INTRODUCTION

In vertebrates, addition of a methyl group at the 5-carbon position of cytosine occurs specifically on the cytosines that precede guanines in DNA strands (CpG dinucleotides). This DNA methylation is maintained by heritability after DNA replication (1). Distinct DNA methylation patterns are developmentally and tissue specific, both in overall 5-methylcytosine content and in the sites modified at specific genes (2, 3). DNA methylation fulfills an important function in both inactivation of the X chromosome and in genomic imprinting (4, 5). Subsequently, it has also been hypothesized that this process controls tissue-specific gene expression and immobilizes mammalian transposons (5). Considering that cancer occurs on the basis of a change in gene expression, and DNA methylation partially controls gene expression, it is presumed that aberrant DNA methylation is implicated in tumorigenesis (6, 7). Indeed, alteration of global DNA methylation pattern have been depicted as important factors in multistep carcinogenesis in colorectal cancers (27, 40–42). Genomic-wide hypomethylation in HCC has been observed (18). However, the mechanism eliciting genome-wide demethylation in HCC and the implications of alteration of genomic methylation in hepatocellular carcinogenesis remain to be clarified.

In the current study, the global DNA methylation status in HCC was studied and correlated to both the clinical data of patients and to histological HCC grades. The mechanism and consequences of changes of genome-wide methylation in HCC were elucidated.

MATERIALS AND METHODS

Tissue. Samples, including HCC and matched non-HCC liver tissues, were obtained from 17 patients who had surgery at Chang Gung Memorial Hospital. Three liver samples were obtained from three patients without liver disease and were included as normal controls. The samples were frozen immediately in liquid nitrogen after resection and stored at −80°C until processing. Diagnoses of HCC and matched nonhepatoma liver tissues were based on histopathological findings. Furthermore, the Ethics and Science Committee of Chang Gung Memorial Hospital approved specimen collection procedures, and informed consent was obtained from each subject or subject’s guardian.

Global DNA Methylation Assay. DNA from surgical resection samples was extracted with a standard method (43). Differences in the DNA 5-methylcytosine contents were determined by analyzing the DNA methyl-accepting capacity with [methyl-3H]SAM and S-adenosylmethionine (16). S-Adenosylmethionine specifically catalyzes the transfer of methyl groups from SAM to cytosine residues within CpG dinucleotides. In this regard, it shared the same specificity as eukaryotic DNA methyltransferase. In brief, 2 μg of DNA were observed in several cancers (20–29). It remains to be seen, however, when, how, and why genome-wide hypomethylation occurs in cancers.

More than 90% of all 5-methylcytosine lies within the transposons, including Alu, SINE, and LINE, which are comparatively rich in CpG dinucleotides and represent more than one-third of the human genome. Cytosine methylation within these CpG dinucleotides is thought to limit the ability of retrotransposons to be activated and transcribed and to participate in recombination. It is therefore proposed that genome-wide demethylation endangers genome stability (5). LINE-1 retrotransposons constitute a maximum of 15% of the human genome (30). Gene disruption by insertion of LINE-1 retrotransposons has been discovered in human cancer and genetic diseases (31–35). In addition, LINE-1 sequences have been identified at or near chromosomal translocation sites (36–38). On the other hand, methylation of LINE-1 promoter sequences has been shown to repress their activity (39). It is therefore tempting to speculate that genome-wide hypomethylation in human cancer activates LINE-1 retrotransposons.

HCC is one of the most abundant neoplasms in the world. Although HCC conventionally occurs in a liver with underlying disease such as chronic hepatitis B and/or C, it is generally believed that genetic alterations are the basis for this. Alterations in the DNA methylation pattern have been depicted as important factors in multistep carcinogenesis in colorectal cancers (27, 40–42). Genomic-wide hypomethylation in HCC has been observed (18). However, the mechanism eliciting genome-wide demethylation in HCC and the implications of alteration of genome-wide methylation in hepatocellular carcinogenesis remain to be clarified.

ABSTRACT

Aberrant genome-wide hypomethylation has been thought to be related to tumorigenesis. However, its mechanism and implications in hepatocellular carcinogenesis remain to be elucidated. Samples of hepatoma (hepatocellular carcinoma, HCC) and paired non-HCC liver tissues were obtained from 17 HCC patients. Normal liver tissues obtained from three individuals were used as controls. Compared with the paired non-HCC liver tissues, genome-wide 5-methylcytosine content in HCC was reduced in all of the tested HCC samples (P < 0.001). Conversely, genome-wide 5-methylcytosine content did not significantly differ among normal, non-cirrhotic, and cirrhotic liver tissues. Moreover, the degree of reduced DNA methylation was related to late histopathological HCC grade (P = 0.005) and large tumor size (P = 0.079). Compared with the paired non-HCC liver tissues, expression of DNA methyltransferases DNMT-1, DNMT-3A, and DNMT-3B and the DNA methyltransferase-like gene, DNMT-2, was up-regulated in 53, 41, 59, and 47% of the HCC samples, respectively. Surprisingly, small amounts of LINE-1 retrotransposon transcripts were detected in HCC and non-HCC as well as normal liver tissues, and the expression levels were not significantly different in HCC compared with the paired non-HCC or normal liver tissues. Of interest, the 3' ends of these LINE-1 transcripts were truncated. Our findings suggest that genome-wide hypomethylation in HCC is a continuing process that persists throughout the lifetime of the tumor cells rather than a historical event occurring in precancer stages or in cell origins for HCC. Up-regulation of DNA methyltransferases might simply be a result of increased cell proliferation in cancer. In addition, our results did not support the hypothesis of activation of transposable elements in HCC via genome-wide hypomethylation.

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2 The abbreviations used are: HCC, hepatocellular carcinoma; DNMT, DNA methyltransferase; SAM, S-adenosylmethionine; RT-PCR, reverse transcription-PCR; RT-PCR-MAC, RT-PCR based on multiplex amplification.
incubated with 5 μCi of [methyl-3H]SAM (3–10 μCi/μmol; Amersham, Buckinghamshire, United Kingdom) and then soaked in 50 mM sodium chloride, 10 mM Tris (pH 8.0), 10 mM EDTA, and 1 mM DTT] for 3 h at 37°C. The reaction was stopped by incubation at 65°C for 20 min. The incubation mixtures were washed onto discs of Whatman DE-81 paper (Fisher Scientific, Springfield, NJ) and then soaked in 50 μl of 5% dibasic sodium phosphate. After drying, the radioactivity representing the amount of incorporated 3H-methyl group onto the DNA was assessed by scintillation counting (LSC-6000; Beckman). All of the samples and measurements were conducted in duplicate, and samples derived from normal liver tissues were included as controls in each assay. For each sample, the measurement was conducted at least three times. This assay produces a reciprocal correlation between the endogenous DNA cytosine methylation status and the exogenous 3H-methyl incorporation. Therefore, a higher incorporation of 3H-methyl groups into DNA reflects a lower state of intrinsic methylation. Furthermore, the differences in endogenous DNA methylation can be represented by the differences in radioactivities measured between HCC and its matched nonhepatic liver tissue.

RNA Extraction and Quantitative RT-PCR. Total RNA was prepared by the one-step acid-phenol method described previously (44). Comparison of the amount of mRNAs for DNMT-1, DNMT-2, DNMT-3A, and DNMT-3B between the paired samples was conducted. For this, a standardized semi-quantitative RT-PCR modified from quantitative RT-PCR-MAC (45) was used. The target gene was amplified simultaneously with the β-actin mRNA as an internal control. The PCR products were analyzed by gel electrophoresis at the ends of a series of amplification cycles (typically 20, 25, 30, and 35 cycles). To construct an amplification curve, the PCR product yields were plotted against the cycle numbers. The cycle difference between the logarithmic phase of the amplification curves for the target sequence and for the β-actin sequence correlated linearly with the initial concentration of the sample over a broad range, up to 10-fold (45). Two μg of total RNA were converted to cDNA. The first-strand cDNA was synthesized via a random-primer method as described previously (44). One-fiftieth of the cDNA was subjected to amplification with the primer-gene primers, 50 μl of each of both the β-actin primers and the target-gene primers, 50 μl of each deoxynucleotide triphosphate, and 2 units of Taq DNA polymerase in a buffer containing 1.5 mM MgCl₂ and 50 mM KCl. Amplification was performed using 20, 25, 30, and 35 cycles of 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min. The PCR products were subjected to electrophoresis through a 3% agarose gel and staining with ethidium bromide. Also, to yield the levels of gene expression relative to that of β-actin, the intensity of the target sequence and the β-actin sequence was quantified by densitometry.

The expression levels for LINE-1 retrotransposons were also determined via the aforementioned semi-quantitative RT-PCR strategy. However, the β-actin sequence was amplified in separate collateral tubes simultaneously with target sequence amplification. This was attributable to the similar sizes of the PCR products of LINE-1 sequence and β-actin sequence.

To confirm the specificity for each PCR reaction, some of the nucleotide sequences of the amplified products were determined by automatic PCR sequencing analysis as described previously (46). Table 1 lists the primers used in the semi-quantitative RT-PCR.

### Table 1: Sequences for primers used in this study

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Targets</th>
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<tr>
<td>5'-GTG AGC AAC ATA ACC AGG TGG AGC-3'</td>
<td>DNMT-1</td>
</tr>
<tr>
<td>5'-ATA GAG GCC AGC CCA GTC TGT GTG-3'</td>
<td>DNMT-2</td>
</tr>
<tr>
<td>5'-GCC CTT GAG AGA AGG TCT TAT ACC TG-3'</td>
<td>DNMT-3A</td>
</tr>
<tr>
<td>5'-TTG ACC CTT GCC CCA CTB CAT ACC-3'</td>
<td>DNMT-3B</td>
</tr>
<tr>
<td>5'-TCA GGC CTA CGT TAT AGC AGA GGA GAT-3'</td>
<td>LINE-1 (5'-ORF)</td>
</tr>
<tr>
<td>5'-TCT GGT TCG ATC GTC TGG GCT CTT GA-3'</td>
<td>LINE-1 (5'-NCR)</td>
</tr>
<tr>
<td>5'-GCC GTG TAT GCT TCC GAG CTT GA-3'</td>
<td>LINE-1 (3'end)</td>
</tr>
</tbody>
</table>

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4 S. Y. Hsieh, unpublished data.
HCC), we analyzed age, sex, cause of liver disease, and histology of the paracancerous liver tissues, as well as tumor size, histological HCC grades, and presurgical serum α-fetoprotein levels. Table 2 shows that the differences in 3H-methyl group incorporation between HCC and paired non-HCC liver tissues were significantly correlated to the histopathological HCC grades and to the size of the HCC. That is, changes in genome-wide hypomethylation were more prominent in poorly differentiated HCC (P = 0.005) and in larger HCC (P = 0.079). The significance of histopathological HCC grading and tumor sizes in the degree of HCC hypomethylation was further confirmed by multivariate analysis (data not shown).

Expression of DNMTs. To examine whether DNMT deregulation contributes to genome-wide hypomethylation in HCC, semiquantitative RT-PCR assays (modified RT-PCR-MAC) were used to compare the expression levels of DNMT-1, DNMT-2, DNMT-3A, and DNMT-3B between the 17 pairs HCC and matched non-HCC liver tissues. Fig. 4 demonstrates the results for 7 HCC cases, and the results for the 17 HCC patients are summarized in Table 3. Up-regulation of DNMT-1, DNMT-2, DNMT-3A, and DNMT-3B expression in HCC compared with paired non-HCC liver tissues was noted in 9 (53%), 8 (47%), 7 (41%), and 10 (59%) of the 17 HCC patients, respectively. None had down-regulation of DNMT-1 expression in HCC compared with non-HCC liver tissues, whereas 1 had a relative down-regulation of DNMT-2 expression in HCC compared with matched non-HCC liver tissues (Table 3). In addition, expression of DNMT genes was not correlated to genome-wide demethylation in the HCC samples (data not shown).

Activation of LINE-1 Retrotransposons. To examine whether genome-wide demethylation in HCC activates transposons, the transcript levels of LINE-1 in HCC were assayed and compared with those in the paired non-HCC and normal liver tissues. Fig. 5 demonstrates the semiquantiative RT-PCR results derived from open reading frame 2 of LINE-1 RNA in three pairs of HCC and matched non-HCC liver tissues. Surprisingly, low levels of LINE-1 transcripts were detected in all of the examined HCC and paired non-HCC samples. The amplified LINE-1 products were not derived from residual chromosomal DNA contamination because they became un-

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>P*</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>≤60 years</td>
<td>9</td>
<td>27841</td>
<td>24015</td>
</tr>
<tr>
<td></td>
<td>&gt;60 years</td>
<td>8</td>
<td>17086</td>
<td>21062</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>11</td>
<td>28545</td>
<td>26264</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
<td>13011</td>
<td>8014</td>
</tr>
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<td>Cirrhosis</td>
<td>Non-cirrhosis</td>
<td>9</td>
<td>16221</td>
<td>14089</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis</td>
<td>8</td>
<td>30759</td>
<td>28468</td>
</tr>
<tr>
<td>B°</td>
<td>&lt;=100</td>
<td>10</td>
<td>31033</td>
<td>26373</td>
</tr>
<tr>
<td></td>
<td>&gt;100</td>
<td>7</td>
<td>27203</td>
<td>15257</td>
</tr>
</tbody>
</table>

* P, statistical significance was tested via the Mann-Whitney nonparametric U test.

° B, chronic infection with hepatitis B virus; non-B, including 4 cases with chronic hepatic C, 1 case with concurrent chronic hepatitis B and C, and 2 cases without known cause for liver disease.

Table 2. Correlation of genome-wide demethylation to clinical data during hepatocarcinogenesis
detectable when the samples were treated with RNase before reverse transcription (Fig. 5, right half). Likewise, low expression of LINE-1 retrotransposons was found in all of the tested HCC and paired non-HCC liver tissues, as well as in normal liver tissues from controls (data not shown). To examine sequence integrity of these LINE-1 transcripts, RT-PCR was also performed to amplify the 5'-end region and the open reading frame 2 region, as well as the 3' end region, as shown in Fig. 6. Small amounts of the transcripts were detected within the 5'-noncoding region and the open reading frame 2 region in both the HCC and paired non-HCC liver tissues (Fig. 6). However, the 3' end region was undetectable in the HCC, paired non-HCC liver tissues, and normal liver tissues (Fig. 6). These findings suggest that the 3' ends of the LINE-1 transcripts are truncated.

### DISCUSSION

Herein we report that genome-wide hypomethylation was present in all of the examined HCC samples compared with the paired non-HCC liver tissues and similar to that found in other cancers. It is tempting to speculate that genome-wide hypomethylation represents a general characteristic of most human cancers. However, because tumors may arise from stem cells that represent only a minor component of the normal tissue, a comparison of tumor tissues with the surrounding normal tissues may create misleading results (47, 48). It is therefore important to ascertain when demethylation occurs during tumorigenesis. In the colon, Feinberg et al. (14) demonstrated a significant reduction in 5-methylcytosine content in adenoma and adenocarcinoma compared with normal, paired colonic mucosa, whereas no difference existed between benign adenoma and malignant adenocarcinoma. In the stomach, Cravo et al. (16) depicted a serial of sequential decreases of 5-methylcytosine content before malignant transformation with no further change of DNA hypomethylation in gastric carcinoma. In cervical cancer, incremental increases in DNA hypomethylation were observed from epithelial dysplasia to cancer (15).

The findings in the colon, stomach, and cervix suggest that genomic demethylation occurs in precancer or early cancer stages or may be attributable to different methylation patterns of tumor cell origins. In contrast, our studies revealed that there was no serial of sequential increases of DNA hypomethylation from normal liver and noncirrhotic liver to cirrhosis. Instead, genome-wide hypomethylation occurred primarily in HCC. Furthermore, higher degrees of genome-wide hypomethylation were found to be strongly associated with late HCC grades and with larger tumor sizes, suggesting that genome-wide demethylation occurs simultaneously with tumor progression. Similar findings of hypomethylation association with tumor progression were also observed in prostate adenocarcinoma (13, 19) and other cancers (11). The reason for the discrepancy between the findings in colon, gastric, and cervical cancers and in prostate and liver cancers is not clear. However, the findings of continuous genome-wide demethylation during tumor progression are reminiscent of the recent observations by Lengauer et al. (47). In their study, methylation of exogenous sequences differed dramatically among various colorectal cancer cell lines. In summary, the findings reported by others and herein suggest that genome-wide hypomethylation in HCC and other cancers represent a continuing physiological process that persists throughout the lifetime of the tumor cells rather than as merely a historical event that occurs in the cell origins of the cancers.

### Table 3

Comparison of DNMT expressions between the paired non-HCC and HCC liver tissues in 17 HCC cases

<table>
<thead>
<tr>
<th></th>
<th>N &lt; T*a</th>
<th>N = T*b</th>
<th>N &gt; T*c</th>
<th>UD/UDd</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT-1</td>
<td>9</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>DNMT-2</td>
<td>8</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>DNMT-3A</td>
<td>7</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>DNMT-3B</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>17</td>
</tr>
</tbody>
</table>

*a N < T: up-regulated in HCC.
*b N = T: identical expression levels between non-HCC and HCC.
*c N > T: down-regulated in HCC.
*d UD/UD, undetectable by RT-PCR in both non-HCC and HCC.
Although genome-wide hypomethylation is generally observed in cancers, its mechanism remains entirely unclear. Because maintenance of the fidelity of CpG dinucleotide methylation through DNA replication requires a tightly regulated methyltransferase expression in replicating cells as well as a tightly controlled enzyme function near the DNA replication fork (48), deregulation of cytosine methyltransferases might lead to genome-wide hypomethylation. However, in this study, the expression levels of cytosine methyltransferases DNMT-1, DNMT-3A, and DNMT-3B were found to be even higher in HCC than in the matched non-HCC liver tissues. Increased DNMT-1 expression was also observed previously in several cancers (49–51). Eads et al. (52) demonstrated recently that expression of DNMT-1, DNMT-3A, and DNMT-3B in colorectal cancers was up-regulated compared with the housekeeping gene (β-actin) but not compared with the proliferation-associated genes, proliferating cell nuclear antigen and histone H4. This suggests that overexpression of cytosine methyltransferases in cancers is a consequence of rapid cell proliferation, and that deregulation of DNMT gene expression fails to play a direct role in establishing tumor-specific abnormal DNA hypomethylation in cancers. Alternately, it has also been discovered that p21 is coexpressed with DNMT-1 in adenomatous polyps but not in normal colonic epithelium (53, 54). Probably, DNMT-1 and p21 coexpression in proliferating cells may undermine maintenance of DNMT activity via p21 disruption of DNMT-1 and proliferating cell nuclear antigen interactions at replication foci, which in turn yields global hypomethylation (54). Likewise, deregulation of the de novo DNMTs DNMT-3A and DNMT-3B or of 5-methylcytosine demethylases in neoplastic cells could further undermine the fidelity of CpG dinucleotide pattern maintenance. Indeed, in methyl-deficient, diet-induced malignant liver in rats, global DNA hypomethylation was found to be associated with increased DNMT activity (55, 56) and strongly correlated to increased levels of histone acetylation (57, 58). In other words, genome-wide hypomethylation may be caused by alteration of the chromatin structure, which in turn prevents access of the DNMTs to the CpG islands. Up-regulation of DNMT expression in cancer cells may be attributed to rapid proliferation of cancer cells (52, 58).

In this report, we found an up-regulation of the DNMT-2 gene in approximately half of HCCs compared with the matched non-HCC liver tissues. Unlike other members in the cytosine-methyltransferase family, DNMT-2 contains only the catalytic domain but lacks the regulatory elements (59). To date, no DNA methylation activity for DNMT-2 has been proved (60–62). Instead of methyltransferase activity, DNMT-2 might function as a negative regulator in DNA methylation, for instance, by interfering in the methylation activities of other DNMTs.

Retrotransposon reactivation might represent a particularly important consequence of DNA hypomethylation, because nearly 90% of all 5-methylcytosine is within these elements, which portray one-third of the human genome (5). Furthermore, transposition of LINE-1 sequences results in a highly unstable branched DNA structure, which is prone to recombination with accessible elements located proximately or even elsewhere in the genome (63–65). Indeed, association of retrotransposon transposition with chromosome deletions and translocations has been verified in several human cancers (31, 33, 36–38). Moreover, LINE-1 sequences have been shown to be hypomethylated and transcribed in human testicular carcinoma cell lines (66), urorhabil carcinoma cell lines, and teratocarcinoma cell lines (67, 68). However, direct evidence of genome-wide hypomethylation eliciting retrotransposon activation in human cancer samples does not currently exist. Surprisingly, small and comparable amounts of LINE-1 transcripts were present in HCC and paired non-HCC liver tissues, as well as normal liver tissues. Apparently, our studies do not provide evidence demonstrating activation of LINE-1 retrotransposons via genome-wide demethylation in HCC. However, tumorigenesis and tumor progression are long-term processes, whereas our assays represent only a single time point during these processes. Obviously, the hypothesis of activation of LINE-1 retrotransposon via genome-wide demethylation, which in turn causes genome instability in HCC and other human cancers, remains to be further investigated.

Because most of the LINE-1 elements in the genome are truncated at the ends, it is essential to examine the sequence integrity of the LINE-1 transcripts detected in HCC, non-HCC, and normal hepatocytes. Indeed, our results indicated that the 3′ ends of the identified LINE-1 transcripts were truncated. Because LINE-1 transposition in normal subjects is a rare event (69), it is plausible to speculate that these 3′-end truncated LINE-1 transcripts detected in HCCs as well as in normal hepatocytes are incapable of transposition. Nevertheless, we do not know the physiological roles of these 3′ truncated LINE-1 transcripts in normal hepatocytes or in HCC. In plants, aberrant transposon transcripts resulting from genome-wide hypomethylation have been shown to cause local de novo methylation and silencing of the cellular genes (70). The findings in plants are reminiscent of the observations in human cancers that genome-wide hypomethylation is usually associated with local de novo hypermethylation (8, 71). Further studies to examine this speculation in human cancers are warranted.

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