestion. In parallel, all cases were analyzed by CGH. A strong correlation between hypomethylated Sat2 sequences and 1q copy gain with a 1q12 breakpoint was found ($P < 0.001$). We postulate that such hypomethylation alters the interaction between the CpG-rich satellite DNA and chromatin proteins, resulting in heterochromatin decondensation, breakage and aberrant 1q formation. Spectral karyotyping further supported the presence of fragile 1q12 in HCC. Of particular interest was the finding of Sat2 DNA hypomethylation in 5 of 24 adjacent nontumorous liver tissues examined. These tissues showed no evidence of malignancy on histological examination nor did they display any CGH abnormalities. Our findings suggest a role for Sat2 demethylation in the early stages of the stepwise progression of liver carcinogenesis. *(Am J Pathol 2001, 159:465–471)*

Hepatocellular carcinoma (HCC) is a highly malignant tumor that is prevalent in China, Southeast Asia, and sub-Saharan Africa. Associated genetic aberrations include common loss of heterozygosity on chromosomes 4q, 8p, 13q, and 16q,[1–3] and frequent gains of 8q, 17q, and 20q.[4–7] By comparative genomic hybridization (CGH),[8] we and others have identified a high incidence of 1q copy number gain in HCC (60 to 80%).[4–7] Karyotypic studies on HCC, on the other hand, indicated consistent structural abnormalities of chromosome 1.[9–14] In our recent spectral karyotyping (SKY) analysis on short-term cultured HCCs, unbalanced translocations of 1q that involved fusion at the centromeric heterochromatin region were found to be the most frequent karyotypic abnormality.[15] Current cytogenetic evidence therefore confers considerable importance of numerical 1q imbalance in liver carcinogenesis.

In cultures of normal human cell, DNA methylation inhibitors have been reported to induce peri-centromeric rearrangements of chromosome 1 at a very high frequency.[16,17] This may suggest that DNA demethylation activity is selective and preferentially targets the centromeric region of chromosome 1. Furthermore, classical satellite 2 (Sat2) hypomethylation has been detected in individuals with the rare recessive ICF syndrome (immunodeficiency, centromeric heterochromatin instability, facial anomalies), in which the chromosome 1 peri-centromeric rearrangements in mitogen-stimulated lymphocytes are

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Table 1. Heterochromatin Hypomethylation and CGH Finding on HCC and Adjacent Nontumorous Liver Tissues

Case no.	Age/sex	Viral status	Cirrhosis	Tumor diameter (cm)	TNM stage	CGH analysis		Sat2 hypomethylation	
						Tumor	Adjacent tissue	Tumor	Adjacent tissue[†]
H1	69/M	HBV	−	11.0	$T_2N_0M_0$	**+1q,** +2, −4, +5q31-qter, +6pter-p22, −9, +10p, +14q, −15q, +16, −17pter-p13, +17q11-q21, +17q22-qter, +20, −21q	Balanced	++	+
H2	37/M	HBV	−	6.9	$T_2N_0M_0$	**+1q,** +2q21-q24, +3p, +4p, −4q, +5, −6q22-q26, +7p22-p11.2, +7q21-qter, −8pter-p12, +8p11.2-qter, −9pter-p21, +9p13-p12, +10q, −11q14-qter, −12pter-p12, +12p11.2-q22, −13q, −14q, −17p, +18p, −18q21-qter, +Y, +Xq13-qter	Balanced	++	+
H3	59/M	HBV	+	4.0	$T_3N_0M_0$	**−1p36, +1q,** +3pter-p13, −4q13-qter, +6pter-p22, +8q, −12q24-qter, −16q13, −16q24	Balanced	+	+
H4	58/M	HBV	−	4.5	$T_2N_0M_0$	**+1q12-q32,** +3q12-q23, −4q, −5q, +8q, +10pter-q21, −11q21-qter, −13q12-q21, +13q32, +14q11.2, +17p12-q21, +18q11.2, +19p13-q13.2, +20q12-q13.2	Balanced	+	+
H5	58/F	HBV	−	7.0	$T_2N_0M_0$	**−1p35-p21, +1q,** −3p, −4q12-q21, −6p12-qter, −9q13-q34, +11q12-q13, −13q, −14q22-qter, +15q, +20, +21q, −Y, +X	Balanced	+	+
H6	30/F	HBV	+	8.0	$T_2N_0M_0$	**+1q,** −2q32-qter, −4q28-qter, −7q32-qter, −8p, +8q, +9q32-qter, +10p, −10q, −11q, −12p, −13q12-q31, −14q, −16q, +16pter-p11.2, −Xpter-q22	Balanced	++	−
H7	74/F	HCV	+	3.0	$T_2N_0M_0$	**+1q,** +11q12-q14	Balanced	++	−
H8	69/F	HBV	+	8.0	$T_2N_0M_0$	**−1pter-p35, +1q,** +2pter-p21, −4q, −5q, +7pter-q33, +7q21-q31, −9pter-p13, −12p, +13q22-qter, −16q, −Xpter-p21	Balanced	++	−
H9	51/M	HBV	+	7.0	$T_2N_0M_0$	+7p, −8p22-p12, +8p11-qter, +11pter-p14, −14q, −15q, −17pter-q11, +17q12-qter, −Y	Balanced	++	−
H10	61/M	HCV	+	2.5	$T_2N_0M_0$	**+1q,** −2q11-q32, −4q13-qter, +5q31-qter, +6p, −6q12-q22, −8p, +10pter-p12, −16q, +17q22-qter, +20q11.2-q13.2, +Xq21-q27	Balanced	++	−
H11	51/M	HCV	+	4.4	$T_2N_0M_0$	**+1q12-q42,** +8q, +Xq	Balanced	++	−
H12	55/M	HBV	+	5.8	$T_3N_0M_0$	**+1q,** +3q22-qter, −4q, −5q, +6p, −8p, −14q22-qter	Balanced	+	−
H13	55/M	HBV	+	7.0	$T_2N_0M_0$	**+1q,** −4, +5pter-q33, +8q, +10pter-q21, −13q21-qter, −14q21-qter, −16q +18q21-qter, +20p, +20q, +X	Balanced	+	−
H14	51/M	N/A	+	3.5	$T_2N_0M_0$	**+1q,** +5q15-qter, +7, −8pter-p12, +8q21-qter, −10q23-qter, −16q, −21q, −22q, +X	Balanced	+	−
H15	61/F	HBV	+	11.0	$T_2N_0M_0$	**+1q,** +8q13-qter	Balanced	+	−
H16	69/M	HBV	+	2.0	$T_1N_0M_0$	**+1q,** +5q14-qter, −8p21-q13, +8q14-qter, −17p, −22q	Balanced	+	−
H17	48/M	HBV	−	7.0	$T_2N_0M_0$	**+1q12-q25,** +2pter-p12, −4q21-q28, −10q25-qter, −13q14-q21, +X	Balanced	+	−
H18	47/M	HBV	+	3.9	$T_2N_0M_0$	**+1q,** −6q24-qter, +12q15-q24.1, −13q, −15q13-qter, −17p, +18q12-q21, −19p, −22q, +X	Balanced	+	−

Thirty-six tumor samples and 24 paired adjacent nontumorous liver tissues were studied by CGH and analyzed for Sat2 hypomethylation. ++, Indicates extensive hypomethylation of the Sat2 DNA in the heterochromatic region of chromosome 1; +, indicates moderate Sat2 hypomethylation; and −, indicates absence of Sat2 hypomethylation.

[†]Moderate hypomethylation with ratio values ranging from 0.7 to 0.9 was detected in five adjacent nontumorous livers (H1 to H5).

Genetic changes detected by CGH are listed as gains and losses. Chromosome 1 imbalances are highlighted in bold and underlined.

HBV, hepatitis B surface antigen positive; HCV, anti-hepatitis C positive; N/A, not determined.

Table 1. Continued

Case no.	Age/sex	Viral status	Cirrhosis	Tumor diameter (cm)	TNM stage	CGH analysis		Sat2 hypomethylation	
						Tumor	Adjacent tissue	Tumor	Adjacent tissue[†]
H19	51/M	HBV	+	8.5	$T_2N_0M_0$	−4q12-q32, −5q11-q14, +6q25-qter, +8q22-qter, −10p11-qter, −11q14-qter, +12q12-q21, −12q23-qter, −13q, −16q, −17p, +19q, −21q, +Xq22-qter	Balanced	−	−
H20	34/F	HBV	+	3.2	$T_2N_0M_0$	+1q25-qter, +5, −6q12-q24, +7q21-q31, −8pter-q21.3, −9pter-p22, +12q24.1, +15q23-qter, −17p11.2-q24, −18q	Balanced	−	−
H21	52/M	HBV	+	4.9	$T_4N_0M_0$	+7, −8p, +8q	Balanced	−	−
H22	44/F	HBV	−	2.7	$T_2N_0M_0$	−8p, +8q, −9pter-p22, −13q, −16q, −17p13, −18, +Xq	Balanced	−	−
H23	54/M	HBV	+	2.7	$T_2N_0M_0$	−1pter-p21, +1p21-qter, +4p, −4q, +6pter-q14, −6q15-qter, −8pter-p21, +8p12-qter, −16	Balanced	−	−
H24	60/M	HBV	+	2.4	$T_3N_0M_0$	+1p13-qter, −4, +7q21-qter, −8p, +8q, −12q21-qter, −13q21-qter, −16q, −17p13, −19q, −20q13, −22q	Balanced	−	−
H25	67/F	HBV	+	3.0	$T_2N_0M_0$	+1q, +2q32-qter, +6pter-p21, +7, +17q, +Xq	N/A	++	N/A
H26	60/M	HBV	−	11.0	$T_2N_0M_0$	+1q, +5q13-qter, −8p, +8q, +10p, −13q12-q22, +13q22-q34, −16q, +X	N/A	++	N/A
H27	33/M	HBV	+	2.0	$T_1N_0M_0$	−4pter-p14, −4q, +5, +6pter-p21.1, +8pter-q23, −11pter-p12, +12q13-q22, −13q12-q22, +14q13-q22, −16q, −17p, +18p, −18q, −19p, −20pter-p11.2, −22q	N/A	++	N/A
H28	69/M	HBV	+	6.5	$T_2N_0M_0$	+1q, −4, +6p, −8p21-p22, −9, +16p, −16q, +17pter-q11.2, +18p11.2-qter, +19q13.1, −19q13.2-qter, −Y, +Xq22-qter	N/A	++	N/A
H29	40/M	HBV	+	5.5	$T_4N_0M_0$	−8pter-p21, −8p12-qter, +10p, +12q, +17q, +Xpter-p21	N/A	++	N/A
H30	74/F	HBV	+	3.6	$T_2N_0M_0$	+1q, +5p, +8q	N/A	+	N/A
H31	60/M	HBV	+	2.6	$T_2N_0M_0$	−1p, +2q21-qter, −6q13-qter, +8q	N/A	+	N/A
H32	39/M	HBV	+	8.5	$T_2N_0M_0$	+1p13-qter, +2q14.1-q33, +5p, +8q11.2-q22, +11q14-qter, −16p	N/A	−	N/A
H33	68/M	HBV	+	4.8	$T_2N_0M_0$	−4p21-qter, +6q24-qter, −11q22-qter, +17q23-qter	N/A	−	N/A
H34	65/M	HBV	+	1.4	$T_1N_0M_0$	−1p22-pter, +3, +4p, −4q24-qter, +5p, −5q, −8p, +11, +12, −13q12-q21, +13q22-qter, −14q21-qter, −15q22-qter, −16, −17p, −18q, +19, +20, −21q, +22q	N/A	−	N/A
H35	57/M	HBV	+	4.0	$T_3N_0M_0$	+1p21-qter, +8q21.2-qter, +13q22-qter	N/A	−	N/A
H36	71/M	HBV	+	6.5	$T_2N_0M_0$	+1q, +6p, −14q13-qter	N/A	−	N/A

characteristic. The Sat2 DNA is the major sequence of the unusually long heterochromatic region adjacent to the centromere of chromosome 1.

Several other human cancers also exhibit frequent chromosome 1 rearrangements that fuses in the vicinity of the heterochromatic region (1q12). The occurrence of chromosome 1q aberration in many different cancers suggests the likelihood of a common underlying mechanism. Hypomethylation of the constitutive heterochromatic region on chromosome 1 has been postulated as the cause for such chromosome 1 instability. Although the molecular mechanism that causes satellite DNA demethylation is still unclear, studies in breast cancer and Wilms' tumor supported the hypothesis that common heterochromatin breakage on 1q12 is attributable to satellite hypomethylation and that this is likely to be the precursor to subsequent whole chromosome 1q translocations.

In this study, we investigated the role of heterochromatin DNA hypomethylation in the formation of aberrant 1q in HCC and its possible involvement in the stepwise progression of HCC development.

Materials and Methods

Patients

Tumorous liver tissues from 36 HCC patients (age 30 to 74 years; 75% male), who underwent surgical resection with curative intent and 24 paired adjacent nontumorous liver tissues were collected. Thirty-five patents were

chronic carriers of viral hepatitis (97%), 32 cases of type B (HBV) and 3 cases of type C (HCV), with 81% arising from a cirrhotic liver. The disease stage of each case was classified according to the TNM staging criteria.[18] Of the 36 patients recruited, 3 cases (8%) were classified as stage I ($T_1N_0M_0$), 27 (75%) as stage II ($T_2N_0M_0$), 4 (11%) as stage III ($T_3N_0M_0$), and 2 (6%) as stage IV ($T_4N_0M_0$). An experienced liver pathologist confirmed the diagnosis of HCC and the nonmalignant status of adjacent liver tissues. The macroscopic and microscopic features of resected specimens were also reviewed for the presence or absence of underlying liver cirrhosis and the maximum diameter of each tumor was recorded.

Heterochromatin Hypomethylation by Southern Blot Analysis

Satellite DNA hypomethylation was examined by the Southern blot analysis described by Narayan and colleagues.[19] Two μg of DNA were digested in 20 U of CpG methyl-sensitive restriction enzyme *Bst*BI (New England Biolabs, Beverly, MA) for 16 hours. Complete DNA digestion was indicated with the aid of an internal DNA control, λ*Hin*dIII. Fractionated DNA blotted on Hybond N membrane (Amersham-Pharmacia, Arlington, Heights, IL) was probed against satellite 2 (Sat2), a major DNA component of the chromosome 1 heterochromatin. The Sat2 probe used was a single-stranded oligonucleotide of 18 mer that has a consensus sequence of 5′-TCGAGTCCATTCGATGAT-3′. Blotting hybridization in Rapid-Hyb buffer (Amersham-Pharmacia) was performed at 50°C using 5′-[γ-^{32}P]-end radiolabeled dATP Sat2 probe. In each blot, normal liver and sperm DNAs were included as the methylated and hypomethylated standards, respectively. Posthybridization washes were performed in 5× sodium saline citrate/0.1% sodium dodecyl sulfate for 30 minutes at room temperature, followed by 1× sodium saline citrate/0.1% sodium dodecyl sulfate for 30 minutes at 50°C.

Using a phosphoimager (Instantimager; Packard, Australia), the approximate extent of hypomethylation in each lane was quantitated by comparing the ratio intensities of hybridized fragments <4 kb to those >4 kb molecular weight. DNA samples with ratios <0.7 were considered to have a normal level of Sat2 methylation, whereas those with values >1.1 were considered to be extensively hypomethylated. Ratio values between 0.7 to 1.1 were considered to display a moderate level of hypomethylation. The cut off value of 0.7 for the presence of hypomethylation was assigned based on the degree of methylation obtained from four normal liver tissues (mean plus 1 SD). These tissues were neither cirrhotic nor viral infected, and had no apparent malignant morphology on histological examination. The ratio of 1.1 for extensive hypomethylation was established from four positive sperm controls (mean minus 1 SD). Ratio values for moderate hypomethylation were those that were between the normal level of methylation and extensive hypomethylation. Statistical analysis for the association between 1q copy gain and Sat2 hypomethylation was performed by the Fisher's exact test.

Comparative Genomic Hybridization

The CGH protocol was performed according to the method of Kallioniemi and colleagues[8] with modifications described in Wong and colleagues.[5] Briefly, differentially labeled tumor and normal DNA with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany) and dig-11-dUTP (Boehringer Mannheim) were co-hybridized onto normal metaphase chromosomes. After hybridization, biotin signals were detected through avidin conjugated-fluorescein isothiocyanate antibody (Sigma, St. Louis, MO), and dig-labeled DNA visualized by tetramethylrhodamine isothiocyanate-conjugated antibody (Sigma). Chromosomes counterstained with 4′,6-diamidino-2-phenylindole (DAPI) were captured through a cooled charge-coupled device camera mounted on a Leitz DM RB (Leica, Wetzlar, Germany) fluorescence microscope. Three band-pass filter sets (DAPI, fluorescein isothiocyanate, and tetramethylrhodamine isothiocyanate) arranged in an automated filter-wheel were used for image acquisition. CGH software ver 3.1 on Cytovision (Applied Imaging Ltd., Sunderland, UK) was used for digital image analysis of fluorescence intensity. Average ratio profiles of 10 to 12 metaphases were calculated based on chromosome identification of the inverted DAPI.[3] Thresholds for gains and losses were defined as the theoretical value of 1.25 and 0.75, respectively.

Spectral Karyotyping

SKY analysis was performed on the aberrant metaphases of cases H25, H26, H29, H32, and H34. The short-term culture of primary tumors was performed according to the procedure described in Wong and colleagues,[15] and subsequent SKY analysis according to the method described by Schröck and colleagues.[20] Briefly, tumorous liver tissues digested by collagenase (type II) were seeded in RPMI 1640 medium supplemented with 16% fetal bovine serum, 35 U/ml penicillin, 35 μg/ml streptomycin, 10 ng/ml selenium, 10 μg/ml transferrin, and 10 μg/ml insulin. At 80% confluency, which took 3 to 5 days, cells were harvested for metaphase chromosomes by colchicine. Labeled SKY probe mixture (Applied Spectral Imaging Ltd., Migdal Haemek, Israel) was applied onto the tumor metaphases. After posthybridization washes, indirectly labeled probes were visualized using fluorescence-conjugated antibodies. Chromosomes counterstained in DAPI was acquired using a SD200 Spectracube (Applied Spectral Imaging,) mounted on a Leica DMRXA microscope (Leica). Spectral information obtained on each chromosome was analyzed by the SkyView software ver 1.6.

Results

Heterochromatin Hypomethylation and 1q Gain

Sat2 DNA hypomethylation was detected in 25 of 36 HCC tissues studied (69%) (Table 1). Examples of hypomethylated Sat2 cases are shown in Figure 1a. Twenty-two of the 25 cases with Sat2 hypomethylation displayed 1q

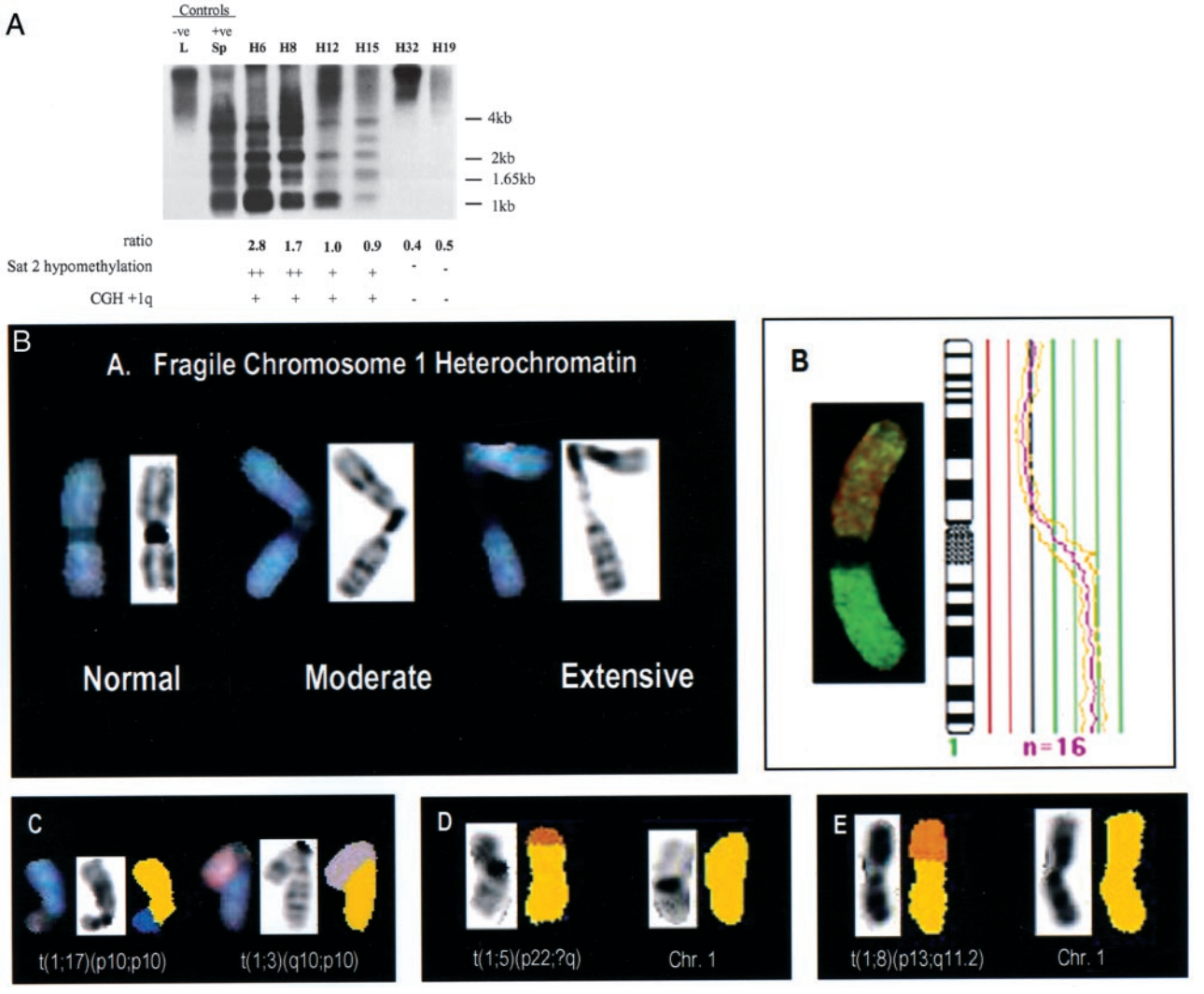

Figure 1. Top: Methylation status of chromosome 1 heterochromatin DNA in HCC. Hypomethylation status of satellite 2 (Sat2) DNA of chromosome 1 was studied by Southern blot. DNA extracted from HCC was digested by methyl-sensitive enzyme *Bst*B1 and probed against Sat2. Liver (L) and sperm (Sp) DNAs were used as the methylated (negative control) and hypomethylated (positive control) standards, respectively. The degree of hypomethylation in each lane was quantitated by comparing the ratio intensities of hybridized fragments smaller than 4 kb to those larger than 4-kb molecular weight. DNA samples with ratios <0.7, 0.7 to 1.1, and >1.1 were considered to have a normal level of methylation, moderate level of Sat2 hypomethylation, and extensive hypomethylation, respectively. **Bottom:** Molecular cytogenetic analysis on HCC. **A:** SKY analysis on H29 revealed copies of chromosome 1 with moderate to extensive decondensation of the heterochromatic region. Repetitive DNA sequences within the heterochromatic region had been suppressed by Cot-1 DNA but the reverse DAPI image indicated fragility of the segment. **B:** CGH analysis on HCC revealed frequent 1q copy number gain. Visual inspection of hybridized chromosome often indicated a strong staining green region of tumor DNA on the q-arm and a suggested breakpoint within 1q12. The figure shown illustrates the 1q copy gain detected in case H2. Fluorescent ratio intensities is plotted alongside the chromosome ideogram and the ratio profile of 16 chromosomes ($n = 16$, **pink line**) is depicted with 95% confidence interval (**gold lines**). **C:** SKY analysis on H29 supported CGH finding for the presence of balanced chromosome 1. The finding of chromosome 1p and 1q translocation, t(1;17)(p10;p10) and t(1;3)(q10;p10), suggested no net gain or loss of chromosome 1 material. **D:** Chromosome 1 translocation in case H34. Classified image is displayed alongside the reverse DAPI image. SKY analysis indicated a translocation between chromosomes 1 and 5, t(1;5)(p22;?q), with a p-arm breakpoint on band 1p22. **E:** Classified karyotype of chromosome 1 in case H32. Reverse DAPI-banding pattern indicated a translocation between chromosomes 1 and 8, t(1;8)(p13;q11.2), with a chromosome 1 breakpoint on p13.

copy number gain as suggested from CGH analysis (88%). Hybridized chromosome from CGH indicated a consistent breakpoint within the heterochromatic region, band 1q12, in these 22 cases (Figure 1b). Although chromosome 1 instability was not suggested in the remaining three cases (H9, H27, and H29), the finding of extensive heterochromatin hypomethylation prompted us to undertake further karyotypic investigation (Table 1). SKY analysis on H29 indicated an unbalanced translocation of chromosome 1p and 1q, t(1;17)(p10;p10) and t(1;3)(q10;p10), respectively (Figure 1b), suggesting no net gain or loss of chromosome 1 material. Of particular interest, SKY analysis in cases H25, H26, and H29 indicated copies of chromosome 1 with much extended heterochromatic region, suggesting fragility of the 1q12 segment (Figure 1b).

In 11 HCC cases with a normal level of Sat2 methylation, 4 showed no evidence of an unbalanced chromosome 1 (H19, H21, H22, and H33). Gain on chromosome 1 was detected in six cases (H20, H23, H24, H32, H35, and H36), and one case exhibited loss of regional 1p (H34). All but one case displayed a breakpoint outside the region of 1q12 (Table 1). The assignment of a breakpoint was further supported by SKY analysis, which indi-

Table 2. A Correlation between Heterochromatin Hypomethylation and 1q12 Breakpoint in 1q Copy Number Gain

	Chromosome 1 heterochromatin hypomethylation	
	Positive	Negative
Breakpoint within 1q12 with evidence of 1q gain	22	1
Breakpoint outside 1q12 with evidence of 1q gain	0	6
Absence of chromosome 1q12 breakpoint with no evidence of 1q gain	3	4

Fisher's exact test, $P < 0.001$.

cated a p-arm breakage in cases H32 and H34 (Figure 1b). A 3×2 contingency statistical analysis was performed to examine the correlation between 1q gain, Sat2 hypomethylation, and the significance of the 1q12 breakpoint (Table 2). Fisher's exact test indicated a strong correlation between heterochromatin DNA hypomethylation and 1q copy gain with a breakpoint at 1q12 ($P < 0.001$). In 36 HCC tissues studied, no obvious relation between the degree of Sat2 hypomethylation and clinical staging, tumor size, or viral infection could be discerned.

Heterochromatin Hypomethylation in Adjacent Hepatitis-Infected Liver Tissues

Five of 24 adjacent liver tissues studied displayed a moderate level of Sat2 hypomethylation (H1 to H5). All cases were viral hepatitis B related, and arose from a noncirrhotic liver except for case H3. Histological examination revealed no apparent malignant phenotype in these tissues, and CGH analysis did not indicate genomic imbalances in any of the 24 adjacent liver tissues examined (Table 1).

Discussion

Methylation of CpG dinucleotides functions to maintain the stability of chromosome structures.[21] Satellite DNA in the heterochromatin, in particular, is more heavily methylated at the CpG islands than the rest of the genomic DNA.[22,23] It has been postulated that under-methylated satellite sequences confer abnormal chromatin structures and predispose to chromosomal instability by enhancing chromosome recombinations.[24] A high frequency of chromosome rearrangements with pericentromeric or heterochromatic breakpoints has been observed in many tumors, which may suggest a relationship between satellite hypomethylation, chromosome instability and carcinogenesis.[25,26]

Our current finding supported a relationship between hypomethylated Sat2 sequences and recurrent aberrant 1q formation. We investigated the methylation status of Sat2 DNA, rather than the centromeric satellite (Sat-α), as Sat2 is a more CpG rich region than Sat-α (AT-rich region). In this series, methyl-sensitive endonuclease analysis showed a reduced methylation of classical Sat2 in 76% of HCC cases that displayed 1q copy number gain. In particular, we found Sat2 hypomethylation to be strongly associated with a 1q12 breakpoint ($P < 0.001$) (Table 2). In our recent karyotypic study on human HCCs, SKY analysis did not identify nonrandom chromosome 1 rearrangements, but rather frequent unbalanced 1q translocations with breakage in the vicinity of 1q12. Consistent localization of breakpoints within the heterochromatic region in HCC therefore suggests an important pathogenic consequence of Sat2 hypomethylation in 1q abnormalities. Structural decondensation of 1q12 is likely to result in centromeric fragility, somatic pairing, and the formation of jumping 1q translocations. We were able to support the presence of 1q12 segment decondensation by the finding of a fragile heterochromatic region in three cases (H25, H26, and H29) that displayed extensive Sat2 hypomethylation (Figure 1b).

DNA methylation patterns are often altered in cancer. In a number of human malignancies, regional hypermethylation of the promoter region of critical tumor suppressor gene(s) results in silencing of transcriptional activity, and global DNA hypomethylation leading to activation of proto-oncogenes and re-expression of provirus sequences has been described.[27,28] Given the multistep nature of liver carcinogenesis, cancer-associated genetic and epigenetic alterations are probable in the putative precancerous liver lesions, the surrounding viral hepatitis-infected cirrhotic tissues. Indeed, microsatellite instability and aberrant DNA methylation of *E-cadherin*, *p16*, and *c-myc* have been reported in the noncancerous liver tissues of HCC.[29–32] In our current series, 24 adjacent nonmalignant liver tissues had been examined for heterochromatin hypomethylation. Similar to our previous report[5] and that of a recent study from Taiwan,[33] CGH aberrations were not found in any of the adjacent liver tissues. However, a moderate level of Sat2 demethylation was detected in 20% of the viral hepatitis B-related surrounding liver. A viral origin in the induction of peri-centromeric fragility in human neoplasms has been previously suggested.[34,35] Although the role of hepatitis B infection, a DNA virus, in the demethylation of repeat sequences is unclear, gene products of cancer-associated DNA virus, such as SV40 and HPV, are known to alter cellular proteins and affect cell-cycle checkpoints, thereby inducing karyotypic instability.[36]

Genome-wide hypomethylation facilitates tumor progression. Demethylation of the repetitive sequences, such as LINE1, alphoid repeats, and Alu, constitute a major part of the global hypomethylation in tumor development. Although LINE1 hypomethylation has not been suggested in surrounding liver tissues of HCC,[37] methylation-sensitive representational differential analysis[38] has indicated that global hypomethylation is not homogenous throughout the entire genome. Instead, hypomethylated regions are scattered in the genome. Our present finding therefore suggests that heterochromatin demethylation precedes genome-wide hypomethylation, whereby heterochromatin fragility results in the clonal evolution of

cells with extra copies of 1q. Chromosome 1q copy number gain may confer proliferative advantages that contribute to the natural evolution of HCC progression.

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