SPANDIDOS PUBLICATIONS

DNA cytosine methylation profile in various cancer-related genes is altered in cultured rat hepatocyte cell lines as compared with primary hepatocytes

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Abstract. We determined the DNA cytosine methylation status in the promoter CpG islands of eight cancer-related genes (*p16*, *Socs-1*, *Rassf1A*, *Hic-1*, *Dlc-1*, *Timp-1*, *Timp-2*, and *Timp-3*) in five rat hepatocyte cell lines, including normal cell lines (Clone 9 and CWSV-1) and tumor cell lines (H4-II-E-C3, MH1C1, and McA-RH7777). The experimental methods used to assess the methylation profile were methylation-specific PCR (MSP) and methylation-sensitive digestion combined with PCR. The results were compared with the methylation status of rat primary hepatocytes. To evaluate methylation-mediated gene induction/silencing, the expression of gene transcripts was semi-quantitatively assessed using RT-PCR. In primary cells, the CpG islands of all genes tested were unmethylated. In contrast, there was at least one hypermethylated gene in the cultured cell lines. Three genes (*p16*, *Socs-1* and *Rassf1A*) were hypermethylated in Clone 9 cells; among the other five genes, three genes (*Hic-1*, *Timp-1* and *Timp-3*) were hypermethylated in the CWSV-1 cell lines and two genes (*Dlc-1* and *Timp-2*) were hypermethylated only in the tumor cell lines. The methylation status in some of the tested genes was altered at an early stage of cell culture as compared to primary cells. It is also noteworthy that hypermethylation in *Socs-1*, *Rassf1*, *Hic-1*, and *Timp-3* was widespread among the cell lines tested, but not in the primary cells and Clone 9 cells. This study suggests that a cautious approach is required when cell lines are utilized to study methylation-related carcinogenic, metastatic or tumoricidal mechanisms.

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Introduction

Established cell lines have been employed in the study of carcinogenic, metastatic, and tumoricidal mechanisms (1-3). However, it is possible that the carcinogenic mechanism found in cell lines may not be the same *in vivo* (4,5). Also, tumoricidal agents that are effective in cancer cell lines may not necessarily be effective cancer-killing agents *in vivo* (6). We speculate that the difference in epigenetic DNA cytosine methylation status in cell lines and primary cells may play a role in such a discrepancy.

Epigenetic DNA cytosine methylation is believed to be one of the root causes of cancer. Indeed, in hepatocellular carcinoma (HCC), some cancer-related genes have been shown to be aberrantly methylated. For example, in human HCC, epigenetic changes of tumor suppressor genes have been demonstrated in *p16*/retinoblastoma (RB) and *p53*/*p21* pathways (7,8). In a rat model of HCC, i.e. dietary choline deficiency-induced HCC, it has been shown that promoter CpG islands in several tumor-suppressor genes and oncogenes are aberrantly methylated in tumor tissues (9-15). There is a cellular model of rat HCC, where CWSV-1 cells have been shown to become tumorigenic when cultured in choline-deficient medium (16,17). In our preliminary experiment using CWSV-1 cells, we found that the DNA methylation profiles of some cancer-related genes are altered as compared with primary cells even before culturing in choline-deficient conditions. Such observation prompted us to determine DNA cytosine methylation profiles in available rat hepatocyte cell lines.

We selected three normal rat hepatocyte cells and cell lines (primary, Clone 9, and CWSV-1) and three tumor hepatocyte cell lines (H4-II-E-C3, MH1C1, and McA-RH7777) and determined the methylcytosine content in the promoter CpG islands of eight genes; *p16* (7), *Suppressor of cytokine signaling-1* (*Socs-1*) (18), *RAS association domain family 1A* (*Rassf1A*) (19), *Hypermethylated in cancer-1* (*Hic-1*) (20), *Deleted in liver cancer-1* (*Dlc-1*) (21), and *Tissue inhibitor of metalloproteinases* (*Timp-1*, *Timp-2* and *Timp-3*) (22). Previously, these genes have been shown to be aberrantly methylated and silenced in the tissue of various cancers, and

Table I. Rat hepatocyte cell lines.

Cell line	ATCC no.	Gender	Normal/tumor	Description
Primary		M	N	From adult male Wistar rats
Clone 9	CRL-1439	M	N	Epithelial cell line from a young male rat
CWSV-1		M	N	p53 inactivated by large antigen
H4-II-E-C3	CRL-1600	M	T(HCC)[a]	(55)
MH1C1	CCL-144	F	T(HCC)	Established from the transplantable Morris hepatoma
McA-RH7777	CRL-1601	F	T(HCC)	Established from the transplantable Morris hepatoma

[a]HCC, hepatocellular carcinoma.

the causal relationship to carcinogenesis was demonstrated. We employed methylation-specific PCR (MSP) and methylation-sensitive digestion combined with PCR for the assessment of methylation. The results indicated that there were significant alterations in methylation status compared with primary cells in all cell lines tested. Considering that such alterations may not be limited to rat hepatocytes, we believe that these data would serve as a cautionary note when established cell lines are used to study methylation-related carcinogenic, metastatic or tumoricidal mechanisms.

Materials and methods

Cell lines and genomic DNA/total RNA extraction. The primary cells and cell lines used in this study are listed in Table I together with brief descriptions. Primary rat hepatocyte cells were isolated from adult male Wistar rats by following the animal experiment protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the Oklahoma Medical Research Foundation. Rat livers were cut into pieces in the medium and partially digested with collagenase, and cells were isolated with filtration. The five rat hepatocyte cell lines used in this study are listed in Table I. The CWSV-1 cell line, a cellular model of choline deficiency-induced hepatocarcinogenesis, was a gift from Dr Hariett Isom, Pennsylvania State University, University Park, PA, USA. Other cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in the medium as specified in the previous report or ATCC's instructions. Chemicals for medium preparation were purchased from Sigma Chemical Company (St. Louis, MO). Cells were harvested after less than 10 passages when stable division was accomplished. Some cells were harvested after a prolonged culture period to determine the age dependence of methylation status. Genomic DNA was extracted using a Qiagen DNeasy kit (Qiagen, Crawley, UK), and total RNA was extracted with Tri reagent (Sigma Chemical Co.). The genomic structures of the eight tumor-related genes are illustrated in Fig. 1.

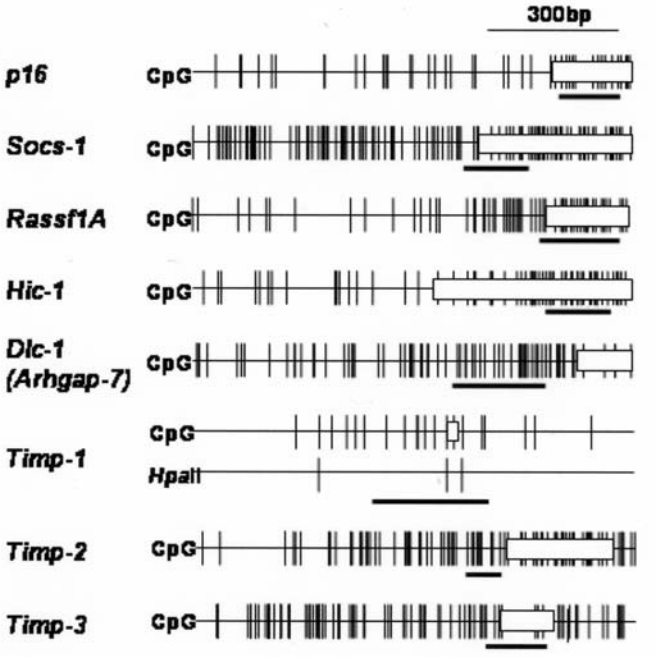

Figure 1. Genomic structures of tumor-related genes. Each short tick shows a single CpG site or *Hpa*II-sensitive site, and blank boxes show Exon1. PCR (MSP and PCR after digestion) was performed at the region marked with thick horizontal lines.

Methylation-specific PCR. Methylation analysis for selected genes (except *Timp-1*) was performed by methylation-specific PCR (MSP) of sodium bisulfite-treated DNA as described previously (23). Briefly, genomic DNA was digested by restriction enzyme into shorter fragments and PCR-amplified followed by chemical treatment with sodium bisulfite. To confirm the specificity, genomic DNA from the primary cells was artificially methylated by *Sss*I methylase and used as a positive control primer for the methylated sequence. All primer sets for unmethylated sequences can amplify PCR products from primary cell DNA. Primers were custom synthesized by the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA. PCR conditions, primer sequences and GenBank accession numbers are shown in Table II. Primer sequences for the *p16* gene are the same as previously reported (24). Densitometric analysis was performed using an imaging workstation (NucleoTech Corporation, Hayward, CA, USA).

Methylation-sensitive digestion and PCR. This method was applied specifically to determine *Timp-1* gene methylation. After genomic DNA was digested by the restriction enzyme *Hpa*II (methylation-sensitive) or *Msp*I (methylation insensitive), the product was PCR amplified using primers specific for the methylated sequence. The primer sequences of *Timp-1* were

Primer sequences for MSP.

Gene	GenBank no.	U/M	Primer sequence (5'→3')	Annealing temperature (˚C)
p16	AB081658	U	GTGAATTTGAGGAGAGTGATTTG	55
		U	CAAAACATTTAATAAAACCCCAA	
		M	AATTCGAGGAGAGCGATTCG	60
		M	AACGTTTAATAAAACCCCGA	
Socs-1	AJ243123	U	TTTTTATTAGTGTAGTTTTGGAT	55
		U	AAAAATAACTCTAACCACCA	
		M	TTTTTATTAGCGTAGTTTCGGAC	55
		M	GAAAATAACTCTAACCGCCG	
Rassf1A	NW_047801	U	TTTGATTGGGTTATGTTGGT	55
		U	AATAACCACAACCCAAAACA	
		M	TTTGATCGGGTTATGTCGGC	60
		M	GATAACCACGACCCGAAACG	
Hic-1	NW_047336	U	TGTGTTGTTTTTTGTTTGTT	55
		U	ACATCTACCAATTCAAACCA	
		M	TGCGTTGTTTTTCGTTCGTC	60
		M	ACGTCTACCGATTCGAACCG	
Dlc-1 (*Arhgap-7*)	NW_047473	U	GTTTTTGTTGTTGGAGTTGT	55
		U	ATAATCAAAATCCCACATCA	
		M	GTTTTCGTTGTTGGAGTCGC	60
		M	AATAATCGAAATCCCACGTCG	
Timp-2	NW_047344	U	GGTAGTGGAGGAGTTGAGTT	60
		U	ACAAAACAATACACCCAACA	
		M	GGTAGCGGAGGAGTCGAGTC	60
		M	GCAAAACAATACACCCGACG	
Timp-3	NW_047773	U	AAAGAATGGTAGTTTTTGTAGT	55
		U	CAAACATATACACACTTCCA	
		M	AAAGAGCGGTAGTTTTCGTAGC	55
		M	CGAACATATACACGCTTCCG	

forward, 5'-CAAGTTGAATGGTTAGTCTGGC-3', and reverse, 5'-GCTGGCAGAAGAGGGAGTAC-3'; and PCR conditions were 94˚C for 10 min, 30 cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec, then 72˚C for 5 min. The GenBank accession number of *Timp-1* is NW_048034.

Semi-quantitative RT-PCR. In order to evaluate methylation-mediated gene silencing/induction, gene transcripts were quantified using semi-quantitative RT-PCR with the Super-Script III One-Step RT-PCR system (Invitrogen, Carlsbad, CA). The PCR conditions, primer sequences and GenBank accession numbers of each gene are shown in Table III. The primer sequences for *Timp1,2* and the glyceraldehyde phosphate dehydrogenase gene (*Gapdh*) were the same as previously reported (25). The expression level of *Gapdh* was used to determine and confirm the quality of total RNA from each cell line.

Results

Methylation status in normal rat hepatocyte cell lines. The methylation status of eight tumor-related genes in five cell lines was analyzed by MSP and methylation-specific digestion. Gel fluorograms are shown in Fig. 2, and analyzed results are listed in Table IV. There were discrepancies in the methylation status between normal hepatocyte cell lines (Clone 9 and CWSV-1) and primary cells for the genes, *p16*, *Socs-1*, *Rassf1A*, *Hic-1*, *Timp-1*, and *Timp-3*. The *p16*, *Socs-1* and *Rassf1A* genes are hypermethylated in both Clone 9 and CWSV-1 cells, while primary cells showed no signs of hypermethylation in these genes.

Methylation status in rat tumor cell lines. The most frequently methylated tumor-related genes in rat tumor cell lines were *Rassf1A* (3/3, 100%), *Hic-1* (3/3, 100%), *Timp-3* (3/3,

Table III. Primer sequences for RT-PCR.

Gene	GenBank no.	Primer sequence (5'→3')	Annealing temperature (˚C)	Cycles
p16	L81167	GCCGTGAGCACGAGGTG CCCAGCGGAGGAGAGTAGAT	60	23
Socs-1	NM_145879	CCGCTCCCACTCTGATTACC TAGTGCTCCAGCAGCTCGAA	60	29
Rassf1A	AB202124	TTCATCTGGGGCGTCGT CTGCAAGGAAGGCGGTTTCT	60	26
Hic-1	XM_220706	CCTGTGACAAGAGCTACAAG ATATGACTGATGAGGTTGCG	55	27
Dlc-1 (*Arhgap-7*)	XM_341444	GCCGCCCTTAATGTGTAGAG GTCCACTTGCCGCTTATG	60	28
Timp-1	NM_053819	ACAGCTTTCTGCAACTCG CTATAGGTCTTTACGAAGGCC	56	20
Timp-2	NM_021989	ATTTATCTACACGGCCCC CAAGAACCATCACTTCTCTTG	56	20
Timp-3	NM_012886	GGAGCCTTGGGCACTG CATCTTGCCTTCATACACGC	60	23
Gapdh		AAACCCATCACCATCTTCCA GGCAGTGATGGCATGGACTG	55	20

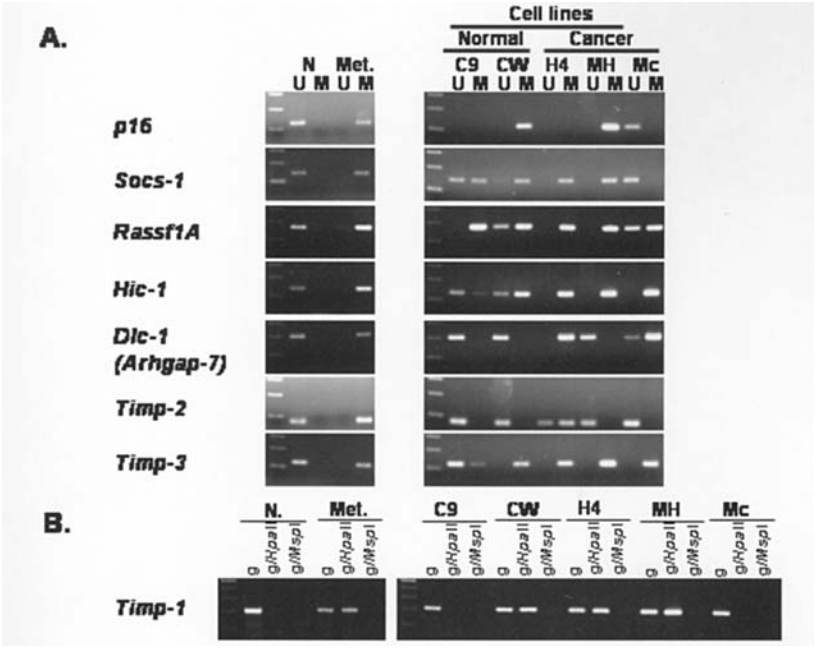

Figure 2. MSP and methylation-specific digestion/PCR results of tumor-related genes in rat hepatocytes. (A) Methylation-specific PCR and (B) methylation-specific digestion/PCR for *Timp-1*. N, primary cells; Met., primary cells, *in vitro* methylated by *Sss*I; C9, Clone 9; CW, CWSV-1; H4, H4-II-E-C3; MH, MH1C1; Mc, McA-RH7777; U, PCR with unmethylated sequence-specific primers; M, PCR with methylated sequence-specific primers; g, PCR from genomic DNA; g/*Hpa*II, PCR from *Hpa*II-digested DNA; g/*Msp*I, PCR from *Msp*I-digested DNA.

100%), *Socs-1* (2/3, 67%) and *Dlc-1* (2/3, 67%) (Table IV). The *p16* gene was hypermethylated in one of the two hepatocellular carcinoma cell lines and was deleted in H4-II-E-C3 cells. The *Dlc-1* and *Timp-2* genes were exclusively hyper-

Methylation status and expression of tumor-related genes.

		Normal			Tumor		
		Prim.	C 9	CW	H4	MH	Mc
p16	Methylation	U	ND	M	ND	M	U
	Expression	ND	-	-	-	-	+
Socs-1	Methylation	U	U=M	M	M	M	U
	Expression	ND	+	++	+	+	+
Rassf1A	Methylation	U	M	U<M	M	M	U<M
	Expression	ND	-	+	-	-	+
Hic-1	Methylation	U	U>M	U<M	M	M	M
	Expression	ND	+	-	-	-	-
Dlc-1	Methylation	U	U	U	M	U	U<M
(*Arhgap-7*)	Expression	ND	+	++	-	+	-
Timp-1	Methylation	U	U	M	M	M	U
	Expression	ND	+	-	-	-	+
Timp-2	Methylation	U	U	U	U<M	U	U
	Expression	ND	++	+	-	+	-
Timp-3	Methylation	U	U>M	M	M	M	M
	Expression	ND	+	-	-	-	-

Normal, normal cells; tumor; tumor cells; prim., primary cells in normal liver tissue; C9, Clone 9; CW, CWSV-1; H4, H4-II-E-C3; MH, MH1C1; Mc, McA-RH7777; U, unmethylated; ND, not determined; M, methylated. The population difference between unmethylated and methylated sequences are shown by >, <, or = when both unmethylated and methylated primers amplify products in MSP. The expression level of each gene is shown from - to ++.

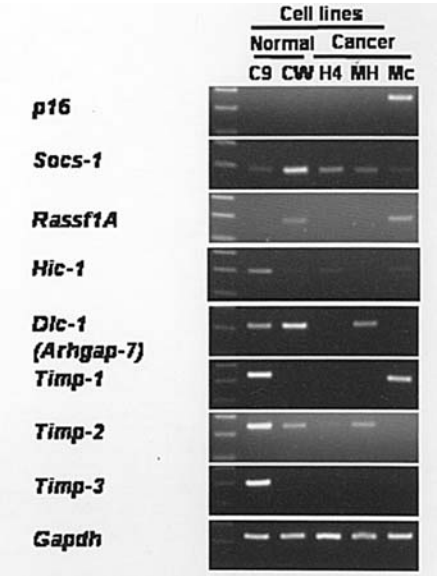

Figure 3. Expression of tumor-related genes as determined by semi-quantitative RT-PCR. C9, Clone 9; CW, CWSV-1; H4, H4-II-E-C3; MH, MH1C1; and Mc, McA-RH7777.

methylated in hepatocellular carcinoma cell lines compared to normal hepatocyte cell lines. The *Timp-2* gene was aberrantly and solely hypermethylated in H4-II-E-C3 cells.

Methylation status and expression of tumor-related genes. The gel fluorogram for RT-PCR analysis of gene transcripts indicated that expression level and methylation status correlated well, except for that of the *Socs-1* and *Timp-2* genes (Fig. 3 and Table IV). In CWSV-1 cells, expression of the *Socs-1* gene was slightly higher than that of the other genes but it did not correlate with methylation status. The *Timp-2* gene was unmethylated but not expressed in McA-RH7777 cells.

Discussion

DNA methylation plays a crucial role in repression of tumor suppressor genes in many cancers, including hepatocellular carcinoma. Thus, a growing number of genes have been shown to be hypermethylated and repressed in hepatocarcinogenesis. Several investigators have reported the methylation profiles of human HCC (26-28), demonstrating the accumulation of aberrant DNA methylation during multistep hepatocarcinogenesis. In rat HCC models, both exogenous and endogenous factors are committed to carcinogenesis; however, the underlying mechanism is still largely obscure. Rat genome sequencing has been completed (29), and it is possible to study the rat counterpart of human tumor-related genes.

Established cell lines have been conveniently used to study carcinogenic and tumoricidal mechanisms, and cancer epigenetic studies are no exception. Therefore, the purpose of

this study was to provide data illustrating that cell lines employed in rat cancer model studies may not always represent the situation in cancer tissues *in vivo* in terms of DNA methylation status. Previously, such a discrepancy has been pointed out in human cancer cells (30). The present study shows that, in primary liver cells isolated from rat liver tissue, none of the selected eight genes were hypermethylated in their promoter CpG islands; however, there was at least one hypermethylated gene in the cell lines (Table IV). It is also noteworthy that hypermethylation in *Socs-1*, *Rassf1A*, *Hic-1*, and *Timp-3* was widespread among the cell lines tested but not among primary and Clone 9 cells, suggesting that Clone 9 cells are close to normal cells in terms of DNA methylation.

Although the primary objective of this report was to provide methylation profile data for selected cell lines, it is necessary to discuss the cause and outcome of aberrant methylation in each gene. *p16*: in human HCC, two major tumor-suppressor pathways of p16/retinoblastoma (RB) and p53/p21 are frequently altered (7,8). The *p16* gene is known to be inactivated by mutations, homozygous deletion, or promoter hypermethylation. The homozygous deletion of this gene was found in 7-30% of human HCC tissues and 0-29% in human HCC cell lines (7,31). In our study, the probable homozygous deletion of *p16* was found in the normal Clone 9 hepatocyte cell line and one of three HCC cell lines (33%), i.e. H4-II-EC3 cells. Promoter hypermethylation of the *p16* gene was found in one of the three HCC cell lines (33%), i.e. MH1C1 cells. It is possible that hypermethylation of the *p16* gene in the normal hepatocyte cell line was achieved during *in vitro* growth, similar to the case in a previous study on normal breast epithelial cells (32).

Socs-1, *Rassf1A*, *Hic-1*, *Timp-1*, and *Timp-3* genes in normal Clone 9 and CWSV-1 hepatocyte cell lines are altered from the early stage of culture. The possible reasons are as follows: 1) normal tissue is a mixture of various kinds of cells, such as epithelial cells and interstitial cells, each of which could have different methylation patterns; 2) in developing cell lines, some genes become silenced by methylation because the expression of genes is no longer essential (33); and 3) aberrant DNA methylation of some genes, such as *p16,* is critical for autonomous growth *in vitro* (34,35). The SOCS-1 protein was discovered to be involved in a negative feedback loop of cytokine signaling (18,36,37). This gene was found to be frequently hypermethylated and repressed in human HCC (38). In our study, promoter hypermethylation of the *Socs-1* gene was found in many cell lines, however, the correlation between methylation and gene expression was poor (Table IV). Because DNA methylation in exon 2 of the *SOCS-1* gene has been shown to regulate its expression in human HCC (39), methylation might also be important in rat HCC cell lines. Our data indicate that *Socs-1* and *Rassf1A* were aberrantly hypermethylated in both normal hepatocyte cell lines, Clone 9 and CWSV-1. Supporting our data, hypermethylation of the *SOCS-1* gene was even shown in normal liver tissue (27). Previously, *Rassf1A* has been shown to be heterogeneously hypermethylated in non-tumor tissue (40).

Hic-1 was identified by Wales *et al* in 1995 (20), and DNA hypermethylation at the D17S5 locus and its reduced expression have been shown in human HCC (41). The present study shows that *Hic-1* is frequently hypermethylated in rat liver cell lines, including the normal CWSV-1 hepatocyte cell line. Because the *p53* gene is inactivated in CWSV-1 cells, *Hic-1* may cooperate with the p53 but not the *p16* locus (42). However, the fact that both *Hic-1* and *p16* are hypermethylated and inactivated in this study is not consistent with this notion.

Timp-1, *-2*, and *-3* are physiologic tissue inhibitors of the metalloproteinases called *Timp,* of which four different forms are known in rats. An imbalance between matrix metalloproteinases (MMPs) and *Timp* is linked to the degeneration of the extracellular matrix associated with angiogenesis, invasion and metastasis (22). Because *Timp-4* is known to not be expressed in the liver (43), we studied *Timp-1*, *-2,* and *-3*. In *Timp-2*, there was only a fair correlation between methylation status and gene expression. However, in the human *Timp-2* gene, it was thought that methylation of the promoter CpG island has no influence on gene expression (44). Aberrant methylation in the human *Timp-3* gene is well documented in many cancers (45,46). In rat HCC cell lines, we showed that *Timp-3* was frequently hypermethylated (3/3, 100%). However, in a normal CWSV-1 hepatocyte cell line, *Timp-3* was also aberrantly hypermethylated. In human HCC, the Timp-1 protein concentration is reported to be higher or similar in HCC as compared to normal tissue (47-49). It was also shown that the Timp-1 protein has the ability to reduce metastasis (50). Our study showed that *Timp-1* was frequently hypermethylated and repressed in rat HCC cell lines (2/3, 67%), and this is the first report describing the aberrant hypermethylation of the *Timp-1* gene in HCC. Furthermore, *Timp-1* has been shown to exist on the X chromosome, and DNA methylation regulates its expression (51). The gender of the MH1C1 and McA-RH7777 cell lines that we used was female. Thus, they should have had one methylated X chromosome and one unmethylated X chromosome before developing cancer, while the MH1C1 cell line only has a methylated X chromosome and McA-RH7777 only has an unmethylated X chromosome. In the present study, we saw a distinct change in aberrant methylation in each HCC cell line. These cells could be good cellular models to study aberrant gene methylation on the X chromosome during hepatocarcinogenesis.

Dlc-1 was identified by Yuan *et al* and previously shown to be frequently hypermethylated or deleted in human HCC (21,52). It has 80% homology with rodent *Arhgap-7*, a GTPase-activating protein for Rho family proteins. It was also demonstrated that the ectopic expression of *Dlc-1* inhibits cell growth and migration *in vitro* and induces apoptosis and reduces tumorigenecity *in vivo* (53). In this study, the *Dlc-1* gene was not deleted but frequently and specifically hypermethylated in HCC cell lines, suggesting that this gene may play a critical role in rat hepatocarcinogenesis. Although CWSV-1 cells are not transplantable to nude mice (54), they become tumorigenic and transplantable when cultivated in choline-deficient medium (17). *Dlc-1* could be a candidate gene involved this process. In summary, we report a cautionary note that DNA methylation profiles in established cell lines may not be the same *in vivo*.

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