Detection of metallothionein 1G as a methylated tumor suppressor gene in human hepatocellular carcinoma using a novel method of double combination array analysis

MITSURO KANDA, SHUJI NOMOTO, YUKIYASU OKAMURA, YOKO NISHIKAWA, HIROYUKI SUGIMOTO, NAOHITO KANAZUMI, SHIN TAKEDA and AKIMASA NAKAO

Department of Surgery II, Graduate School and Faculty of Medicine, University of Nagoya, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

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Abstract. Gene expression profiling or karyotyping analysis has made it possible to identify novel genes with altered expressions or copy numbers that have not been previously reported in liver cancer. On the same HCC sample, we performed double array analysis, both expression profiling and karyotyping analysis using a single nucleotide polymorphism (SNP) array in an attempt to find a novel tumor suppressor gene for its prognostic marker. We conducted expression array and SNP chip array using tumor and corresponding non-tumor tissues from the resected liver specimen of a 68-year-old woman who had chronic hepatitis type C. Additionally, we performed quantitative real-time reverse transcription polymerase chain reaction (PCR) and methylation-specific PCR (MSP) for gene detection using specimens from 48 patients with HCC, and investigated their correlation with the prognosis. Metallothionein (MT) 1G gene located on 16q13 showed a decreased expression in tumor tissue. The copy number by SNP chip array revealed no loss of heterozygosity since no deletions were detected in 16q13, and HCC tissue showed AB call in both SNPs next to MT1G. In quantitative real-time PCR using 48 HCC clinical samples, mRNA expression of MT1G decreased significantly compared with that in corresponding non-cancerous liver tissues ($p<0.0323$). Twenty-nine (60.4%) of 48 HCCs gave a positive result in MSP, indicating a poorer prognosis than the negative group, although the difference was not significant ($p<0.0978$). Our results indicated that MT1G acts as a tumor suppressor gene in HCC. Moreover, findings suggested that the mechanisms of MT1G silencing are related to promoter hypermethylation.

Correspondence to: Dr Shuji Nomoto, Department of Surgery II, Graduate School and Faculty of Medicine, University of Nagoya, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
E-mail: snomoto@med.nagoya-u.ac.jp

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and has an adverse prognosis (1). It is known that hepatitis B or hepatitis C viral infection causing chronic liver disease, inflammation and cirrhosis play an important role in the etiology of HCC (2). Also, external factors such as alcohol and aflatoxin can cause hepatocarcinogenesis as well as free radicals from chronic inflammation due to the hepatitis virus (3,4). Most HCC patients were found to be progressing to an advanced stage, with no effective systemic treatment being available at present (4,5). Therefore, novel treatment strategies, especially new molecular targets, are urgently needed. However, the molecular mechanisms contributing to hepatocarcinogenesis are still not completely understood.

In the field of molecular-biological research, microarray techniques have offered considerable advances (6,7), the functions of various genes in malignant tumors have been investigated through the use of microarray (8,9), which also contributes to detecting candidates for tumor suppressor genes in HCC (10,11). Furthermore, the advent of single nucleotide polymorphism (SNP) arrays has enabled us to detect a large number of DNA polymorphic loci simultaneously and to analyze both copy number changes and copy-neutral loss of heterozygosity (LOH) events (12-14). In this study, we developed an innovative method of integrating these two arrays in order to discover new candidates for tumor suppressor genes in HCC and to elucidate their gene silencing mechanism more effectively.

Using an expression array, we selected MT 1G as candidate tumor suppressor gene. We then examined the mRNA expression of MT1G to determine its functions in HCC. Moreover, to explore the mechanism of MT1G gene silencing in HCC, we conducted karyotyping analysis using an SNP chip array and methylation-specific polymerase chain reaction (MSP). Thanks to an innovative combination of two arrays, this is to our knowledge, the first definitive report describing the

expression and methylation status of MT1G with clinical samples in HCC.

Materials and methods

Sample collection and DNA preparation. Five HCC cell lines (HLE, PLC/PRF/5, HuH1, HuH2 and HepG2) were obtained from the American Type Culture Collection (Manassas, VA). They were grown in RPMI-1640 supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37˚C.

A 68-year old woman with chronic hepatitis type C was discovered to have a hepatocellular carcinoma, 3 cm in diameter, on the right lobe of her liver. The tumor was enhanced homogeneously using the abdominal CT scan. She underwent partial hepatectomy in 2007. The specimens of her tumor and corresponding non-cancerous tissues were immediately cut, and done the procedure for the extraction of total RNA and genomic DNA was initiated. The tumor was pathologically diagnosed as hepatocellular carcinoma, and the area containing more than 80% of the cancer cells was selected for the DNA and RNA extraction. The total RNA was used for the expression array, and the genomic DNA was used for the SNP chip array.

Forty-eight primary HCC tissues and corresponding non-cancerous tissues were collected at the Nagoya University Hospital from HCC patients during liver-resection surgery. Their ages ranged from 39-77 years (62.4±7.9 years), and the male-to-female ratio was 43:5. All these patients had undergone liver resection between 1994 and 2001, including 38 patients with hepatitis C and 7 patients with hepatitis B. The median length of follow-up was 64.5 months (range 17.9-105.9 months). All tissues were diagnosed histologically as HCC. Written informed concent, as required by the institutional review board, was obtained from all patients. Collected samples were stored immediately in liquid nitrogen at -80˚C until analysis. Genomic DNA was obtained from these samples by digestion with proteinase K, followed by phenol/chloroform extraction (15).

RNA isolation and microarray procedure. Total RNA was isolated from each of the frozen samples with the RNeasy mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Gene expression profiles were determined using Affymetrix HGU133A and HGU133B GeneChips (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations. In brief, double-stranded cDNA was synthesized with 8 μg of total RNA with oligo (dt)24 T7 primer and transcribed into biotinylated cRNA using the IVT labeling kit (Affymetrix). Biotinylated cRNA (20 μg) was fragmented at 94˚C for 35 min and hybridized to human Genome U133 Plus 2.0 gene chip array (Affymetrix), which contains >54,000 probe sets. The hybridized cRNA probes to oligonucleotide arrays were stained with streptavidin R-phycoerythrin and were processed for signal values using Micro Array Suite 5.0 software (Affymetrix). All data used for subsequent analysis passed the quality control criteria.

GeneChip Affymetrix platform. Next, we performed SNP chip array for double array analysis following expression array in order to detect new candidates for tumor suppressor genes and explore their gene silencing mechanism more effectively. SNP chip array experiments were done according to the standard protocol for Affymetrix GeneChip Mapping 500K arrays (Affymetrix). Briefly, total genomic DNA was digested with a restriction enzyme (*Xba*I or *Hin*dIII), ligated to an appropriate adapter for each enzyme and subjected to PCR amplification using a single primer. After digestion with DNase I, the PCR products were labeled with a biotinylated nucleotide analogue using terminal deoxynucleotidyl transferase and hybridized to the microarray. Hybridized probes were captured by streptavidin-phycoerythin conjugates, and the array was scanned and genotypes called as previously described (16). All the examples of copy number analysis with Affymetrix GeneChip Mapping 500K arrays were treated using copy number analyzer for Affymetrix GeneChip Mapping 500K arrays (CNAG) version 2.0.

Reverse transcription polymerase chain reaction (RT-PCR). The expression of MT1G mRNA was analyzed by RT-PCR and real time RT-PCR. Total RNA (10 mg) isolated from HCC cell lines (HLE, PLC/PRF/5, HuH1, HuH2 and HepG2), primary HCC tissues, and corresponding non-cancerous tissues were used to generate complementary DNA (cDNA), they were then amplified by polymerase chain reaction (PCR) primers for MT1G sense (S) (5'-GCCAGCTCCTGC AAGTG CAA-3' in exon1) and antisense (AS) (5'-ATGCA GCCCTGGGCACACTT-3' in exon1), which amplify a 99 base pair (bp) product. The RT-PCR amplification consisted of 31 cycles at 94˚C for 12 sec, 60˚C for 12 sec, and 72˚C for 12 sec, after the initial denaturation step (94˚C for 5 min). RT-PCR of ß-actin was performed to confirm that equal amounts of cDNA were used as templates. Each PCR product was loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Real-time quantitative RT-PCR analysis. PCR reactions were performed with the SYBR-Green PCR core reagents kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) under the following conditions: 1 cycle at 50˚C for 2 min, 1 cycle at 95˚C for 10 min, then 45 cycles at 95˚C for 15 sec and at 60˚C for 30 sec. Real-time detection of the SYBR-Green emission intensity was conducted with an ABI prism 7000 Sequence Detector (Perkin-Elmer, Applied Biosystems). The primers for the PCR reaction were used the same primer pairs for RT-PCR as described above. For standardization, the expression of GAPDH (TaqMan^R, GAPDH control reagents, Applied Biosystems) in each sample was quantified. Quantitative RT-PCR was performed at least 3 times, including no-template samples as a negative control. The expression amount of each sample was shown as the value of MT1G divided by that of GAPDH.

Methylation specific PCR (MSP). DNA from HCC cell lines, primary tumors and corresponding normal specimens was subjected to bisulfite treatment. Briefly, 2 μg of DNA was denatured by NaOH and modified using sodium bisulfite. DNA samples were then purified using Wizard purification resin (Promega Corp., Madison, WI), treated again with NaOH, precipitated with ethanol, and resuspended in water.

Table I. The result of expression array on MT1G gene.

Probe set ID	Gene symbol	Log2 ratio	Normal signal	Detection	Tumor signal	Detection	Probe ID	Chromosomal location
204745_X_at	metallothionein 1G	-3.6	3133.7	P	334.3	P	HU1 33p 2_1 4193	Chr 16q13
210472_at	metallothionein 1G	-3.1	2260.8	P	189.3	P	HU1 33p 2_1 9870	Chr 16q13

Table II. The result of SNP chip array.

Probe set ID	Chromosome	Physical position	1 Call	1 Confidence	2 Call	2 Confidence
SNP_A-2245406	16	55254052	AB	0.003750	AB	0.002813
SNP_A-2314533	16	55261415	AB	0.007813	AB	0.007613

The primer pairs for unmethylated detection were in the MT1G promoter region near exon 1: S (5'-GGGGTTGTTTT GTGGTGTGTG-3') and AS (5'-AAACACCCCACCCCA CCCTT-3'), which amplify a 135-bp product, and those for methylated detection were in the same region: S (5'-TTCG CGAGTCGGTGCGAAAG-3') and AS (5'-CCGCGATCCC GACCTAAACT-3'), which amplify a 96-bp product. The PCR amplification consisted of 30 cycles of 94˚C for 10 sec, 60˚C for 10 sec, and 72˚C for 10 sec, following the initial denaturation step (94˚C for 5 min). Each PCR product was loaded directly on to 15% acrylamide gels, stained with ethidium bromide and visualized under UV illumination.

Sequence analysis. We performed sequence analysis using ABI PRISM 310 genetic analyzer (Applied Biosystems). Genomic bisulfite-treated DNA of HCC cell lines were sequenced, with PCR reactions was performed in all cases. The primer pair for the sequence was in the MT1G promoter region for forward primer and in exon 1 for reverse primer: S (5'-AGGGATTTTGTATTTGGTTT-3') and AS (5'-AAA TAAAACCCAACAACCAA-3'), which amplify a 178-bp product. The PCR amplification consisted of 35 cycle of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec, following the initial denaturation step (94˚C for 5 min). PCR products were purified directly using the QIA quick gel extraction kit (Qiagen, Hilden, Germany).

5-Aza-2'-deoxycytidine (5-aza-dC) treatment. To confirm that the promoter hypermethylation led to a silencing of the gene expression, methylated HCC cell lines (HLE, PLC/PRF/5 and HuH2) and unmethylated HCC cell lines (HuH1 and HepG2) were treated with the DNA methylation inhibitor, 5-aza-dC (Sigma-Aldrich, St. Louis, MO). Cells ($1.5x10^6$) were cultured for 6 days with medium changes on days 1, 3 and 5. After incubation, the cells were harvested, RNA was extracted, and RT-PCR was performed as described above.

Statistical analysis. The relative mRNA expression levels (MT1G/GAPDH) were calculated from quantified data. We used an unpaired t-test to analyze the differences in MT1G expression levels between HCC and the non-cancerous tissues. To analyze the correlation between MT1G expression and clinicopathological parameters, differences in the numerical data between the two groups were evaluated using the Chi-square test. Overall survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. Statistical analysis was performed using SPSS Statistics 17.0 software (SPSS Inc., IL, USA). The presence of a statistically significant difference was denoted by $p<0.05$.

Results

Expression array and SNP chip array. First, we conducted an expression array in order to find new tumor suppressor genes in HCC. We searched for genes whose expression in tumor tissues were reduced further than that of corresponding non-cancerous tissues, and found that the MT1G gene fits the requirements (Table I). The diminished values of the chip were -3.6 and -3.1, and its reduced expression was confirmed by RT-PCR using the cDNA of the samples (Fig. 1A).

Next, we checked the result of the SNP chip array. We observed deletions in 3q, 8p, 11q, 12q, 16p, 17p, 19q, and X chromosomes, and chromosomal gains in 1q, 3q, 11q, 12p and 12q. The copy number of chromosome 16q, that MT1G located in, showed no deletions, whereas chromosome 16p had one (Fig. 2). Additionally, MT1G existed within a very narrow range (55258154-55259478), and there were no SNPs within this SNP array, although there were two SNPs (SNP_A-2245406 and SNP_A-2314533) in both adjacent sides of MT1G. Since both of these SNPs presented AB call, we concluded that there were no LOH between them (Table II). These results suggested that MT1G was suppressed without LOH or deletion.

We then reviewed the sequence of the promoter region of MT1G gene and found many CpG islands in the region. This led us to hypothesize that hypermethylation of the CpG islands was the mechanism for decreasing its expression in tumor tissue.

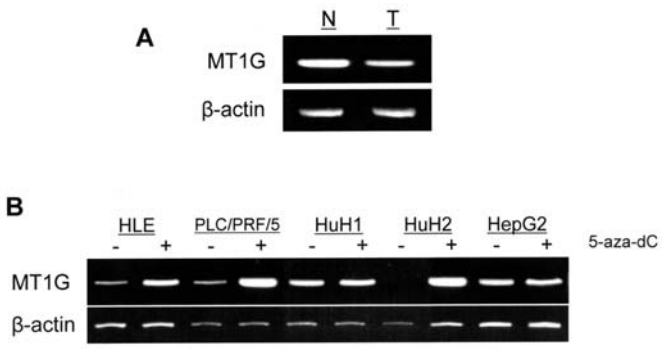

Figure 1. RT-PCR results of MT1G gene. (A) Down-regulation of MT1G was seen in the tumor sample compared with corresponding non-cancerous tissue. Total RNA obtained from normal and tumor samples were used for the analyses of expression profiling and the SNP array. RT-PCT of ß-actin was performed to normalize the quality of the cDNAs. (B) MT1G expression had been reactivated in cells of HLE, PLC/PRF/5 and HuH2 by treatment with 5-aza-dC (+). Expression in cells of HuH1 and HepG2 was not up-regulated by the treatment. RT-PCR of ß-actin was performed to normalize cDNA quality. N, non-cancerous tissue; T, tumor tissue.

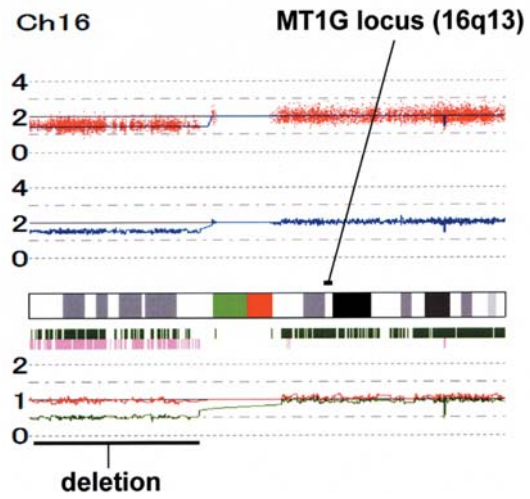

Figure 2. Copy number analysis of chromosome 16 by SNP array in an HCC sample. Whereas a deletion occured at 16p in copy number analysis by 500K SNP array in the HCC sample, 16q13 the MT1G locus showed no deletion or amplification.

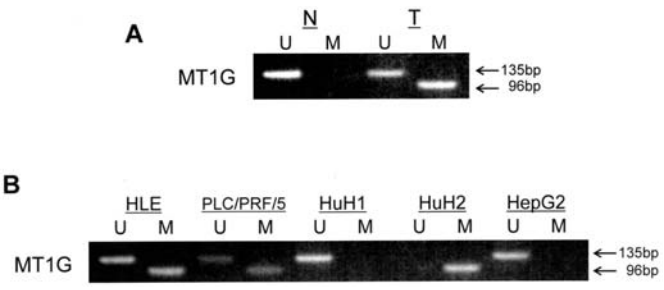

Figure 3. Results of promoter hypermethylation in MT1G gene examined by methylation or un-methylation specific PCR in hepatocellular carcinoma. (A) When normal and tumor samples were examined using expression profiling and SNP array, promoter hypermethylation was seen only in tumor tissue. (B) Methylation status of MT1G gene in HCC cell line. HuH2 showed complete methylation. Complete un-methylation was observed in HuH1 and HepG2 cells. Other cells showed only partial methylation. N, non-cancerous tissue; T, tumor tissue; M, methylation specific PCR; U, un-methylation specific PCR.

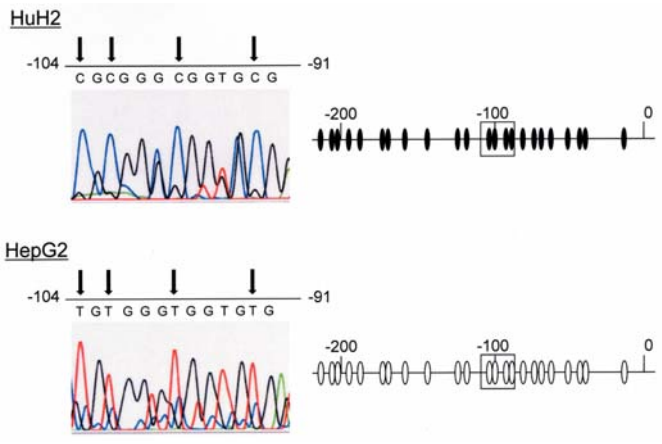

Figure 4. Sequencing analysis of HuH2 and HepG2 cells.

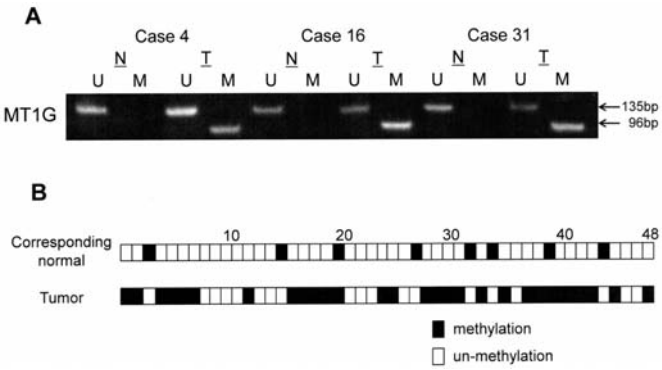

Figure 5. (A) Representative results of methylation status in primary HCC tissues. (B) 29 of 48 tumors showed hypermethylation of MT1G gene. Only 8 cases showed methylation in corresponding non-cancerous samples. Black box depicted hypermethylation and open box un-methylation. N, non-cancerous tissue; T, tumor tissue; M, methylation specific PCR; U, un-methylation specific PCR.

5-Aza-2'-deoxycytidine (5-aza-dC) treatment of five cell lines. To explore whether promoter hypermethylation leads to the suppression of MT1G, we examined the expression of MT1G before and after 5-aza-dC treatment of the mRNA of HCC cell lines. After treatment with the DNA methylation inhibitor, 5-aza-dC, three methylated cells (HLE, PLC/PRF/5 and HuH2) showed a reactivation of MT1G mRNA expression. In HuH1 and HepG2 cells, where no mehtylation was detected, the expression did not increase after 5-aza-dC treatment (Fig. 1B).

MSP and un-methylation specific PCR. To verify the hypothesis described above, we designed primers for methylation specific and un-methylation specific PCR. Then we checked the methylation status of the samples used in the arrays and 5 cell lines derived from hepatocellular carcinoma. Among the samples used in the arrays, promoter hypermethylation was seen only in the tumor sample (Fig. 3A). By electrophoresis of methylation specific PCR, we confirmed the bands in appropriate size in the lanes of HLE, PLC/PRF/5 and HuH2. In un-methylation specific PCR, there were bands in lanes of HLE, PLC/PRF/5, HuH1 and HepG2 (Fig. 3B). We

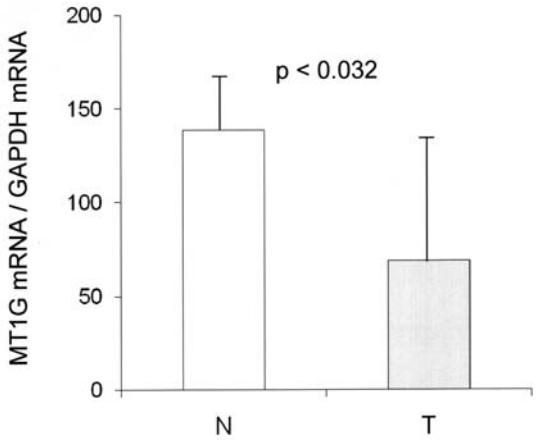

Figure 6. We examined expressions of MT1G by real-time quantitative RT-PCR analysis of 48 clinical samples. Mean MT1G/GAPDH in HCCs was significantly lower than that in non-cancerous tissues (p<0.032).

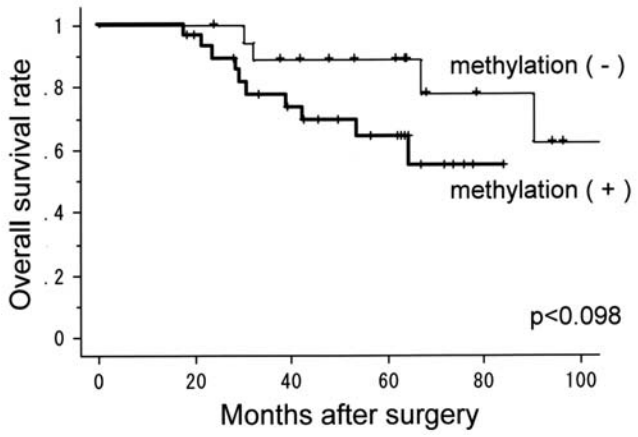

Figure 7. Overall survival rate assessed by Kaplan-Meier curves via classification of 48 patients into methylation positive and negative groups in tumor samples. Patients with positive MSP in HCCs tended to have poorer 5-year survival rates than those in negative MSP group, although the difference was not significant (p<0.098).

concluded that complete methylation existed in HuH2, complete un-methylation in HuH1 and HepG2, and partial methylation in HLE and PLC/PRF/5.

Sequence analysis. To ascertain whether the amplification was done correctly, we conducted a direct sequencing analysis of HuH2 and HepG2, and found that all CpG islands in the fragment of HuH2 were CG, while all those of HepG2 were TG (Fig. 4), thus confirming that the MSP and un-methylation specific PCR had worked correctly.

MSP and un-methylation specific PCR of 48 HCC samples. Next, we conducted MSP and un-methylation specific PCR using human clinical samples. Twenty-nine of 48 (60.4%) HCCs and 8 of 48 corresponding non-cancerous tissues showed promoter hypermethylation of the MT1G gene (Fig. 5).

Real-time quantitative RT-PCR analysis of 48 HCC samples. We then performed real-time quantitative RT-PCR analysis of clinical samples to investigate the gene suppression of MT1G in HCC. We examined the expressions of MT1G and GAPDH in all specimens, then standardized them using the MT1G/GAPDH ratio. We compared the amount of MT1G expression in the tumor tissues with that in the corresponding non-cancerous tissues for each case. In 46 (95.8%) out of 48 cases, the expression level of MT1G in HCC was lower than that in the corresponding non-cancerous tissues. Furthermore, the mean MT1G/GAPDH ratio in HCCs was significantly lower than that in the corresponding non-cancerous tissues (p<0.032, Fig. 6).

Correlation between the MT1G expression and clinicopathological factors. Having analyzed the correlations between the expression levels of MT1G and clinicopathological factors (Pugh-Child classification, hepatitis virus, serum α fetoprotein value, tumor multiplicity, tumor size, tumor differentiation, vascular invasion, serosal infiltration, formation of capsule and pathological stage), we found no significant relation (data not shown). We then analyzed overall survival rates to assess the prognostic significance of the promoter hypermethylation of MT1G. The 5-year overall survival rate of the 48 HCC patients was 77.1%. When assessed by Kaplan-Meier curves, patients with positive MSP in HCCs tended to have poorer 5-year survival rates than those in negative MSP group, 63.8% (positive MSP) and 88.9% (negative MSP), respectively, although the difference was not significant (p<0.098, Fig. 7).

Discussion

Metallothioneins (MTs) are cysteine-rich proteins with a molecular weight of approximately 6000 Da, and have a specific binding capacity to group II metal ions (17,18). Human MTs are encoded by a family of genes clustered on chromosome 16, containing at least 10 functional members, and consisting of four major isoforms: MT1 (L, E, M, CP, B, F, G, H, IP and X), MT2, MT3 and MT4 (19). MT1 and MT2 are expressed ubiquitously in various tissues (17,20). Expressions of MT3 and MT4 are restricted to brain and reproductive organs, as well as to stratified squamous epithelia of the skin and tongue, respectively (21,22). While MT3 and MT4 are constitutively expressed, MT1 and MT2 are coordinately regulated by a variety of developmental and environmental signals, such as metals, oxidative stress, cytokines and glucocorticoid hormones (23,24). The major function of MT is to preserve the homeostasis of biologically essential metals such as zinc and copper, and to detoxify poisonous metals, such as cadmium and mercury (17,18,25). Additionally, MT purges free radicals by using its unique metal-thiolate clusters that act as redox sensors and are rapidly oxidized by reactive oxygen species releasing apo-MT and the metal ions (26,27). Therefore, a diminished expression of MT can cause hepatocarcinogenesis through the insufficient purging of free radicals from chronic inflammation. MT1G is one of the major isoform of MTs, with the MT1G gene being located on chromosome 16, band q13 (28,29). In previous studies, it was reported that MT1G suppression contributes to carcino-

genesis in colorectal cancer, malignant ovarian epithelial tumors, papillary thyroid carcinoma, esophageal squamous cell carcinoma and prostate cancer (30-34). In papillary thyroid carcinoma, esophageal squamous cell carcinoma and prostate cancer, the mechanism of MT1G gene silencing was demonstrated to be promoter hypermethylation (32-34).

On the association between HCC and MT1G, Datta *et al* reported a very interesting result in 2007 (35). They first observed that MT expression is suppressed in mouse and rat cell lines and in a transplanted rat hepatoma through promoter methylation (36). Subsequently, they tried unsuccessfully to prove that the mechanism of gene silencing was the same in human as in rat HCC. They then found another mechanism whereby MT is suppressed in primary HCC by transcriptional repression rather than by promoter methylation, and is mediated through activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, inducing dephosphorylation of the transcription factor CCAAT/enhancer binding protein (C/EBP).

Dramatic improvements in microarray techniques have contributed significantly to genetics research (8). Lau *et al* reported on the first study of an expression array for HCC in 2000 (37). Subsequently, many studies were performed to discover novel candidates of tumor suppressor genes in HCC using expression array technology (38-40). Moreover, SNP chip array analysis, which now enables us to simultaneously detect a large number of DNA polymorphic loci in a simple way, was further developed for genotyping.

We recently devised a novel method of combining these two array techniques to detect a new suppressor oncogene and its mechanism of modulation. In this study, we confirmed that MT1G is a tumor suppressor gene in HCC and the silencing mechanism was promoting hypermethylation. It was reported in other types of tumors, however, to our knowledge this is the first study regarding HCC. Based on this study, double array analysis method seems to be a concise and efficient procedure for detection of tumor suppressor genes and presumption of their silencing mechanisms, although it requires complementary examinations such as real-time quantitative RT-PCR and MSP to verify the validity.

In summary, our results indicated that MT1G acts as a tumor suppressor gene, and that the mechanisms of MT1G-silencing are related to promoter hypermethylation in human HCC, suggesting that down-regulation or promoter hypermethylation of MT1G may be proposed as a novel biomarker for detecting HCC and predicting prognosis. Finally, the novel method of double array analysis, which we devised, potentially is useful in detecting new suppressor oncogenes and their mechanisms.

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