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Array-based profiling of the differential methylation status of CpG islands in hepatocellular carcinoma cell lines

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Abstract. Alterations in the DNA methylation status particularly in CpG islands are involved in the initiation and progression of many types of human cancer. A number of DNA methylation alterations have been reported in hepatocellular carcinoma (HCC). However, a systematic analysis is required to elucidate the relationship between differential DNA methylation status and the characteristics and progression of HCC. In the present study, a global analysis of DNA methylation using a human CpG-island 12K array was performed on a number of HCC cell lines of different origin and metastatic potential. Based on a standard methylation alteration ratio of ≥2 or ≤0.5, 58 CpG island sites and 66 tumor-related genes upstream, downstream or within were identified. This study showed a series of CpG island methylation alterations in the HCC cell lines. The expression of various oncogenes, tumor suppressor genes and other key genes were up- or downregulated, respectively, resulting in CpG island hypomethylation or hypermethylation accordingly. To conclude, a foundation has been provided for screening CpG island methylation profiles as HCC biological markers.

Introduction

DNA methylation plays an essential role in various physiological processes such as mammalian development, genomic imprinting, X chromosome inactivation and aging (1). Alterations in the DNA methylation status were also found to be involved in the initiation and progression of human cancer. Among these alterations, the aberrant methylation of CpG islands located in the promoter regions or first exons of various genes is the best-categorized epigenetic change (2). By silencing tumor suppressor genes or activating oncogenes, epigenetic modifications can affect many important cellular processes, such as cell cycle control, DNA repair and apoptosis. Previously, the aberrant methylation status of specific genes was linked to the pathogenesis of various types of cancer, and tumor-specific methylation changes were established as prognostic markers in numerous tumor entities (3). Unlike genetic modifications, such as mutations or genomic imbalances, epigenetic changes are potentially reversible, making these changes particularly important therapeutic targets in cancer and other diseases (4).

In recent years, different techniques have been developed for the genome-wide screening of CGI methylation status. Included is differential methylation hybridization (DMH) which is a high-throughput DNA methylation screening tool that utilizes methylation-sensitive restriction enzymes to profile-methylated fragments by hybridizing them to a CpG island microarray. This method was introduced by Huang and coworkers in 1999 (5), further developed and has since been widely used (6).

Human hepatocellular carcinoma (HCC) is the sixth most common type of cancer worldwide and is the second cause of cancer-related death in China. The main obstacles to improving the outcome of HCC patients is the high frequency of recurrence and metastasis. Various genetic and epigenetic abnormalities in HCC have been identified, suggesting a multi-step nature of hepatocarcinogenesis (7). A number of genetic and epigenetic alterations have been noted in HCC (8). However, each alteration appears to be implicated in a limited fraction of HCC. Thus, the systematic analysis of the genetic and epigenetic alterations underlying the origin and evolution of HCC has attracted the interest of scientists worldwide (9-13). The present study employed the DMH method to analyze the differential methylation status of 12K CpG islands in a number of HCC cell lines. Tumor cell lines are commonly used as experimental tools in cancer research as such cell lines exhibit a number of the same epigenetic and genetic aberrations that are noted in primary carcinomas. The HCC cell lines selected in this study are of different origin, and among these, the MHCC97 series cell lines have the same genetic backgrounds but exhibit different metastatic potential. These characteristics render them useful for screening epigenetic alterations underlying the mechanism of metastasis.

Materials and methods

Cell culture. Human HCC cell lines HepG2, Hep3B, PLC/RPF/5 and human liver cell line L02 were purchased from

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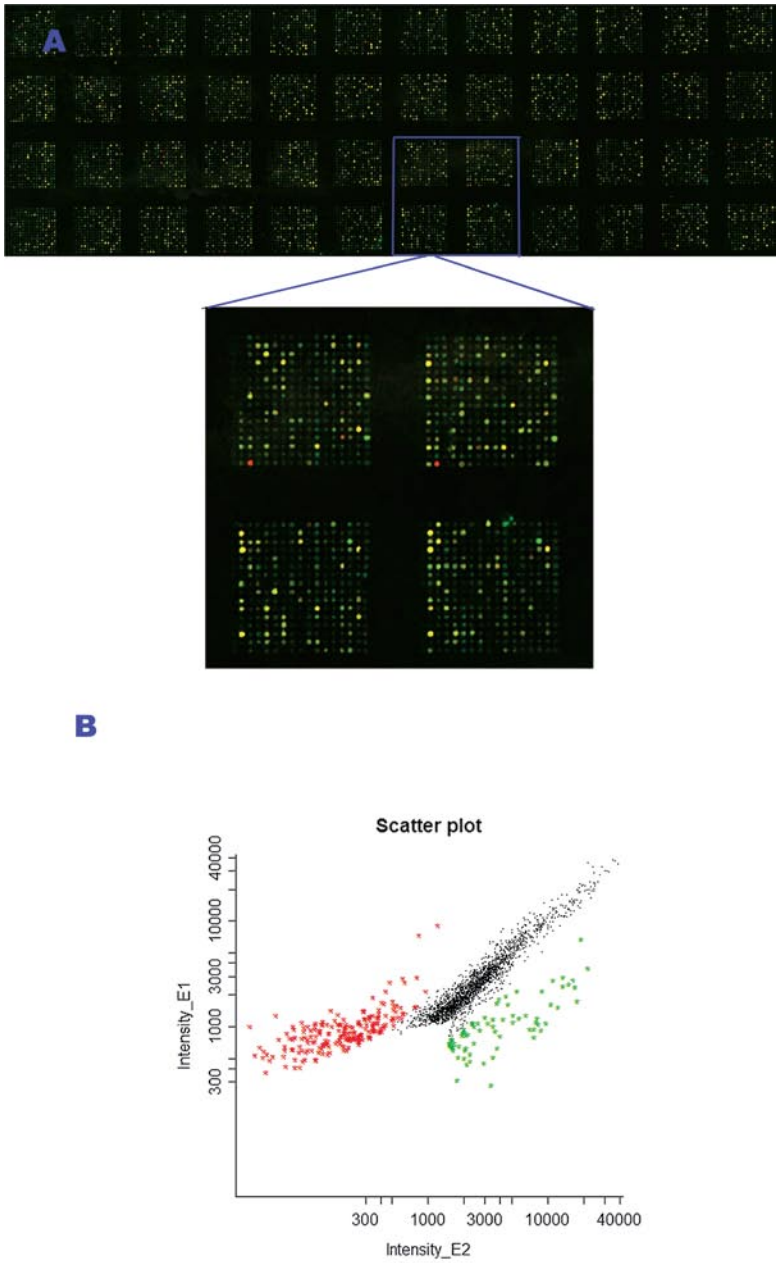

Figure 1. Representative results of differential methylation hybridization. (A) The DNA amplicons of HCC and control cell lines were fluorescently labeled with Cy5 and Cy3, respectively, and cohybridized to a UHN 12K human CpG island microarray. The hybridization output was measured in intensities of the two fluorescence reporters. The inset is an expanded view of the box area. The yellow spots indicate no methylation differences between the tumor and normal genomes. The red spots show hypermethylated CpG island loci present in the tumor genome. The green spots indicate loss of methylation in the tumor genome. (B) Scatter diagram of the hybridization results of the two samples. Each point in the scatter diagram represented the hybridization signal of corresponding CpG islands in the chip. The results show obvious differentiation of CpG island methylation between HCC and the control cell lines.

American Type Culture Collection (ATCC). SMMC-7721 and BEL-7402 cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The MHCC97-H, MHCC97-L, HCCLM3 and HCCLM6 cell lines, which have similar genetic backgrounds but different metastatic potential, were established at our institute. Cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin under humidified conditions in 5% CO_2.

CpG island microarray hybridization and data analysis. Total DNA of the cells was extracted using the DNeasy Kit (Qiagen, Germany). The CpG island array containing 12,192 CpG island clones was purchased from Canada UHN Microarray Center. Hybridization and signal scanning were conducted by the Capitalbio Corporation of Beijing, China. The hybridization images were analyzed using GenePix Pro 4.0 software (Axon Instruments). The data were smoothed using the Lowess method which was subsequently followed by cluster analysis. The ratios of Cy5 to Cy3 were calculated for each location on each microarray. A ratio (Cy5/Cy3) of ≥2 or ≤0.5 was considered to indicate differential methylation loci. Additionally, the hybridization output was measured in intensities of the two fluorescence reporters (Fig. 1).

Table I. Hypermethylated CpG islands and associated genes in the HCC cell lines.

ID of CpG island	Associated genes	
UHNhscpg0011158	PPP2CA	(upstream)
	CDKL3	(downstream)
UHNhscpg0009735	SRPK1	(downstream)
UHNhscpg0004629	CP110	(downstream)
UHNhscpg0004752	GJB2	(within)
	GJB6	(downstream)
	GJA3	(upstream)
UHNhscpg0004878	BCL2	(downstream)
UHNhscpg0002925	SMAD7	(downstream)
UHNhscpg0008205	IRS4	(upstream)
UHNhscpg0008444	FGFR2	(downstream)
UHNhscpg0003663	LHX5	(within)
UHNhscpg0007707	PIK3CB	(upstream)
UHNhscpg0004799	VRK2	(upstream)
	BCL11A	(downstream)
	FANCL	(within)
UHNhscpg0009327	TM4SF5	(upstream)
UHNhscpg0008203	PIM1	(upstream)
UHNhscpg0000104	BCL11A	(upstream)
UHNhscpg0008444	BRWD2	(upstream)
UHNhscpg0005488	PDCD4	(downstream)
UHNhscpg0008226	ST5	(downstream)
UHNhscpg0004835	CBX4	(within)
	CBX8	(upstream)
UHNhscpg0003460	CDH18	(upstream)
UHNhscpg0009830	EPHB4	(upstream)
UHNhscpg0003252	ATF2	(within)
UHNhscpg0004910	HNF4G	(downstream)
UHNhscpg0004957	LRP1	(upstream)
UHNhscpg0004834	GSTA4	(upstream)
	ICK	(within)
UHNhscpg0004596	RYK	(upstream)
UHNhscpg0003693	FZD3	(upstream)
UHNhscpg0001904	SPRY3	(downstream)
UHNhscpg0004926	MAPK8IP3	(downstream)
UHNhscpg0011292	SLIT2	(downstream)
UHNhscpg0003315	HAND2	(within)

Analysis of the genes associated with the differentially methylated CpG islands. The official website (http://data.microarrays.ca/cpg/) of the UHN human CpG island database was accessed, and the upstream, within and downstream genes associated with significant CpG islands were located according to their ID numbers. The references were searched and the associated genes were classified according to their function.

Bisulfite treatment of DNA- and methylation-specific PCR. The bisulfite treatment of DNA was adapted from Frommer *et al* with slight modifications (14). Briefly, 2 μg DNA was diluted with 50 μl distilled H_2O, and 5.5 μl 3 M NaOH was added. Incubation was carried out at 37˚C for 15 min to create single-stranded DNA. Freshly prepared 10 mM hydroquinone (30 μl) (Sigma) and 520 μl 3 M sodium bisulfite (pH 5.0) (Sigma, S-8890) were added to each tube. After thorough mixing, a layer of 200 μl mineral oil was added. Incubation was carried out at 50˚C for 16 h, and the oil was then removed. The modified DNA was purified using the Promega Wizard Cleanup DNA kit and precipitated at -20˚C overnight. Centrifugation was performed at 12,000 rpm for 30 min at 4˚C. The supernatant was removed using a pipette, and the precipitate was washed with 500 μl 75% ethanol and centrifuged at 12,000 rpm for 5 min at 4˚C, and repeated once. The supernatant was then removed using a pipette, and the dried pellet was resuspended in 30 μl distilled water and preserved at -20˚C until use.

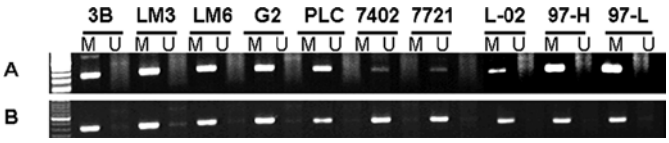

Figure 2. MSP results of two randomly selected CpG islands: (A) UHNhscpg0009830 and (B) UHNhscpg0004835. M and U are the methylated and unmethylated PCR products, respectively.

Two differential CpG islands were randomly selected and methylation-specific PCR (MSP) was performed to verify the microarray results. PCR primers were designed using MethPrimer (http://www.ucsf.edu/urogene/methprimer/index1.html). The PCR program involved an initial denaturation at 95˚C for 10 min, followed by 35 cycles of amplification as follows: denaturation at 95˚C for 30 sec, annealing at 48˚C for 30 sec, and extension at 72˚C for 30 sec, with an additional extension at 72˚C for 4 min. The PCR products were analyzed with agarose gel electrophoresis and DNA sequencing.

Results

The DNA amplicons of different HCC cell lines, with Chang's liver cell line as a control, were fluorescently labeled with Cy5 and Cy3, respectively, and cohybridized to a UHN 12K human CpG island microarray. Findings showed obvious differentiation of CpG island methylation between HCC and the control cell lines (Fig. 1). After further cluster analysis, we detected 58 differentially methylated CpG islands and 66 related tumor-associated genes that exhibited a similar trend in alteration (6 of the 9 cell lines had an identical trend) in the 9 HCC cell lines compared with the normal control. Among these, 37 CpG islands showed hypermethylation (Table I) and 29 showed hypomethylation (Table II). In addition, when compared with the MHCC97-L cells, 16 differentially methylated CpG islands that may be associated with metastatic potential were detected in the MHCC97-H cells (24 related genes) (Table III).

Two randomly selected differential CpG islands were further analyzed with MSP and DNA sequencing in the HCC cell lines. The results showed a high correlation with the microarray results (Fig. 2). The methylation status of a number of other significant CpG islands and expression of the associ-

Table II. Hypomethylated CpG islands and associated genes in the HCC cell lines.

ID	Associated genes	
UHNhscpg0006172	SEC13L1	(upstream)
UHNhscpg0002224	PRC1	(upstream)
UHNhscpg0008081	RAE1	(within)
UHNhscpg0007289	HNF4G	(upstream)
UHNhscpg0002482	CDCA7L	(upstream)
UHNhscpg0006623	EPHA7	(within)
	MAP3K7	(upstream)
UHNhscpg0005013	PECAM1	(within)
UHNhscpg0003756	PA2G4	(upstream)
UHNhscpg0002482	CDCA7L	(upstream)
UHNhscpg0001077	EGR3	(downstream)
UHNhscpg0002020	NAB2	(downstream)
UHNhscpg0007707	PIK3CB	(upstream)
UHNhscpg0002943	PPIAL4	(upstream)
UHNhscpg0009335	MAF	(upstream)
UHNhscpg0010477	ID2	(downstream)
UHNhscpg0005863	ZNF278	(within)
UHNhscpg0001373	MAPRE2	(downstream)
UHNhscpg0005140	PTPNS1	(downstream)
UHNhscpg0000025	VCL	(downstream)
UHNhscpg0002244	DPYSL2	(within)
UHNhscpg0004830	THBS1	(within)
UHNhscpg0001262	NR5A2	(within)
UHNhscpg0009002	DYNLRB2	(downstream)
UHNhscpg0002482	RAPGEF5	(downstream)
UHNhscpg0004867	RIN3	(downstream)
UHNhscpg0009717	IRS4	(upstream)
UHNhscpg0008232		
UHNhscpg0001274	ROBO3	(within)
UHNhscpg0005005	SV2B	(within)

Gene function determined from GeneCards at http://www.genecards.org.

ated gene were also detected with MSP and RT-PCR with the aim of analyzing the association of gene expression with DNA methylation status.

Discussion

Tumorigenesis and tumor metastasis are complex, multi-step processes involving genetic and epigenetic alterations. The roles of epigenetic alterations, particularly that of DNA methylation status, in the progression and metastasis of HCC recently attracted the interest of numerous investigators. Due to HCC sensitivity to the influence of external factors as opposed to the DNA sequence, alterations in the DNA methylation status may be the earliest event in tumorigenesis and metastasis. The reversible characteristics of the DNA methylation status make it attractive in tumor intervention (15,16).

The present study employed the differential methylation hybridization method to assess the alteration of CpG island methylation in a number of HCC cell lines. The results showed that alteration of the CpG island methylation is a common event in HCC cell lines of different origin and metastatic potential. The genes associated with these CpG islands included pro-oncogenes and oncogenes, tumor suppressor genes, apoptosis genes, proliferation and cell cycle regulatory genes, as well as tumor angiogenesis- and immuno-escape-associated genes. The concept of a CpG island methylator phenotype (CIMP) was first proposed by Issa *et al* (17) and is currently widely accepted as a novel type of biomarker employed to classify different types of cancer or cancers of different stages (17,18). The present array-based study profiled the CpG island methylation status in HCC cell lines of different origin and metastatic potential. The potential of an HCC-specific CIMP was also noted, particularly since numerous liver cell-specific genes were involved in the differences. As a 'marker pattern', CIMP has a marked superiority in reflecting the clinical complexity and variability of cancer versus the use of a single marker.

Recently, molecular alterations of various signaling pathways (e.g., Wnt/β-catenin, Ras/Raf/MEK/ERK and PI3K/Akt/mTOR) were identified in liver carcinogenesis, providing novel molecular targets for new treatment modalities (19,20). Sorafenib, the first FDA-approved drug for advanced liver cancer, is a multi-kinase inhibitor that targets the Ras/Raf signaling pathway (21). A DNA methylation mechanism was involved in the modification of these signaling pathways. Hypermethylation in the promoters of the Wnt pathway associated with *adenomatous polyposis coli* and E-cadherin was recently reported (22-24). Based on our data, a number of other members involved in the Wnt pathway also displayed alterations in the methylation status in associated CpG islands. These members included frizzled 3 (FZD3), receptor

Table III. Genes associated with the differentially methylated CpG islands in the MHCC97H and MHCC97L cell lines[a].

Cell line	Associated genes
97H/97L	
With hypermethylation in the CpG island	BCL11A, VRK2, FANCL, GJB2, GJB6, GJA3, CBX4, CBX8, ICK, ATF2, LRP1, GSTA4, TM4SF5
With hypomethylation in the CpG island	THBS1, ZNF278, IRS4, HNF4G, CDCA7L, RAPGEF5, PPIAL4, PA2G4, PIK3CB, MAF, DYNLRB2

[a]The cell lines have the same genetic background but different metastatic potential.

tyrosine kinase (RYK), c-myc, ID2 and MAPRE2, which showed hypomethylation in the associated CpG islands in the HCC cell lines compared with the control cells, suggesting that DNA methylation is a crucial mechanism that adjusts the Wnt signaling pathway to favor HCC. On the other hand, alterations in the methylation of factors involved in the Ras/Raf pathway were also found in this study, including RAPGEF5, which serves as a Ras activator and Ras and Rab interactor 3 (RIN3).

Numerous other important CpG islands and the associated genes previously investigated in other solid tumors were also analyzed and verified in our data. EPHB4 is a member of the Eph family of receptor tyrosine kinases. Its interaction with the ligand ephrinB2 contributes to the growth of various types of tumors and can influence the clinical outcome of cancer patients (25-27). According to our data, the EPHB4-associated CpG island was hypermethylated, and the expression of the EPHB4 gene was downregulated in the HCC cell lines. The role of the EPHB4 gene in HCC progression warrants further investigation. Slit and Roundabout (Robo) were first identified as guidance molecules for neurons and leukocytes (28). More recently, their roles were expanded to include the mediation of angiogenesis, heart morphogenesis and tumor metastasis (29-32). In vertebrates three Slit (Slit1, Slit2 and Slit3) and four Robo (Robo1, Robo2, Robo3/Rig-1 and Robo4/Magic Robo) genes have been identified (33). Certain members of the Slit/Robo signaling pathway also showed frequent alterations of the CpG island methylation status in our data. Further investigations of the methylation status of CpG islands in the promoter region and mRNA expression levels of Slit1, Slit2, Slit3, Robo1 and Robo3 were subsequently carried out in several HCC cell lines of different metastatic ability (data not shown). A number of differences in the methylation and expression of the genes were detected in different HCC cell lines. In particular, Slit2 expression may be associated with metastatic potential, indicating a potential involvement of Slit/Robo in the progression or metastasis of HCC.

Various genes of unknown function also appeared in the list due to regular alterations between HCC and the control cell lines. The potential roles of these genes in the progression and metastasis of HCC warrant further investigation.

In conclusion, the differentially methylated CpG islands and associated genes screened by microarray in the HCC cell lines in this study have provided the foundation for understanding HCC-specific CIMP and for developing potential biomarkers of significance for the prognosis and metastasis of HCC. Furthermore, the reversible characteristic of DNA methylation status offers suitable targets for the interference therapy of HCC.

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