ELSEVIER

European Journal of Cancer 41 (2005) 1185–1194

European Journal of Cancer

www.ejconline.com

Identification and investigation of methylated genes in hepatoma

Tetsuhiro Chiba [a], Osamu Yokosuka [a,*], Kenichi Fukai [a], Yuichi Hirasawa [a], Motohisa Tada [a], Rintaro Mikata [a], Fumio Imazeki [a], Hideki Taniguchi [b], Atsushi Iwama [c], Masaru Miyazaki [d], Takenori Ochiai [e], Hiromitsu Saisho [a]

[a] *Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo Ward, Chiba 260-8670, Japan*
[b] *Department of Regenerative Medicine, Faculty of Medicine, Yokohama City University, 3–9 Fukuura, Kanazawa Ward, Yokohama 236-0004,Japan*
[c] *Department of Cellular and Molecular Medicine, Graduate School of medicine, Chiba University, 1–8–1 Inohana, Chuo Ward, Chiba 260-8670, Japan*
[d] *Department of General Surgery, Graduate School of Medicine, Chiba University, 1–8–1 Inohana, Chuo Ward, Chiba 260-8670, Japan*
[e] *Department of Academic Surgery, Graduate School of Medicine, Chiba University, 1–8–1 Inohana, Chuo Ward, Chiba 260-8670, Japan*

Received 21 October 2004; received in revised form 18 February 2005; accepted 22 February 2005
Available online 27 April 2005

Abstract

Gene silencing due to aberrant DNA methylation plays an important role in carcinogenesis. Previous microarray analysis demonstrated that 14 genes, including *hepatocyte growth factor activator inhibitor 2/placental bikunin* (*HAI2/PB*) gene, showed particularly high inductions after 5-aza-2′deoxycytidine (5Aza-dC) treatment in multiple hepatoma cell lines. In the present study, we studied all of these genes except for the *HAI2/PB* gene and examined DNA methylation status and levels of acetylated histones using bisulphite genomic sequencing and the chromatin immunoprecipitation (ChIP) assay, respectively. Aberrant methylation in primary hepatoma tissues was also examined using methylation-specific polymerase chain reaction (MSP). Genes for *E-cadherin*, *collagen type I alpha 2* (*COL1A2*), *insulin-like growth factor binding protein 2* (*IGFBP2*), *connective tissue growth factor* (*CTGF*) and *fibronectin 1* exhibited aberrant methylation in several hepatoma cell lines. The ChIP assay showed that DNA methylation and deacetylation of histones generally coexist except for *fibronectin 1*. In further studies of 24 primary hepatoma tissues, methylation signals for *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* were detected in 13, 18, 4 and 10 patients, respectively. In conclusion, aberrant methylation of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* genes were detected in hepatoma cell lines. We also demonstrated that the methylation of 5′CpG islands and histone deacetylation generally coexisted in the regulation of gene expression except for *fibronectin 1*. The results of MSP in hepatoma tissues suggested that some of these genes might be involved in the development or progression of hepatoma.

Keywords: 5-aza-2′deoxycytidine; DNA methylation; Histone acetylation; Hepatoma; cDNA microarray; Epigenetics

1. Introduction

Epigenetic modifications, such as DNA methylation and histone acetylation, in promoter areas are estimated to play a crucial role in the control of gene expression and chromosome structure in mammalian cells [1,2]. The cytosine methylation of CpG dinucleotide sequences is a common occurrence and roughly 70% of all CpG dinucleotides in mammalian genomes seem to be methylated. It is reported that 40–60% of human genes possess CpG-rich sequences in 5′ regulatory regions, so called '5′CpG islands', and normally remain unmethylated [3]. The methylation of CpG islands is deeply associated with inactivation of the X-chromosome in females and genomic imprinting [4,5]. Moreover, transcriptional silencing of tumour suppressor

* Corresponding author. Tel.: +81 43 2262083; fax: +81 43 2262088.
E-mail address: yokosukao@faculty.chiba-u.jp (O. Yokosuka).

0959-8049/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2005.02.014

and mismatch repair genes due to hypermethylation of 5′CpG islands is observed in a variety of malignancies [6,7].

Hepatoma is one of the most common malignancies worldwide and still has a poor prognosis. It is considered that genetic alterations, such as loss of heterozygosity (LOH) and the mutation of tumour suppressor genes, accumulate and play an important role in multistep hepatocarcinogenesis [8,9], however, this does not completely explain the mechanisms underlying hepatocarcinogenesis. Molecular approaches have recently demonstrated interesting epigenetic alterations in various malignancies. It is noted that microsatellite instability and LOH following DNA methylation might contribute to hepatocarcinogenesis [10,11]. Decreases in the expression levels of p15, p16, E-cadherin and 14-3-3 sigma caused by aberrant DNA methylation have already been reported in hepatoma [12–14], although only a small number of genes silenced by hypermethylation have been identified.

Previously, we detailed the gene expression profile altered after exposure to a DNA methyltransferase inhibitor, 5-aza-2′deoxycytidine (5Aza-dC), in six hepatoma cell lines using a cDNA microarray spotted with 557 distinct cDNA of cancer-related genes and reported high frequency of hypermethylation of the *hepatocyte growth factor activator inhibitor 2/placental bikunin* (*HAI2/PB*) gene in both hepatoma cell lines and primary hepatoma tissues [15]. The expression of 14 genes, namely *HAI2/PB*, *E-cadherin*, *collagen type I alpha 2* (*COL1A2*), *insulin-like growth factor binding protein 2* (*IGFBP2*), *connective tissue growth factor* (*CTGF*), *fibronectin 1*, *cyclin A2*, *integrin alpha V* (*ITGAV*), *hyaluronan-mediated motility receptor* (*RHAMM*), *chromosome segregation 1-like* (*CSE1L*), *lumican*, *Rho GDP dissociation inhibitor beta* (*Rho-GDI β*), *collagen type III alpha 1* (*COL3A1*) and *secreted protein*, *acidic*, *cysteine-rich* (*SPARC*) was increased (more than fivefold) in at least two cell lines and these genes were considered candidates for genes silenced by aberrant methylation in hepatoma cells. Transcriptional start sites of these genes were determined utilising the human genome database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) and it was found that 10 genes, those for *HAI2/PB*, *E-cadherin*, *COL1A2*, *IGFBP2*, *CTGF*, *fibronectin 1*, *cyclin A2*, *ITGAV*, *RHAMM* and *CSE1L*, possess 5′CpG islands according to the criteria reported by Gardiner-Garden and colleagues [16].

In the present study, we examined the methylation status of 5′CpG islands in all these genes except the *HAI2/PB* gene for the further detection of novel genes silenced by aberrant methylation in hepatoma cells. We further estimated the association between aberrant methylation and levels of acetylated histones in some of these genes. The frequency of aberrant methylation of several genes showing aberrant methylation of 5′CpG islands in hepatoma cell lines was also examined in primary hepatoma tissues.

2. Materials and methods

2.1. Cell culture and treatment with 5Aza-dC

Human hepatoma cell lines HLE, HuH7, Hep3B, HepG2, PLC/PRF/5 and HuH6 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, United States of America (USA)) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). The hepatoma cells were incubated at 37 °C in an atmosphere containing 5% CO_2 and were treated with 1 μM of 5Aza-dC (Invitrogen) for 96 h. The medium including 5Aza-dC was replaced every 24 h.

2.2. Source of hepatoma tissue samples

Hepatoma tissues were obtained from 24 patients who underwent hepatic resection or autopsy for primary hepatoma. The specimens were immediately frozen and stored at −70 °C for the subsequent isolation of DNA and RNA. There were 22 men and 2 women with a mean age of 65 ± 8 (mean ± SD) years (range 42–79 years). Numbers of patients positive for hepatitis B surface antigen (HBsAg), positive for hepatitis C antibody (HCVAb) and negative for HBsAg and HCVAb were 8, 11 and 5, respectively. Pathological examination of non-tumour tissues revealed that 11 patients had chronic hepatitis and 13 patients had cirrhosis. Normal liver tissues were also obtained from 2 patients with colon cancer and a patient with bile duct cancer.

2.3. Quantitative real-time reverse transcriptase-polymerase chain reaction

Total RNA was extracted from hepatoma cell lines and primary hepatoma tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) was carried out using First strand superscript (Invitrogen) after pre-treatment with amplification grade DNase I (Invitrogen). Polymerase chain reaction (PCR) was carried out by TaqMan technology using ABI PRISM 7000 Sequence detection System (Applied Biosystems, Foster City, CA, USA). Assays-on-Demand primer and probe mixes were used for *E-cadherin*, *COL1A2*, *IGFBP2*, *fibronectin 1*, *cyclin A2*, *ITGAV*, *RHAMM*, *CSE1L* and β-*actin* (assay IDs Hs00170423_m1, Hs00164099_m1, Hs00167151_m1, Hs00170014_m1, Hs00277509_m1, Hs00153138_m1, Hs00233790_m1, Hs00234864_m1, Hs00169158_m1and Hs99999903_m1, respectively; Applied Biosystems). The thermal cycling was performed using 40 cycles (hepatoma

cell lines) or 45 cycles (hepatoma tissues) of 95 °C for 15 s and 60 °C for 1 min with duplicates of interest. β-actin was used for normalisation. Relative quantitation was performed using the comparative cycle threshold (C_T) method, as described by the manufacturer.

2.4. Sodium bisulphite genomic sequencing

The methylation status of 5′CpG islands of *E-cadherin*, *COL1A2*, *IGFBP2*, *fibronectin 1*, *cyclin A2*, *ITGAV*, *RHAMM* and *CSE1L* was examined using sodium bisulphite genomic sequencing, because the analysis of *CTGF* in 5′CpG was demonstrated previously [18]. Bisulphite treatment was performed for 18 h at 50 °C on 1 μg of the genomic DNA extracted from six hepatoma cell lines, according to the method of Herman and colleagues [19]. Next, nested PCR was carried out using specific primers to amplify the sequences of 5′CpG islands (Table 1). The number of cycles was 35 for the first PCR and 40 for the second PCR. The PCR products were subcloned into the TOPO TA cloning vector (Invitrogen). Sequencing of 10 clones in each cell was performed together using an ABI PRISM Dye Deoxy Terminator Cycle sequencing Kit (Applied Biosystems) and then the sequences were analysed with an ABI 377 DNA Sequencer.

2.5. Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed as described previously [17]. The PCR of immunoprecipitated DNA was performed using primers for *E-cadherin* (5′-CAGGTCCCATAACCCACCTA-3′ and 5′-TCACAGGTGCTTTGCAGTTC-3′) and *fibronectin 1* (5′-AGGCATTAGAAGGGATTTTTCC-3′ and 5′-GTACTCACGCTTGCTTTGACTG-3′). PCR conditions were 95 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 58 °C (E-cadherin) or 50 °C (fibronectin 1) for 30 s, and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. The primer sequences and PCR conditions for *COL1A2*, *IGFBP2*, *CTGF* and β-*actin* were the same as those described previously [17,18]. The PCR bands were normalised by the β-*actin* expression and quantified using NIH Image 1.62.

2.6. Methylation-specific PCR

Genomic DNA was isolated from human hepatoma specimens using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. The DNA was modified with sodium bisulphite, as described above, and was amplified with a set of primers for the methylated reaction and another set of primers for the unmethylated reaction (Table 2), as described previously [15].

3. Results

3.1. Expression levels of mRNA based on quantitative real-time RT-PCR

We analysed changes in the mRNA expression of nine genes, including *E-cadherin*, *COL1A2*, *IGFBP2*, *CTGF*, *fibronectin 1*, *cyclin A2*, *ITGAV*, *RHAMM* and *CSE1L* by quantitative real-time RT-PCR (Table 3). These genes showed an up-regulated expression in multiple cell lines, as detected in the cDNA microarray analysis, although a difference in the detection sensitivity might exist between the cDNA microarray and RT-PCR. *IGFBP2* in Huh6 cells exceptionally exhibited an inconsistent result between microarray and quantitative real-time RT-PCR.

3.2. Methylation status of 5′CpG islands

To examine the methylation status of 5′CpG islands in the eight genes, namely *E-cadherin*, *COL1A2*, *IGFBP2*, *fibronectin 1*, *cyclin A2*, *ITGAV*, *RHAMM* and *CSE1L*, we performed bisulphite genomic sequencing (Fig. 1). The number of CpG dinucleotides was 29, 23, 65, 24, 38, 25, 15 and 30, respectively. The methylation status was classified as 'methylated' or 'demethylated'. 5′CpG islands of *fibronectin 1* in six cell lines studied were heavily methylated, although those of *cyclin A2*, *ITGAV*, *RHAMM* and *CSE1L* were fully demethylated. Those of *E-cadherin*, *COL1A2*, *IGFBP2* and *CTGF* showed various methylation patterns in each hepatoma cell line. *CTGF* in 5′CpG islands showed a comparatively heavy methylation in HepG2 and HuH6 cells as reported previously [18]. The levels of aberrant methylation did not necessarily correlate with the fold induction after 5Aza-dC treatment.

3.3. Levels of acetylated histones H3 and H4

The ChIP assay using anti-acetylated histone H3 or H4 antibody revealed alterations in the level of acetylated histones H3 or H4 in the promoter region in *E-cadherin*, *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* (Fig. 2). *E-cadherin* in HLE and HepG2 cells demonstrated no PCR bands. Levels of acetylated histones H4 in these genes were similar to those of acetylated histones H3, but no bands of *IGFBP2* in HLE and HepG2 cells were detected concerning acetylated histones H4.

3.4. Association between DNA methylation and histone acetylation in gene expression

The status of DNA methylation and histone acetylation was shown in association with the mRNA expression of each gene in six hepatoma cell lines (Fig. 3). mRNA expression appeared to be correlated with levels

Table 1
Primers used for sodium bisulphite genomic sequencing

Gene name	Primer	Base pairs	Temperature (°C)
E-cadherin 1st PCR	Fw 5′-GTATTTTAGTTTGGGTGAAAGAGTGAGA-3′	739	60
	Rv 5′-TCACCACCCACCCCCCACTCCCATCACT-3′		
2nd PCR	Fw 5′-AGTAATTTTAGGTTAGAGGG-3′	321	45
	Rv 5′-TCCAAAAACCCATAACTAAC-3′		
COL1A2 1st PCR	Fw 5′-TAAAGTTAGAGAAAAGTTGGAAGGGGTTGG-3′	779	50
	Rv 5′-ACTAAACATAAAATTACTACAAACAACAAC-3′		
2nd PCR	Fw 5′-ATAGTGTTTTTAAATTTGGAAAGGG-3′	460	45
	Rv 5′-AACACTTAAACATACAAACTCCTTA-3′		
IGFBP2 1st PCR	Fw 5′-TATTTTAGGATGGAAGGAGTTGGTATGAGT-3′	990	55
	Rv 5′-CCAAATTTCTCCCAACTACTTATTCCAAAC-3′		
2nd PCR	Fw 5′-ATTGAAATTTATTTGAAGGT-3′	604	48
	Rv 5′-CCCAATAACAACAACAACAA-3′		
Fibronectin 1 1st PCR	Fw 5′-GGGAAAGGTAGTTTTGTTTTGGGATTGAAA-3′	1170	58
	Rv 5′-ATCTTATCATCCCAAACCCTCAAAACCAAC-3′		
2nd PCR	Fw 5′-GAAGGGAAGTAAATTTGGTG-3′	403	50
	Rv 5′-TCTTATCATCCCAAACCCTC-3′		
Cyclin A2 1st PCR	Fw 5′-GGGATATTTGAATTGTAAGAATAGT-3′	391	48
	Rv 5′-AATATTCTCCTAATCCTCTTAAAAC-3′		
2nd PCR	Fw 5′-TTGGGTAGTGTTTGTTTG-3′	281	48
	Rv 5′-CTACTACAATACTAACAAC-3′		
ITGAV 1st PCR	Fw 5′-GTTGATTTAGGTTTAGGAGTTGGGGG-3′	872	58
	Rv 5′-ATTCATTTCCCCAAATATTTCCTCCACCCA-3′		
2nd PCR	Fw 5′-GGTAGTTTTTAGTTTTAGA-3′	369	48
	Rv 5′-CACAAAAATAACAAAAATCC-3′		
RHAMM 1st PCR	Fw 5′-GTTTGGGAAGAGGAAATTGAATATAAGTTT-3′	1109	44
	Rv 5′-TCCTTAACATCCTAATTAATACTTA-3′		
2nd PCR	Fw 5′-GGGAATGATTGGGTGGGTGG-3′	333	62
	Rv 5′-CCCAACTCTTTCCCCCTTAC-3′		
CSE1L 1st PCR	Fw 5′-TAAAATAGAAGTTTTAAGAGGGGTAGGAAG-3′	720	55
	Rv 5′-CTCAATTACCCTCAACCCTCACAAACCCTA-3′		
2nd PCR	Fw 5′-AATGGGTTAGAAGTTAGTAG-3′	409	50
	Rv 5′-CCTCATCCCTTCCTCAACTA-3′		

PCR, polymerase chain reaction; Fw, forward; Rv, reverse; COL1A2, collagen type I alpha 2; IGFBP2, insulin-like growth factor binding protein 2; ITGAV, integrin alpha V; RHAMM, hyaluronan-mediated motility receptor; CSE1L, chromosome segregation 1-like.

of acetylated histones H3 and H4, and inversely with DNA methylation in the promoter region in *E-cadherin*, *COL1A2*, *IGFBP2* and *CTGF*, however *E-cadherin* in HLE cells exceptionally showed DNA demethylation with deacetylated histones, which resulted in no mRNA expression. These results also indicated that aberrant methylation and histone deacetylation are closely associated with the silencing of these genes. Although 5′CpG islands of *fibronectin 1* showed heavy methylation in all six cell lines, comparatively high levels of mRNA expression accompanied by acetylated histones were observed in HuH7 and Hep3B cells compared with other cell lines.

3.5. Frequency of aberrant methylation and suppressed gene expression in primary hepatoma tissues

We analysed the methylation status of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* in normal liver tissues

Table 2
Primers used for methylation-specific PCR (MSP)

Gene name	Primer	Base pairs	Temperature (°C)	Cycles
COL1A2 M	Fw 5′-CGTATAAATAGGGTAGATTC-3′ Rv 5′-AAATATCACCTAACGAACGC-3′	95	50	45
U	Fw 5′-TGTATAAATAGGGTAGATTT-3′ Rv 5′-AAATATCACCTAACAAACAC-3′		47	45
IGFBP2 M	Fw 5′-GTCGGTTATAGGGGAAGCGC-3 Rv 5′-GAATTTAAATAAAACCGACGTAACG-3′	104	53	40
U	Fw 5′-GTTGGTTATAGGGGAAGTGT-3′ Rv 5′-AAATTTAAATAAAACCGACATAACA-3′		47	35
CTGF M	Fw 5′-TCGTTTCGGTCGATAGTTTC-3′ Rv 5′-CGAAACCCATACTAACGACG-3′	159	55	40
U	Fw 5′-TTGTTTTGGTTGATAGTTTT-3′ Rv 5′-CAAAACCCATACTAACAACA-3′		50	40
Fibronectin 1 M	Fw 5′-CGGTGTTTTTTACGGGAGTTTC-3′ Rv 5′-GCGCACACACTCGCACACACG-3′	209	55	40
U	Fw 5′-TGGTGTTTTTTATGGGAGTTTT-3′ Rv 5′-ACACACACACTCACACACACA-3′		50	40

M, methylated; U, unmethylated; Fw, forward; Rv, reverse.

Table 3
Fold induction of the 9 genes after 5Aza-dC treatment based on quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Gene name	Acc. no	HLE	HuH7	Hep3B	HepG2	PLC/PRF/5	HuH6
E-cadherin	Z13009	10.25	9.92	1.24	12.22	2.28	0.90
COL1A2	J03464	5.62	7.21	10.34	4.11	0.05	0.28
IGFBP2	X16302	10.32	2.06	4.14	11.63	0.17	0.28
CTGF	X78947	5.29	1.52	1.21	3.89	3.46	4.72
Fibronectin 1	X02761	6.41	4.11	0.77	5.78	1.99	1.43
Cyclin A2	X51688	1.58	5.35	1.56	3.76	5.39	1.25
ITGAV	M14648	6.70	10.13	3.13	1.56	1.69	0.91
RHAMM	AF032862	0.88	3.62	1.19	4.93	4.51	1.20
CSE1L	AF053641	5.06	4.41	0.70	9.09	3.01	0.88

Acc. no., GenBank accession number; COL1A2, collagen type I alpha 2; IGFBP2, insulin-like growth factor binding protein 2; CTGF, connective tissue growth factor; ITGAV, integrin alpha V; RHAMM, hyaluronan-mediated motility receptor; CSE1L, chromosome segregation 1-like.

($n = 3$) and primary hepatoma tissues ($n = 24$) by MSP analysis (Table 4, Fig. 4). No significant association between aberrant methylation and serological or pathological background of the patients was found. No methylation signals were observed in three normal liver tissue samples in these four genes, but aberrant methylation was detected in 13, 18, 4 and 10 hepatoma samples, respectively, and that in non-tumour tissue, showing pathological findings of chronic hepatitis or cirrhosis, were also found in 6, 11, 4 and 8 samples, respectively (Table 5). Methylation in non-tumour tissue samples from the same individuals with hepatoma tissues showing methylation in *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* was detected in 5/13, 8/18, 2/4 and 7/10 samples, respectively. However, the signals for *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* were stronger in hepatoma tissues than non-tumour tissues in 5, 7, 1 and 5 samples, respectively.

Moreover, quantitative real-time RT-PCR revealed that decreased mRNA expression of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* in hepatoma tissue compared with corresponding non-tumour tissue was observed in 10/13, 13/18, 2/4 and 6/10 samples, respectively (Fig. 4).

4. Discussion

The aberrant methylation of 5′CpG islands is a crucial epigenetic alteration in cancers and associated with gene silencing in specific tumours. The suppression of gene activity is in part mediated by a reduction in the binding affinity of methylation-sensitive transcription factors [20,21]. A link between DNA methylation and histone deacetylation through the family of methyl-CpG binding proteins such as methyl-CpG binding protein 2 (MeCP2), methylated-DNA binding domain (MBD) 2 and MBD3 to recruit HDACs was also reported [22,23].

5Aza-dC, a DNA methyltransferase inhibitor, regulates gene expression and cell differentiation. The agent is also a potent anti-tumour agent and has already been used clinically to treat several malignancies [24]. 5Aza-dC exhibits anti-proliferative effects by reactivating tumour suppressor or mismatch repair genes. It is also suggested that the cytotoxic effect of 5Aza-dC is mediated directly by the covalent binding of DNA methyltransferase 1 (DNMT1) to 5Aza-dC-substituted DNA [25].

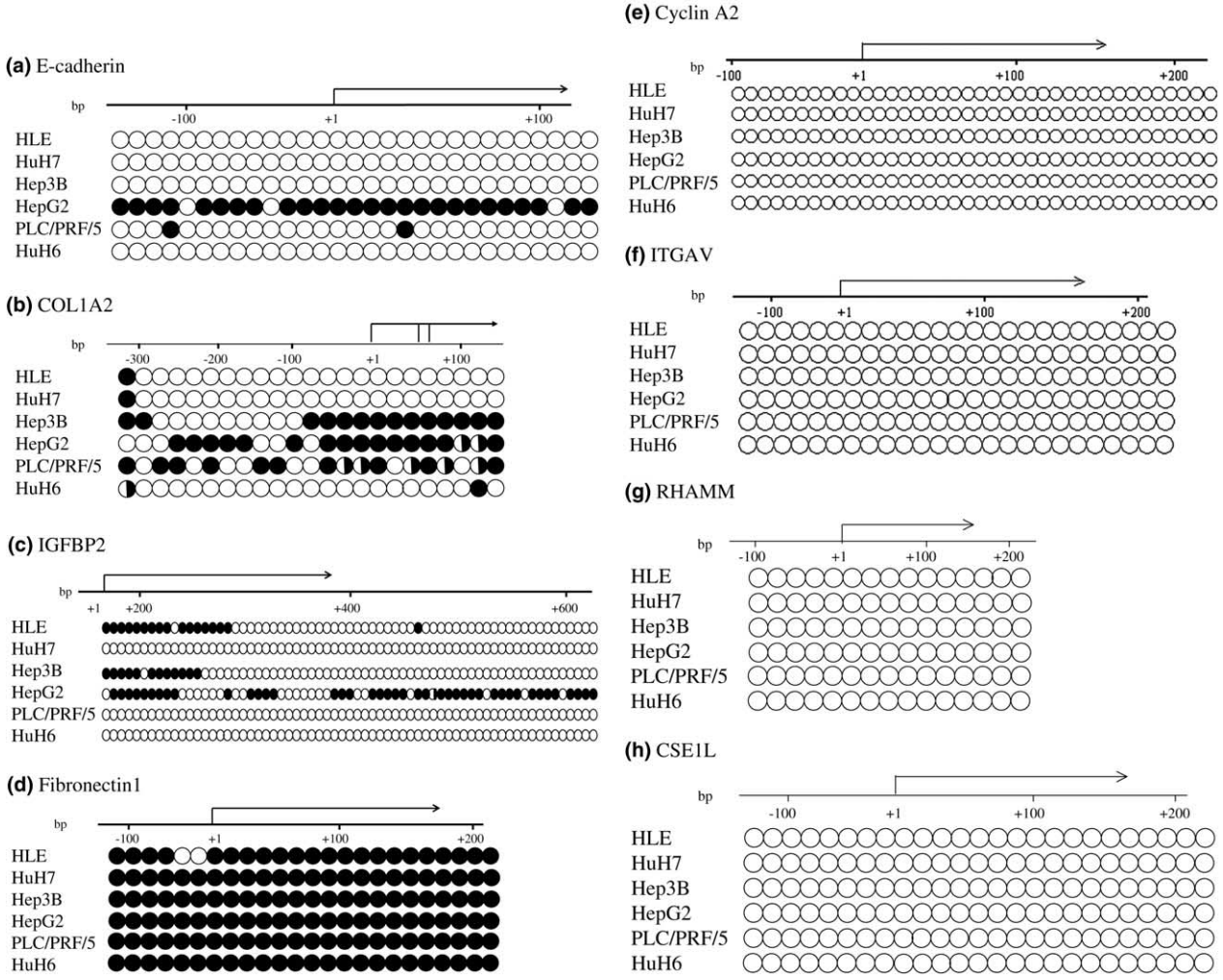

Fig. 1. Methylation analysis of the genes for (a) E-cadherin, (b) COL1A2, (c) *IGFBP2*, (d) *fibronectin 1*, (e) *cyclin A2*, (f) *ITGAV*, (g) *RHAMM* and (h) *CSE1L* using bisulphite genomic sequencing. Each circle shows a CpG site in the primary DNA sequence (circle, non-methylated; filled circle, methylated; half-filled circle, methylated in 50% of clones). Arrows indicate transcription start site.

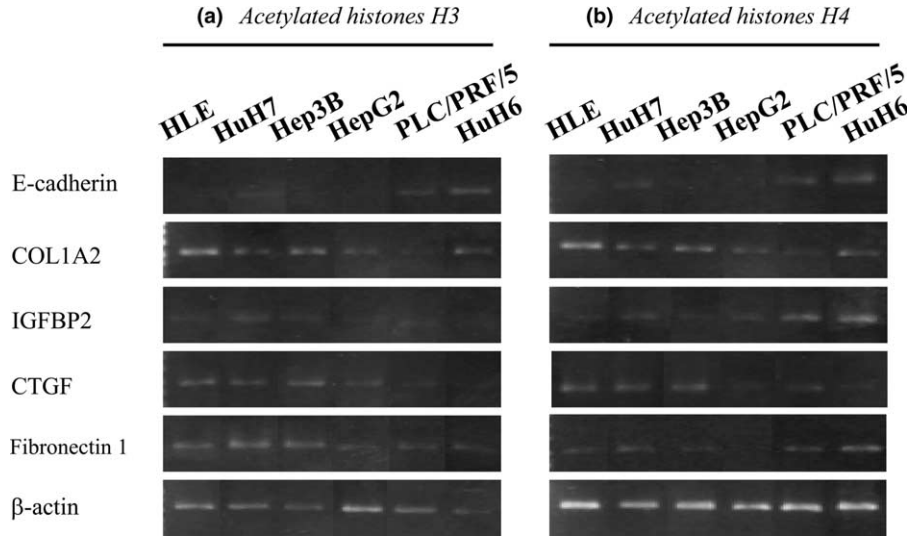

Fig. 2. Levels of acetylated histones based on the chromatin immunoprecipitation (ChIP) assay using (a) anti-acetylated histone H3 antibody and (b) anti-acetylated histone H4 antibody in the genes for *E-cadherin*, *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1*. Levels of acetylated histones H4 in these genes were generally similar to those of acetylated histones H3.

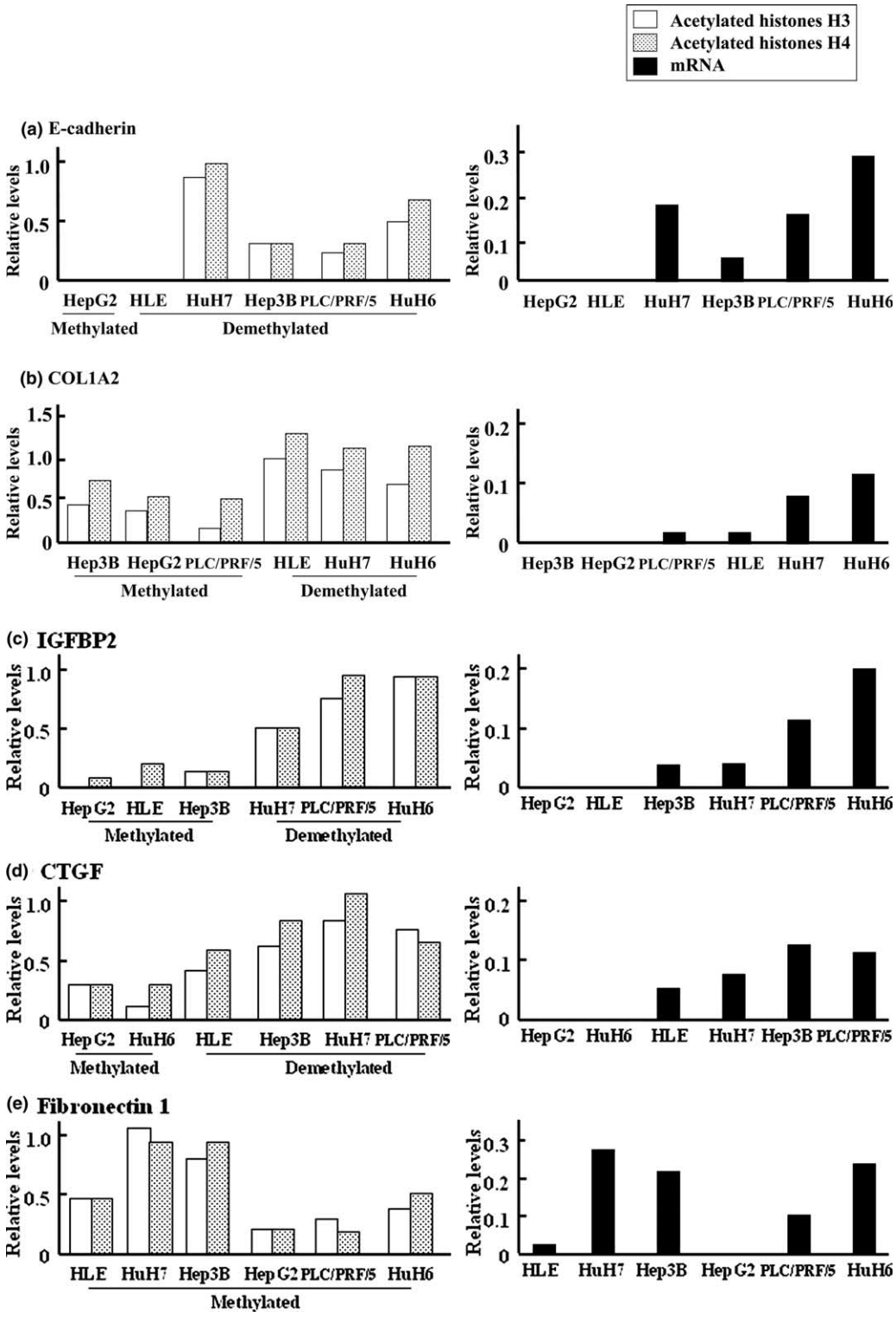

Fig. 3. Association between aberrant DNA methylation and histone acetylation in the expression of (a) *E-cadherin*, (b) *COL1A2*, (c) *IGFBP2*, (d) *CTGF* and (e) *fibronectin 1* in hepatoma cell lines. The intensity of the bands in the chromatin immunoprecipitation (ChIP) assay was quantified with normalisation by that of β−*actin*. mRNA level of the four genes was also quantified by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). DNA methylation and low levels of acetylated histones H3 and H4 were, in general, observed in silenced genes except the *fibronectin 1* gene.

Recently, a genomic screen for genes up-regulated by DNA methyltransferase inhibition and/or histone deacetylase inhibition in several cancers using cDNA microarray was reported [15,26]. Our previous microarray-based profiling led to the identification of 14 candidate genes of which expression was silenced by aberrant

Table 4
Detection of DNA methylation in 24 primary hepatoma tissues

Case	Age	Sex	HBsAg	HCVAb	LC	Differentiation	Aberrant methylation detected			
							COL1A2	IGFBP2	CTGF	Fibronectin 1
1	60	M	+	–	–	Moderate	–	–	+	–
2	70	M	+	–	–	Moderate	–	+	–	+
3	55	M	+	–	+	Well	–	+	+	–
4	42	M	+	–	+	Moderate	+	+	–	+
5	50	M	+	–	+	Moderate	–	+	–	–
6	59	M	+	–	+	Moderate	+	+	–	+
7	74	M	+	–	+	Moderate	–	+	–	+
8	69	M	+	–	+	Poor	+	–	+	–
9	62	M	–	+	–	Well	–	+	–	–
10	67	M	–	+	–	Well	+	+	–	+
11	55	M	–	+	–	Moderate	+	+	–	–
12	73	M	–	+	–	Moderate	+	+	–	+
13	63	M	–	+	–	Poor	–	+	–	+
14	72	M	–	+	–	Poor	+	+	–	–
15	64	M	–	+	+	Well	–	+	+	–
16	62	M	–	+	+	Moderate	–	+	–	–
17	69	M	–	+	+	Moderate	–	–	–	–
18	73	M	–	+	+	Moderate	+	–	–	+
19	65	F	–	+	+	Poor	+	–	–	–
20	71	F	–	–	–	Well	+	+	–	+
21	65	M	–	–	–	Moderate	+	+	–	–
22	68	M	–	–	–	Moderate	–	–	–	–
23	79	M	–	–	–	Moderate	+	+	–	–
24	71	M	–	–	+	Moderate	+	+	–	+

HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C antibody; LC, liver cirrhosis; M, male; F, female; COL1A2, Collagen type I alpha 2; IGFBP2, insulin-like growth factor binding protein 2; CTGF, connective tissue growth factor.

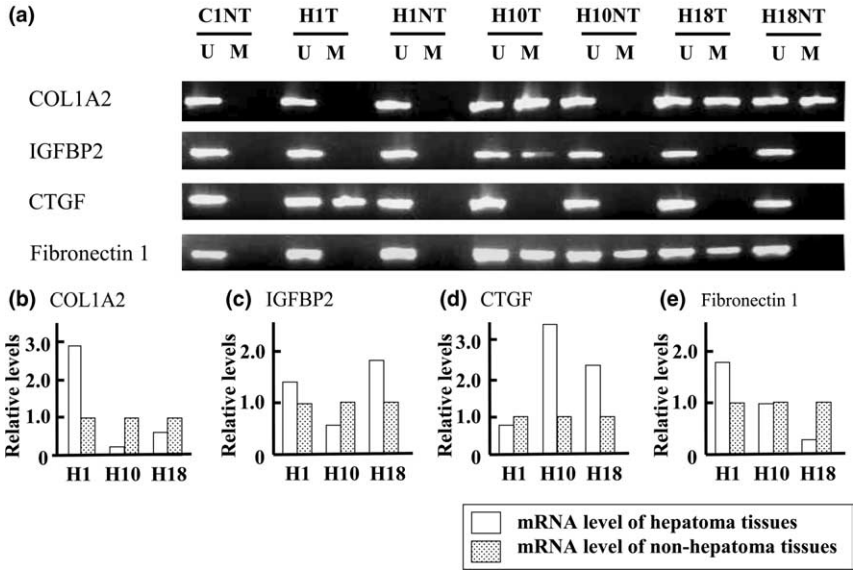

Fig. 4. (a) Representative methylation-specific PCR (MSP) of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* in liver metastasis of colon cancer (C) and hepatoma tissues (H). Numbers of hepatoma samples correspond to those in Table 4. T, tumour tissues; NT, non-tumour tissues; M, methylated; U, unmethylated. (b)–(e) Representative quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1*.

methylation of 5′CpG islands. Because we have already reported aberrant methylation of the *HAI2/PB* gene in hepatoma, we sought to examine the methylation status of 13 genes in this study. *Lumican*, *Rho-GDI* β, *COL3A1* and *SPARC*, do not possess 5′CpG islands according to the available human genome database. Bisulphite

Table 5
Frequency of aberrant methylation in chronic liver disease and hepatoma

DNA methylation detected (%)				
Gene name	NL (n = 3)	CH (n = 11)	LC (n = 13)	Hepatoma (n = 24)
COL1A2	0	2 (18.2)	4 (30.8)	13 (54.2)
IGFBP2	0	5 (45.5)	6 (46.2)	18 (75.0)
CTGF	0	2 (18.2)	2 (15.4)	4 (16.7)
Fibronectin 1	0	3 (27.3)	5 (38.5)	10 (41.7)

NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis; CTGF, connective tissue growth factor; IGFBP2, insulin-like growth factor binding protein 2; COL1A2, collagen type I alpha 2.

genomic sequencing revealed the 5′CpG islands of *E-cadherin*, *COL1A2*, *IGFBP2* and *CTGF* to have aberrant methylation in several cell lines. *Fibronectin 1* showed heavy methylation in all cell lines examined, although *cyclin A2*, *ITGAV*, *RHAMM* and *CSE1L* were fully demethylated. This might be due to sequential activation by the up-regulation of upstream genes after 5Aza-dC treatment.

The aberrant DNA methylation of *HAI2/PB* and *E-cadherin* had already been reported in hepatoma [13,15], but the relationship between other genes and the development or progression of hepatoma had not been well argued. COL1A2 is synthesised in hepatic stellate cells and is a component of the extracellular matrix [27]. Sengupta *et al.* [28] reported the methylation of COL1A2 in both a variety of cancer cell lines and primary colorectal cancer tissues. IGFBP2, which is a member of the IGFBPs, regulates IGF associated with growth control and carcinogenesis [29]. It was reported that CTGF might play an important role in hepatocarcinogenesis and correlated with recurrence and metastasis of hepatoma [30]. Fibronectin 1 is an important adhesive glycoprotein in connective tissue and is reported to be associated with the metastasis of melanoma cells [31]. The present study showed that methylation of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* was detected in 13, 18, 4 and 10 out of 24 hepatoma tissues, respectively. Because decreased mRNA expression of *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* was observed in 10/13, 13/18, 2/4 and 6/10 hepatoma samples, respectively, it appears that aberrant DNA methylation plays a crucial role in gene expression. Moreover, MSP bands in non-tumour tissues from the same individuals with hepatoma showed weaker signals in more than half of these cases. Aberrant methylation in these genes appeared to be observed frequently with the progression of chronic liver damage. Taking into consideration that no methylation was detected in 3 samples of normal liver tissue, hypermethylation of 5′CpG islands in some genes might be an early event in hepatocarcinogenesis.

It was reported that aberrant methylation and histone deacetylation are closely associated with gene silencing, although heavy methylation of 5′CpG islands is dominant for maintaining stability [32,33]. The current PCR analysis of ChIP assay revealed that the genes with heavy methylation of 5′CpG islands generally displayed low levels of acetylated histones and weak mRNA expression with few exceptions. The role of epigenetic modification in the expression of *fibronectin 1* should be further examined. Although mRNA expression of *E-cadherin* in HLE cells was suppressed in spite of DNA demethylation, it might be due to activation of Snail and SIP1 (Smad interacting protein 1), which works as crucial transcription factors in epithelial-mesenchymal transition (EMT) [34].

In conclusion, we showed four genes including *COL1A2*, *IGFBP2*, *CTGF* and *fibronectin 1* as methylated genes in both hepatoma cell lines and primary hepatoma tissues. These genes might be involved in hepatocarcinogenesis or the development of hepatoma. It is also presumed that epigenetic alterations, including DNA methylation and histone deacetylation, are closely associated with the altered gene expression in hepatoma.

Conflict of interest statement

None declared.

References

1. Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. *Science* 2001, **293**, 1068–1070.
2. Grunstein M. Histone acetylation in chromatin structure and transcription. *Nature* 1997, **389**, 349–352.
3. Larsen F, Gundersen G, Lopez R, *et al*. CpG islands as gene markers in the human genome. *Genomics* 1992, **13**, 1095–1107.
4. Riggs AD, Pfeifer GP. X-chromosome inactivation and cell memory. *Trends Genet* 1992, **8**, 169–174.
5. Razin A, Cedar H. DNA methylation and genomic imprinting. *Cell* 1994, **77**, 473–476.
6. Merlo A, Herman JG, Mao L, *et al*. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995, **1**, 686–692.
7. Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002, **21**, 5427–5440.
8. Liao C, Zhao M, Song H, *et al*. Identification of the gene for a novel liver-related putative tumor suppressor at a high-frequency loss of heterozygosity region of chromosome 8p23 in human hepatocellular carcinoma. *Hepatology* 2000, **32**, 721–727.

9. Murakami Y, Hayashi K, Sekiya T. Aberration of the tumor suppressor p53 and retinoblastome in human hepatocellular carcinomas. *Cancer Res* 1991, **51**, 5520–5525.
10. Kondo Y, Kanai Y, Sakamoto M, *et al*. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis – A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology* 2000, **32**, 970–978.
11. Kanai Y, Ushijima S, Tsuda H, *et al*. Aberrant DNA methylation precedes loss of heterozygosity on chromosome 16 in chronic hepatitis and liver cirrhosis. *Cancer Lett* 2000, **148**, 73–80.
12. Roncalli M, Bianchi P, Bruni B, *et al*. Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. *Hepatology* 2002, **36**, 427–432.
13. Yang B, Guo M, Herman JG, *et al*. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. *Am J Pathol* 2003, **163**, 1101–1107.
14. Iwata N, Yamamoto H, Sasaki S, *et al*. Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. *Oncogene* 2000, **19**, 5298–5302.
15. Fukai K, Yokosuka O, Chiba T, *et al*. Hepatocyte growth factor activator inhibitor 2/placental bikunin (HAI-2/PB) gene is frequently hypermethylated in human hepatocellular carcinoma. *Cancer Res* 2003, **63**, 8674–8679.
16. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. *J Mol Biol* 1987, **196**, 261–282.
17. Chiba T, Yokosuka O, Fukai K, *et al*. Cell growth inhibition and gene expression induced by the histone deacetylase inhibitor, trichostatin A, on human hepatoma cells. *Oncology* 2004, **66**, 481–491.
18. Chiba T, Yokosuka O, Arai M, *et al*. Identification of genes upregulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells. *J Hepatol* 2004, **41**, 436–445.
19. Herman JG, Graff JR, Myohanen S, *et al*. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, **93**, 9821–9826.
20. Prendergast GC, Ziff EB. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. *Science* 1991, **251**, 186–189.
21. Clark SJ, Harrison J, Molloy PL. Sp1 binding is inhibited by (m)Cp (m)CpG methylation. *Gene* 1997, **195**, 67–71.
22. Wade PA. Methyl CpG-binding proteins and transcriptional repression. *Bioessays* 2001, **23**, 1131–1137.
23. Ballestar E, Esteller M. The impact of chromatin in human cancer: linking DNA methylation to gene silencing. *Carcinogenesis* 2002, **23**, 1103–1109.
24. Van Groeningen CJ, Leyva A, O'Brien AM, *et al*. Phase I and pharmacokinetic study of 5-aza-2′-deoxycytidine (NSC 127716) in cancer patients. *Cancer Res* 1986, **46**, 4831–4836.
25. Juttermann R, Li E, Jaenisch R. Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci USA* 1994, **91**, 11797–11801.
26. Suzuki H, Gabrielson E, Chen W, *et al*. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. *Nat Genet* 2002, **31**, 141–149.
27. Niki T, Rombouts K, De Bleser P, *et al*. A histone deacetylase inhibitor, trichostatin A, suppresses myofibroblastic differentiation of rat hepatic stellate cells in primary culture. *Hepatology* 1999, **29**, 858–867.
28. Sengupta PK, Smith EM, Kim K, *et al*. DNA hypermethylation near the transcription start site of collagen alpha2 (I) gene occurs in both cancer cell lines and primary colorectal cancers. *Cancer Res* 2003, **63**, 1789–1797.
29. Grimberg A, Cohen P. Role of insulin-like growth factors and their binding proteins in growth control and carcinogenesis. *J Cell Physiol* 2000, **183**, 1–9.
30. Zeng ZJ, Yang LY, Ding X, *et al*. Expressions of cysteine-rich61, connective tissue growth factor and Nov genes in hepatocellular carcinoma and their clinical significance. *World J Gastroenterol* 2004, **10**, 3414–3418.
31. Bittner M, Meltzer P, Chen Y, *et al*. Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 2000, **406**, 536–540.
32. 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* 1991, **21**, 103–107.
33. Stimson KM, Vertino PM. Methylation-mediated silencing of TMS1/ASC is accompanied by histone hypoacetylation and CpG island-localized changes in chromatin architecture. *J Biol Chem* 2002, **277**, 4951–4958.
34. Miyoshi A, Kitajima Y, Sumi K, *et al*. Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *Br J Cancer* 2004, **90**, 1265–1273.