might be involved in the formation of a field defect, independently of aberrant H3K27me3. However, in contrast with H3K27me3, the individual roles of H3K9me2 and H3K9me3 in repression of tumor-suppressor genes are not well understood due to their colocalization with DNA methylation (46), and assessment of their roles in the formation of a field defect seems difficult.

In conclusion, aberrant H3K27me3 was shown to be induced in normal-appearing tissues by exposure to a specific inducer, and its involvement in the formation of a field defect was suggested.

Supplementary material

Supplementary Tables 1–4 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

Funding

Grants-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan H20-2; Global Research Laboratory Program from Korea Foundation for International Cooperation of Science and Technology.

Conflict of Interest Statement: None declared.

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Received June 21, 2012; revised August 18, 2012; accepted September 9, 2012

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Role of Transcriptional and Posttranscriptional Regulation of Methionine Adenosyltransferases in Liver Cancer Progression

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Down-regulation of the liver-specific *MAT1A* gene, encoding S-adenosylmethionine (SAM) synthesizing isozymes MATI/III, and up-regulation of widely expressed *MAT2A*, encoding MATII isozyme, known as *MAT1A:MAT2A* switch, occurs in hepatocellular carcinoma (HCC). Here we found *Mat1A:Mat2A* switch and low SAM levels, associated with CpG hypermethylation and histone H4 deacetylation of *Mat1A* promoter, and prevalent CpG hypomethylation and histone H4 acetylation in *Mat2A* promoter of fast-growing HCC of F344 rats, genetically susceptible to hepatocarcinogenesis. In HCC of genetically resistant BN rats, very low changes in the *Mat1A:Mat2A* ratio, CpG methylation, and histone H4 acetylation occurred. The highest *MAT1A* promoter hypermethylation and *MAT2A* promoter hypomethylation occurred in human HCC with poorer prognosis. Furthermore, levels of AUF1 protein, which destabilizes *MAT1A* messenger RNA (mRNA), Mat1A-AUF1 ribonucleoprotein, HuR protein, which stabilizes *MAT2A* mRNA, and Mat2A-HuR ribonucleoprotein sharply increased in F344 and human HCC, and underwent low/no increase in BN HCC. In human HCC, *Mat1A:MAT2A* expression and MATI/III:MATII activity ratios correlated negatively with cell proliferation and genomic instability, and positively with apoptosis and DNA methylation. Noticeably, the MATI/III:MATII ratio strongly predicted patient survival length. Forced *MAT1A* overexpression in HepG2 and HuH7 cells led to a rise in the SAM level, decreased cell proliferation, increased apoptosis, down-regulation of *Cyclin D1, E2F1, IKK, NF-κB*, and antiapoptotic *BCL2* and *XIAP* genes, and up-regulation of *BAX* and *BAK* proapoptotic genes. In conclusion, we found for the first time a post-transcriptional regulation of *MAT1A* and *MAT2A* by AUF1 and HuR in HCC. Low MATI/III:MATII ratio is a prognostic marker that contributes to determine a phenotype susceptible to HCC and patients' survival. *Conclusion*: Interference with cell cycle progression and I-kappa B kinase (IKK)/nuclear factor kappa B (NF-κB) signaling contributes to the antiproliferative and proapoptotic effect of high SAM levels in HCC. (HEPATOLOGY 2012;56:165-175)

Human hepatocellular carcinoma (HCC) is one of the most common and deadliest tumors worldwide.[1] Better understanding of the pathogenetic mechanisms may hasten identification of new prognostic markers and development of new diagnostic and therapeutic strategies.[1]

Molecular events leading to cell cycle deregulation in HCC include highest up-regulation of iNOS/

Abbreviations: AUF1, AUrich RNA binding factor 1; Chip, chromatin immunoprecipitation assay; COBRA, combined bisulfite restriction analysis; DN, dysplastic nodule; DUSP1, dual-specificity phosphatase 1; ERK, extracellular signal-regulated kinase; ERMA, enzymatic regional methylation assay; FOXM1, forkhead box M1B; FOXO1, forkhead box O1; GI, genome instability; HCC, hepatocellular carcinoma; HCCB, HCC with better prognosis; HCCP, HCC with poor prognosis; HuR, Hu-antigen R; iNOS, inducible nitric oxide synthase; MAT1A, methyl adenosyltransferase 1A; MeDip, methylated DNA immunoprecipitation; Mybl2, v-Myb avian myeloblastosis viral oncogene homolog-like2; qPCR, quantitative real-time reverse-transcription polymerase chain reaction; RAPD, random amplified polymorphic DNA; RASSF1A, Ras-associated factor 1; SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-methionine; SL, surrounding nontumorous liver.

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Received September 9, 2011; accepted January 24, 2011.

NF-κB (inducible nitric oxide synthase/nuclear factor-κB)[2] and RAS/ERK (extracellular signal-regulated kinase) signaling, ubiquitination of ERK inhibitor DUSP1 (dual-specificity phosphatase 1),[3,4] and deregulation of FoxM1 (forkhead box M1B),[5] Mybl2 (v-Myb avian myeloblastosis viral oncogene homolog-like2),[6,7] and cell cycle key genes[8,9] in rapidly progressing HCC of F344 rats, genetically susceptible to hepatocarcinogenesis, and in a human HCC subtype with poorer prognosis (<3 years survival, after partial liver resection, HCCP). These changes were lower/absent in slow-progressing HCC of resistant BN rats and human HCC with better outcome (>3 years survival; HCCB). Furthermore, highest ubiquitination and proteasome degradation of cell cycle inhibitors of WAF/Kip families, P130, RASSF1A (Ras-associated factor 1), and FOXO1 (forkhead box O1) contribute to cell cycle up-regulation in F344 HCC and HCCP.[9,10]

SAM (S-adenosylmethionine) deficiency strongly influences HCC development. Low SAM content favors HCC development in rodents and humans,[11-15] whereas cell proliferation is inhibited by reconstitution of normal SAM levels in rat HCC, *in vivo*, by SAM administration,[11-13] and in human HCC cell lines by *MAT1A* (methionine adenosyltransferase 1A) transfection or SAM addition to culture medium.[14,16] In mammals the liver-specific *MAT1A* gene encodes MATI/III isozymes, whereas the widely expressed *MAT2A* encodes the MATII isozyme.[11] A fall in *MAT1A* expression with concomitant *MAT2A* up-regulation occurs in liver cirrhosis and HCC of rodents and humans.[11,17,18] The MATII isoform is inhibited by reaction product, and its up-regulation does not compensate for the fall in MATI/III isozymes.[11] Insights into the role of SAM deficiency in HCC pathogenesis were provided by HCC development in *MAT1A* knockout mice.[15]

Previous work[18] showed a role of deregulation of methionine metabolism in genome instability (GI) and aberrant DNA methylation in c-Myc transgenic mice and human HCC. The SAM/SAH (SAM/S-adenosylhomocysteine) ratio and liver MatI/III activity progressively decreased in dysplastic and neoplastic liver lesions of c-Myc transgenics and in most human HCC. This resulted in a rise of global DNA hypomethylation positively correlated with GI in mice and humans.[18] *Mat1A* down-regulation in cirrhotic liver of CCl_4-treated rats and in the human HepG2 cell line was associated with CCGG sequence methylation in the *Mat1A* promoter.[19] In Huh7 cells, *MAT1A* down-regulation was attributed to CCGG methylation at +10 and +80 of coding region.[20] *Mat2A* up-regulation in human HCC was associated with CCGG hypomethylation of the gene promoter.[21] Furthermore, recent work[22] showed *Mat1A* mRNA decrease in fetal rat liver, associated with an increase in its interaction with AUF1 (AUrich RNA binding factor 1), enhancing messenger RNA (mRNA) decay,[23] and an increase in *Mat2A* mRNA and its interaction with HuR (Hu-antigen R), which selectively binds to AUrich elements promoting mRNA stabilization.[23,24] Immunofluorescence analysis revealed increased HuR and AUF1 protein levels in human livers with HCC, suggesting post-transcriptional regulation of MAT proteins in HCC.[22]

These observations support the role of epigenetic regulation of MAT isozymes expression during HCC development. Here we tested if genetic susceptibility to HCC contributes to promoter methylation status and posttranscriptional deregulation of *Mat1A* and *Mat2A* during progression of rat hepatocarcinogenesis. Furthermore, because SAM levels influence cell proliferation, we investigated the prognostic role of *MAT1A:MAT2A* ratio and its contribution to the deregulation of signaling pathways involved in human HCC progression.

Materials and Methods

Animals and Treatments. F344 and BN rats were treated according to the "resistant hepatocyte" protocol.[25] Dysplastic nodules (DNs) and HCCs were collected at 32-40 and 50-67 weeks, respectively.

Human Tissue Samples. Six normal livers and 48 HCC and corresponding surrounding nontumorous

Supported by grants from the Associazione Italiana Ricerche sul Cancro (IG8952), Ministero Università e Ricerca (PRIN 2009), Regione Autonoma della Sardegna, Fondazione Banco di Sardegna; and a grant for the Third-term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour, and Welfare, Japan.

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.25643

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

livers (SLs) were used. Supporting Table S1 shows patient clinicopathological features.

Cell Lines and Treatments. Huh7 and HepG2 cell lines were transfected with *MAT1A* complementary DNA (cDNA) in pCMV6-XL expression vector (OriGene Technologies, Rockville, MD).

SAM, SAH, *and Global DNA Methylation Assays.* MatI/III and MatII activities and SAM and SAH levels[12] and global DNA methylation[26] were determined as reported.

Mat1A *and* MAT2a *Promoter Methylation.* Genomic DNA was digested with *HpaII* or MspI for Southern blot analysis. The samples were electrophoresed, transferred to a nylon membrane, and hybridized with a probe, generated by polymerase chain reaction (PCR) from liver gDNA. Bisulfite modified gDNA,[27] was used for combined bisulfite restriction analysis (COBRA),[28] methylation-specific PCR,[9] and enzymatic regional methylation assay (ERMA),[29] with the primers in Table S2.

Quantitative Real-Time Reverse Transcription (RT)-PCR (qPCR). qPCR reactions were performed as published.[5]

Chromatin Immunoprecipitation Assay (Chip). For Chip analysis,[31] chromatin extracted from nuclei was treated with anti-acetyl-histone H4 or rabbit IgG antibodies. Input DNA and immunoprecipitated chromatin were PCR-amplified with primers covering *Mat1a* and *Mat2A* gene promoters.

RNA Protein Interaction. Interaction of AUF1 and HuR proteins with *Mat1A* and *Mat2A* mRNAs, respectively, was determined by ribonucleoprotein immunoprecipitation in tissue extracts.[32]

Western Blot Analysis. Hepatic tissue samples were processed as reported.[5]

Random Amplified Polymorphic DNA (RAPD). Genomic alterations in human HCC were scored using 22 GC-rich arbitrary primers as described.[19]

Statistical Analysis. Differences of rat and human samples were analyzed by Tukey-Kramer, Student, or Mann-Whitney tests. Correlations were evaluated by multiple regression analysis, probability of overall survival by Mantel-Cox, and predictivity of patient survival by the Cox method.

See the Supporting Information for detailed descriptions of Materials and Methods.

Results

General Findings. Thirty-two to 40 weeks after initiation, high- and low-grade DNs developed in nine F344 and BN rats, respectively. At 50 weeks, seven moderately differentiated (ES grade II/III) and two poorly differentiated (ES grade IV) HCC developed in nine F344 rats. At 60-67 weeks, six moderately differentiated (ES grade II/III) and three well-differentiated (ES grade I/II) HCC developed in nine BN rats. Hematoxylin/eosin staining revealed the absence of contaminating normal parenchyma. DNs were distinguished from HCCs on the basis of histological criteria and for the presence of reticulin fibers and absence of glutathione synthase immunostaining[33] (not shown).

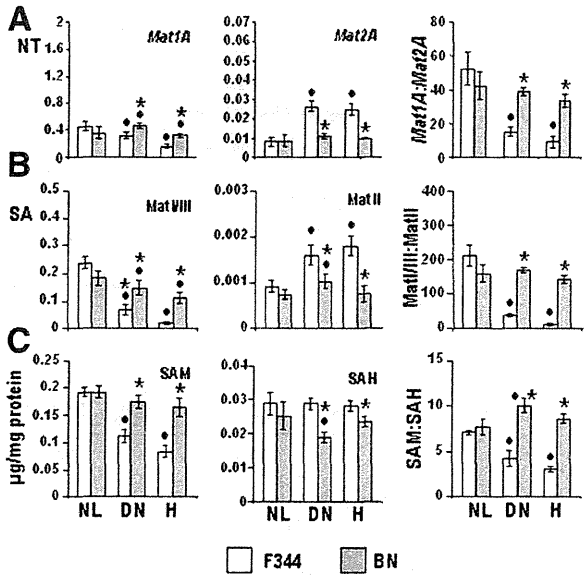

Fig. 1. *Mat1A* and *Mat2A* expression, MatI/III and MATII specific activity, and S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) content in normal liver (NL), dysplastic nodule (DN), and HCC (H) of F344 and BN rats. (A) Gene expression. The results are expressed as N-fold differences in target gene expression relative to the *RNR-18* expression, named N Target (NT). NT = $2^{-\Delta Ct}$, ΔCt of each sample was calculated by subtracting the Ct of the target gene from the Ct of the *RNR-18* gene. Enzymatic activity: (B) specific activity (SA) of MatI/III and MatII: nmol of labeled SAM/min, mg of protein. (C) SAM and SAH content and SAM/SAH ratio. Data are means ± standard deviation (SD) of six experiments. Analysis of variance (ANOVA): $P < 0.0001$ for gene expression, SA, and SAM and SAH content, and corresponding ratios. Tukey-Kramer test: (●) differences from control, at least $P < 0.05$. (*) F344 versus BN: at least $P < 0.05$.

Genetic Control of* Mat1A *and* Mat2A *Expression. No interstrain differences occurred in basic liver levels of *Mat1A* and *Mat2A* expression, MatI/III and MatII activities, and SAM/SAH ratio (Fig. 1). In F344 DN and HCC, *Mat1A* mRNA and MatI/III activity decreased, whereas *Mat2A* mRNA and MatII activity increased. In BN rat lesions, *Mat1A* level and MatI/III activity were higher, and *Mat2A* mRNA level and MatII activity were lower than in F344 rat lesions

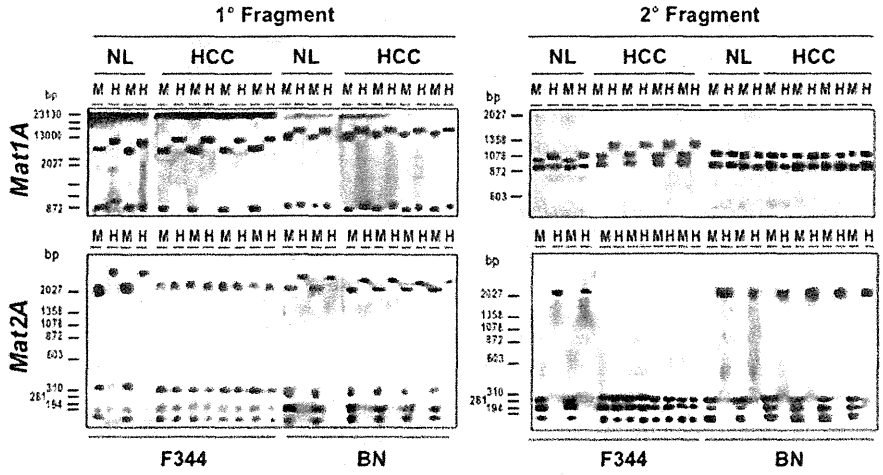

Fig. 2. Southern blot analysis of fragments I and II (cf. Fig. S1) of *Mat1A* and *Mat2A* promoter DNA, digested with *HpaII* (H), which is inhibited when the internal cytosine of CCGG sequence is methylated, or *MspI* (M), insensitive to methylation of the internal cytosine residue, in normal liver (NL) and HCC of F344 and BN rats. Note that digestion with *MspI* of *Mat1A* and *Mat2A* promoters of normal liver and HCC resulted in the same number and size of bands for both of F344 and BN rats. However, digestion of *Mat1A* promoter by *HpaII* only occurred in normal liver of both strains and in BN HCC. Digestion of *Mat2A* promoter by *HpaII* only occurred in F344 HCC. Southern blot hybridization was performed using labeled fragments of rat *Mat1A* and *Mat2A* promoters as described in Table S2.

(Fig. 1A,B). Accordingly, *Mat1A:Mat2A* and MatI/III: MatII ratios and SAM levels decreased in F344 liver lesions, and did not change significantly in BN DN and HCC (Fig. 1C). SAH level underwent low/no change in both strains and the SAM/SAH ratio decreased in F344 DN and HCC, and increased/did not change in BN rat lesions.

Mat1A *and* Mat2A *Promoter Methylation.* Because of the reported role of CCGG methylation in *MAT1A* and *Mat2A* promoters in rats and/or humans,[19-21] we evaluated the interstrain differences in methylation status of MAT genes. Southern analysis of *Mat1A* promoter indicated the presence of unmethylated CCGG sequences in normal liver of F344 and BN rats and HCC of BN rats, and of methylated sequences in F344 HCC (Fig. 2). Hypomethylated CCGG sequences occurred in two fragments of the *Mat2*A promoter of F344 HCC, corresponding to the −839, −791, −713, −622, and −401 positions, and to the −178, and +100 positions, respectively (Fig. S1). CCGG sequences in *Mat2*A promoter of normal liver of F344 and BN rats and BN HCC were methylated (Fig. 2).

According to COBRA, the TCGA sequence of *Mat1A* promoter at −1437 (site I; Fig. S1) was prevalently unmethylated in normal liver of both strains and in BN HCC, whereas it was partially methylated in F344 HCC. TCGA at −1385 (site II) was prevalently methylated in normal liver and HCC of both strains, with highest values in F344 HCC (Fig. S2). No differences in methylation of TCGA and CgCg sites of *Mat2A* promoter (Fig. S1) occurred between normal liver and HCC of both strains (not shown). No differences were found between normal liver and HCC of both strains for CpGs methylation in the −765:−458 fragment of *Mat1A* promoter, analyzed by msPCR, whereas hypomethylation of the −618:−405 CpGs of *Mat2A* promoter occurred in F344 HCC but not in BN HCC (Fig. S2).

CpGs methylation was also tested by ERMA (Fig. 3A). The analysis of three DNA fragments of *Mat1A* promoter, containing GpGs between −1205:−909, −778:−573, and −658:−242, respectively (Fig. S1), showed increased density of methylated CpGs in the first and third fragment of F344 HCC, and no change in BN HCC. The analysis of two DNA fragments of *Mat2A* promoter, with CpGs sites between −837:−333 and −439:+66, respectively (Fig. S1), evidenced hypomethylation of CpGs of F344 HCC, whereas no change and an increase in CpG islands methylation occurred in the first and second fragment of BN HCC, respectively (Fig. 3A). Because promoter fragments covering the transcription start site (Fig. S1) are the most important determinant of gene expression levels,[34] we further examined by methylated DNA immunoprecipitation (meDIP) the methylation of CpGs of Mat gene promoters close to ATG (−124:+10). No change in the methylation status of the single CpG in this fragment of *Mat1A* promoter occurred in either strain (not shown), whereas, in

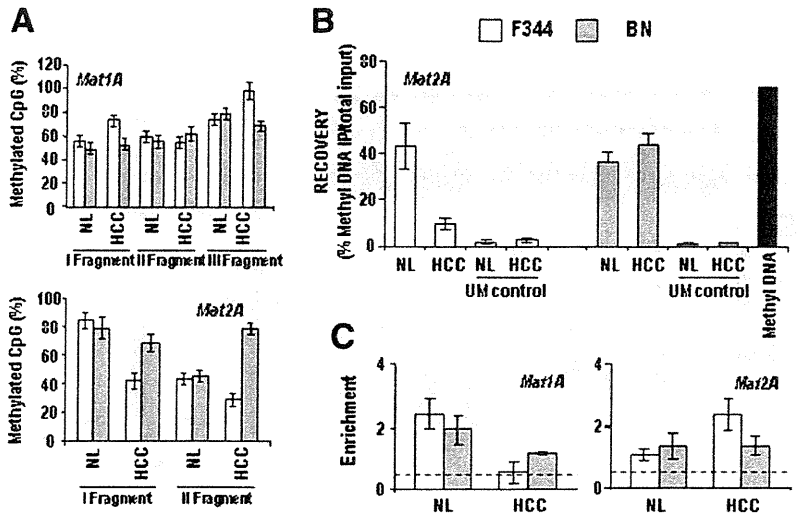

Fig. 3. Methylation status and histone H4 acetylation of *Mat1A* and *Mat2A* promoter in F344 and BN rats. (A) ERMA analysis of fragments I, II, and III of *Mat1A* promoter, and I and II of *Mat2A* promoter of normal liver (NL) and HCC of F344 and BN rats (cf. Fig. S1 and Table S1). Data are means ± SD of nine experiments. ANOVA: $P < 0.0001$ for *Mat1A* and *Mat2A*. Tukey-Kramer test: *Mat1A*, NL versus HCC, $P < 0.001$ for the first and third fragment, and third fragment of F344 and BN HCC, respectively. F344 versus BN, $P < 0.001$ for the first and third fragment. *Mat2A*, NL versus HCC, $P < 0.001$ for the first and second fragment, and the second fragment of F344 and BN HCC, respectively. F344 versus BN, $P < 0.001$ for the second fragment. (B) MeDip analysis of *Mat2A* between −124 and +10 basepairs. Recovery represents the efficiency of immunoprecipitated methyl DNA, calculated from qPCR data, and reported as percentage of the starting material: meDNA-IP/imput = $\hat{2}[Ct^{(input)} - Ct^{(meDNA\text{-}IP)} \times 100]$. Data are means ± SD of three independent determinations. ANOVA: $P < 0.0001$ for F344 an BN. Tukey-Kramer test: NL versus HCC for F344 $P < 0.001$. (C) Histone H4 acetylation. Chromatin immunoprecipitated by anti-acetyl-histone H4 antibody was amplified by PCR in the presence of primers covering the promoters of *Mat1a* and *Mat2A* genes. Negative controls with chromatin immunoprecipitated by anti-IgG antibody showed the absence of enrichment with respect to input. Results were normalized and presented as percentages of input DNA. The dashed line represents the threshold signal of 0.5%, above which significant enrichment for histone acetylation was scored. Data are means ± SD of three independent determinations. ANOVA: $P = 0.0015$ and $P = 0.0195$ for *Mat1A* and *Mat2A*, respectively. Tukey-Kramer test: HCC versus control (NL), *Mat1A*, $P < 0.001$ for F344 and BN; *Mat2A*, $P < 0.001$ for F344. F344 versus BN HCC, $P < 0.001$ for *Mat1A* and *Mat2A*.

agreement with ERMA analysis, an ≈5-fold decrease and no significant change of methyl-DNA recovery occurred in *Mat2A* promoter of F344 and BN rats HCC, respectively (Fig. 3B).

Due to the role of histone acetylation in the regulation of DNA methylation and gene expression,[31] we determined histone H4 acetylation in the core region of *Mat1A* and *Mat2A* promoters (Fig. 3C). No inter-strain difference in histone H4 acetylation of *Mat1A* and *Mat2A* promoters occurred in normal liver. The highest deacetylation of histone H4 occurred in *Mat1A* promoter of F344 HCC, compared with BN HCC. In *Mat2A* promoter the highest histone H4 acetylation was detected in F344 HCC and no change in BN HCC with respect to normal liver.

The above results document differences in Mat gene promoters methylation between HCCs differently prone to progress of F344 and BN rats. Evaluation of methylation status of MATs promoters (−370 to +100) in human HCCB and HCCP and corresponding SLs showed (Fig. S3) a 3.8 to 4.3-fold increase in MAT1A promoter methylation of SLP/HCCP, and no change in SLB/HCCB, compared with normal liver. MAT2A promoter methylation between −440 to −121 decreased 1.9 to 3-fold in both SLB/HCCB and SLP/HCCP. A significant decrease also occurred in HCCB, SLP, and HCCP between −121 and +100.

AUF1 and HuR Proteins Expression and Binding to Mat1A and Mat2A mRNAs. Recent work showed increased levels of HuR and AUF1 proteins in human livers with HCC,[22] suggesting posttranscriptional deregulation of MATs in human HCC. We evaluated this hypothesis by investigating Aufl and HuR interactions with *MAT1A* and *MAT2A* mRNA, respectively, in rat and human HCC. A higher increase in AUF1 protein levels occurred in F344 than BN HCC, whereas HuR up-regulation only occurred in F344 HCC (Fig. 4A,B). Mat1A-AUF1 ribonucleoprotein (Fig. 4C) increased ≈10-fold and ≈2-fold, when compared with normal liver, in HCC of F344 and BN rats, respectively. Mat2A-HuR ribonucleoprotein increased ≈10-fold in F344 HCC and did not change in BN HCC. A progressive increase in AUF1 and HuR proteins and Mat1A-AUF1 and Mat2A-HuR

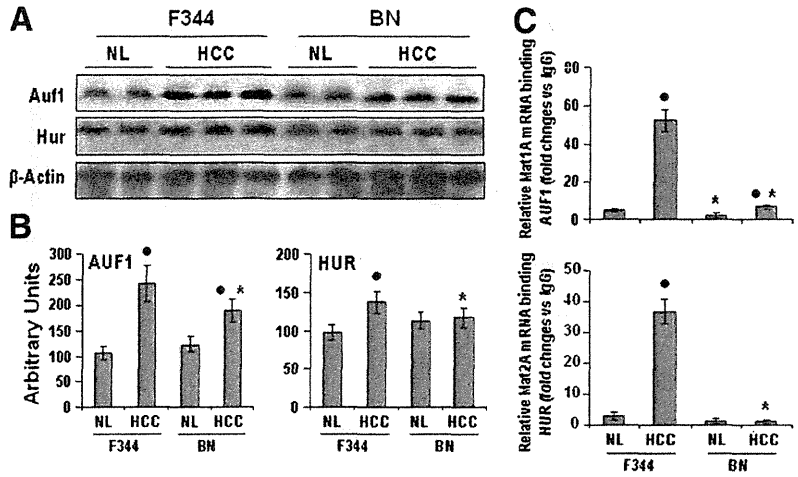

Fig. 4. Posttranscriptional regulation of Mats in normal liver (NL) and HCC of F344 and BN rats. (A) Representative western blot of Auf1 and HuR. (B) Chemiluminescence analysis showing the mean (SD) of three NL and five HCC from each strain. Optical densities of the peaks were normalized to β-actin values and expressed in arbitrary units. ANOVA: $P < 0.0001$ for AUF1, $P = 0.0047$ for HuR. Tukey-Kramer test: (●) HCC versus NL, $P < 0.001$ for AUF1, $P < 0.01$ for HuR. (*) BN versus F344, $P < 0.05$. for AUF1 and HuR. (C) Protein-mRNA complexes and IgG normal control were immunoprecipitated with specific antibodies and RNA was isolated and cDNA were analyzed by qPCR. Auf1- and HuR-ribonucleoproteins were calculated as fold changes versus control IgG. Data are means ± SD of three NL and five HCC from each strain. Tukey-Kramer test: (●) HCC versus NL $P < 0.001$; (*) BN versus F344, $P < 0.001$.

ribonucleoproteins occurred from human SL to HCC compared with normal liver (Fig. 5).

To further evaluate the contribution of promoter methylation and posttranscriptional control in the regulation of *MAT1A:MAT2A* ratio, we analyzed in Huh7 cells the effects of DNA hypomethylation and AUF1 knockdown on expression of *MAT1A*, whose sharp decrease is largely responsible for MAT1A:MAT2A switch. As shown in Fig. S4, a 3 to 9-fold decrease in promoter methylation, in 5-azacytidine-treated cells and a 1.6 to 2.5-fold decrease in AUF1 expression, induced by specific siRNA, are associated with a 6 to 9- and 2.2 to 3.5-fold rise in *MAT1A* expression, respectively. This indicates that both promoter methylation status and AUF1 expression may contribute to deregulation of *MAT1A* expression and the *MAT1A:MAT2A* ratio in HCC cells.

MatI/III Activity Correlates with Prognostic Parameters. The greatest decrease in *Mat1A:Mat2A* expression and MATI/III:MATII activity ratios in more aggressive F344 HCCs suggests that these parameters might both influence tumor progression and act as prognostic markers. To test this hypothesis, we analyzed the correlation of the MatI/III:MATII ratio with various progression-related features of rat and human HCC. Lower DNA synthesis and higher

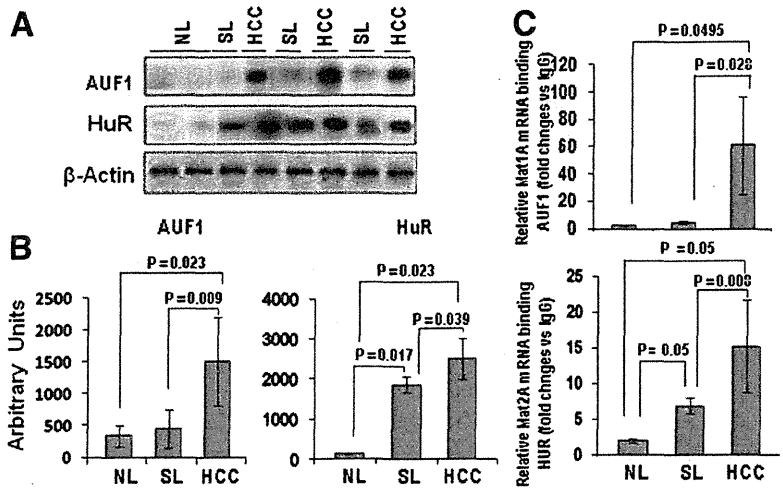

Fig. 5. Posttranscriptional regulation of Mats in human normal liver (NL), SL, and HCC. (A) Representative western blot of Auf1 and HuR. (B) Chemiluminescence analysis showing the mean ± SD of three NL and six HCC and corresponding SL. Optical densities of the peaks were normalized to β-actin values and expressed in arbitrary units. (C) Protein-mRNA complexes and IgG normal control were immunoprecipitated with specific antibodies and RNA was isolated and cDNA was analyzed by qPCR. Auf1- and HuR-ribonucleoproteins were calculated as fold changes versus control IgG. Data are means ± SD of three NL and six SL and HCC. Differences between means were calculated by Mann-Whitney test.

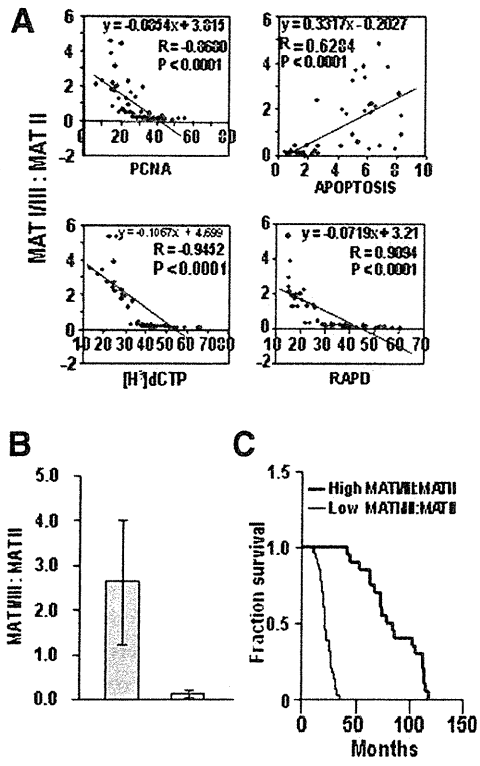

Fig. 6. (A) Relationships between MATI/III:MAT II ratio and proliferation index (expressed as the percentage of PCNA-positive nuclei with respect to total cells counted), apoptotic index (percentage of apoptotic cells), global DNA methylation (expressed as the amount of ^{3}H-dCTP in gDNA), and genomic instability (RAPD; expressed as the percentage of primers showing altered RAPD profiles). The values of ^{3}H-dCTP are inversely proportional to DNA methylation. Because of the nonparametric nature of the data, the Spearman rank correlation coefficient (R) was calculated. (B) MATI/III:MATII ratios in two distinct subgroups of human HCC with high (1.28-5.33) and low (0.029-0.42) ratios. Two-phase decay curves were used to calculate the cutoff value of MATI/III:MATII ratio (0.42-0.5) in correspondence to curve intersection in the correlation analysis of the ratio with PCNA, [H^3]dCTP, and RAPD. Difference between subgroups: Mann-Whitney test, $P < 0.0001$. (C) Kaplan-Meier survival curves of human HCC with high and low MATI/III:MATII ratios. Mantel-Cox statistical analysis: differences between survival curves MATI/III:MATII, $P < 0.0001$.

apoptosis occurred in BN rats DN and HCC than in F344 rat lesions, and methyl acceptance assay showed genomic hypomethylation in F344 DN and HCC but not in BN rat lesions (Fig. S5). The MATI/III:MATII ratio (Fig. S5) and *Mat1A:Mat2A* expression ratio (not shown) were negatively correlated with DNA synthesis and positively correlated with apoptosis of F334 and BN rat liver lesions. A negative correlation of the MATI/III:MATII ratio with [^{3}H]dCTP (Fig. S5) indicates its positive correlation with DNA methylation.

The analysis of 48 human HCCs (Fig. S6) showed a progressive decrease in *Mat1A* expression and MATI/III activity and progressive increase in *MAT2A* expression and MATII activity from SL to HCC as compared with normal liver values. These changes were paralleled by progressive increase in proliferation rate, and decrease in DNA methylation (increase in [^{3}H]*methyl* incorporation) from SL to HCC, compared with normal liver. Apoptotic index increased in SL and HCC (Figs. S6, S7). Correlation analysis revealed (Fig. 6A) an inverse correlation of *MAT1A:MAT2A* expression ratio (not shown) and MATI/III:MATII activity ratio with PCNA expression, [^{3}H]*methyl* incorporation into DNA, and GI, and a direct correlation with apoptosis. Notably, direct correlation of MATI/III:MATII ratio with the proliferation rate ($R = 0.6983$, $P < 0.0001$) and inverse correlation with DNA methylation ($R = 0.9056$; $P < 0.0001$) occurred in SL, whereas no significant correlation with apoptosis index was found.

The results in Fig. 6A clearly indicate the presence of two patient subgroups with significantly different MatI/III:MatII ratios (Fig. 6B) and survival length, with the HCC subset showing the lower MATI/III:MARII ratio being associated with shorter survival (Fig. 6C). Remarkably, Cox analysis showed that MATI/III:MATII ratio significantly predicted patient survival length: hazard ratio, 0.811, 95% confidence interval: 0.663-0.993, $P = 0.042$. In the multivariate analysis model (Table 1), the MATI/III:MATII ratio remained significantly associated with overall survival, together with patients' age, tumor etiology and grade, PCNA expression, DNA methylation, and GI. A significant association of the *MAT1A:MAT2A* expression ratio and etiology, PCNA, and GI with overall survival was also found (not shown).

Effects of* MAT1A *Overexpression in Human HCC Cell Lines. The above results suggest a role of high *MAT1A* expression and *MAT1A:MAT2A* ratio in

Table 1. Multivariate Analysis of Factors Contributing to Overall Survival

	Overall Survival		
Variable	Hazard Ratio	95% Confidence Interval	*P* Value
Sex	2.27	0.85-6.09	0.102
Age	0.96	0.92-1.00	**0.047**
Etiology	0.69	0.49-0.97	**0.031**
Cirrhosis	1.11	0.37-3.31	0.849
Tumor size	1.74	0.59-5.13	0.310
Edmondson-Steiner grade	2.83	1.07-7.50	**0.037**
AFP secretion	1.53	0.65-3.62	0.334
MATI/III:MATII ratio	0.76	0.62-0.94	**0.010**
PCNA	1.06	1.01-1.11	**0.011**
Apoptosis	1.08	0.75-1.55	0.697
Global DNA methylation	1.19	1.02-1.41	**0.032**
Genomic instability (RAPD)	1.22	1.09-1.37	**0.001**

AFP, α-Fetoprotein; PCNA, proliferating cell nuclear antigen; RAPD, random amplified polymorphic DNA.

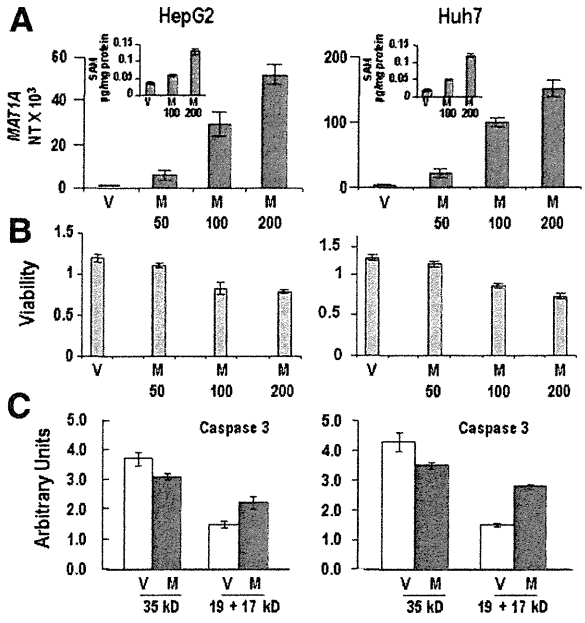

Fig. 7. Effect of *MAT1A* transfection of cell proliferation and gene expression. HepG2 and HuH7 cells were transiently transfected with *MAT1A* cDNA in pCMV6-XL vector. (A) *MAT1A* expression 72 and 96 hours in HepG2 and Huh7 cells, respectively, after transfection with empty vector (V) or the indicated amounts (ng) of *Mat1A* cDNA (M). Data are means ± SD of three independent experiments of N-fold differences in *MAT1A* expression relative to the *RNR-18* expression, and named N Target (NT). $NT = 2^{-\Delta Ct}$, $\Delta Ct = [CT^{(target)} - CT^{(RNR\text{-}18)}]$. Insets: SAM levels. Tukey-Kramer test: *Mat1A* expression and SAM level, M versus V, $P < 0.001$. (B) Cell viability of HepG2 and Huh7 cells 72 and 96 hours after transfection, respectively. Tukey-Kramer test: M versus V $P < 0.001$ for M 100 and 200 ng. (C) Caspase 3 cleavage in HepG2 and Huh7 cells 72 and 96 hours after transfection, respectively, with empty vector (V) or 200 ng of *Mat1A* cDNA. Data are means ± SD of three independent experiments. Tukey-Kramer test: M versus V $P < 0.001$ for 35 and 17+19 kD fragments of both cell lines.

suppression of growth and induction of apoptosis of human HCC. Furthermore, because SAM level modulates IKK-β activity of *in vitro*-growing cells[35] and counteracts NF-κB activation in rat preneoplastic foci,[36] we evaluated the effects of *MAT1A* up-regulation on proliferation, apoptosis, and the IKK-NF-κB cascade in human HCC cell lines transiently transfected with *MAT1A*. *MAT1A* transfection, which resulted in 95- and 70-fold increases in its expression at 96 hours, a 5 to 7-fold rise in SAM level (Fig. 7A), a significant reduction in cell proliferation (Fig. 7B), and increase in apoptosis, as shown by caspase 3 cleavage (Fig. 7C; Fig. S7) in HepG2 and Huh7 cells. This was associated with a significant down-regulation of *Cyclin D1, E2F1, IKK, NF-κB,* and the antiapoptotic *BCL2* and *XIAP* genes, and up-regulation of the proapoptotic *BaK* and *BAX* genes in HepG2 and Huh7 cells, 72 and 96 hours after transfection, respectively (Fig. S8).

Discussion

Our results show a marked decrease in *Mat1A* expression and MATI/III activity, an increase in *Mat2A* expression and MATII activity, and a decrease in *Mat1A:Mat2A* expression ratio and the MATI/III: MATII activity ratio in DN and HCC of F344 rats, genetically susceptible to hepatocarcinogenesis. *Mat1A* and *Mat2A* expression and MAT isozymes underwent low/no changes in DN and HCC of genetically resistant BN rats. Changes in the MATs isozyme pattern, analogous to those of F344 HCC, occur in human HCC,[16,19,21] and have been associated with CCGG methylation of *MAT1A* promoter, and CCGG hypomethylation of *MAT2A* promoter.[19,21] Accordingly, we observed prevalent CpGs methylation and histone H4 deacetylation of the *Mat1A* promoter and prevalent CpGs hypomethylation and histone H4 acetylation in the *Mat2A* promoter of F344 HCC. Considering the ATG flanking regions of the two genes (+10 to −778 and +66 to −837 for *Mat1A* and *Mat2A*, respectively, analyzed by ERMA), ≈82% and ≈60% of CpGs were methylated in the *Mat1A* promoter of F344 and BN HCC, respectively. Methylation of 85%-86% of CpGs occurs in the ATG flanking region of the *Mat1A* promoter of mouse Hepa-1[37] and human Huh7 HCC cells.[20] In the *Mat2A* promoter, ≈68% and ≈20% of CpGs were unmethylated in F344 and BN HCC, respectively. Notably, ≈62% of CpGs in the ATP flanking region of human *MAT1A* were methylated in HCCP against ≈25% in HCCB. The *MAT2A* promoter was largely hypomethylated, with a lower difference between HCCP and HCCB. These changes roughly reproduce interstrain differences between rat HCCs differently prone to progress.

The mechanisms modulating MATs promoter methylation in hepatocarcinogenesis are still unknown. Interaction of DNA methyltransferases (DNMTs) with higher-order chromatin, inducing DNA methylation, may be reduced by histone acetylation.[38] *Dnmt1, Dnmt3a,* and *Dnmt3b* genes are equally overexpressed in HCC from both F344 and BN rats (data not presented). Further research should investigate the influence of interstrain variations in the mutual interplay between DNMTs, histone acetylation, and chromatin on *Mat1A* and *Mat2A* promoter methylation. Moreover, DNMT1 and DNMT3a interaction with histone

deacetylases may repress gene expression by way of a methylation-independent mechanism.[39]

Suppression of gene expression is frequently associated with methylated DNA and deacetylated histones, in a dense chromatin structure, whereas active transcription is associated with unmethylated DNA, hyperacetylated histones, in open chromatin.[40] According to recent results,[20] methylation of CCGG at −997 does not inhibit the *MAT1A* promoter, whereas CCGG methylation at +10 and +88 reduces promoter activity in Huh7 cells. However, no CCGG sequences are present in the first exon of rat *Mat1A* (NCBI AC_000084.1). Recent results indicate that binding to promoter of the transcription factor C/EBPβ, regulated by DNA methylation and histone deacetylation, determines *MAT1A* down-regulation in mouse, rat, and human.[37] Interestingly, *C/EBPβ* expression is ≈4-fold higher in F344 than BN HCC (data not shown), but C/EBPβ protein biding to promoter has not yet been evaluated. Nevertheless, in F344 HCC the highest histone H4 deacetylation was associated with the highest methylation of *Mat1A* promoter and low gene expression, whereas the highest histone H4 acetylation was associated with low methylation of the *Mat2A* promoter and gene up-regulation. These changes are very low/absent in BN HCC. Thus, our observations suggest, in accordance with previous results,[20,21] that changes in MATs promoter methylation and histone acetylation might be involved in *Mat1A:Mat2A* switch, and clearly indicate their association with the capacity of DN and HCC to progress.

Our results also support a role of posttranscriptional regulation of Mats expression in tumor progression and aggressiveness. A rise in AUF1 and HuR protein levels and of their interaction with *MAT1A* and *MAT2A* mRNAs, respectively, occurred in F344 rat and human HCC. Because the protein HuR promotes mRNA stabilization and AUF1 enhances mRNA decay,[22-24] our observations could at least in part explain the decrease in *MAT1A* and increase in *MAT2A* mRNA levels in these HCCs. AUF1 and HuR interaction with *Mat1A* and *Mat2A*, respectively, was very low in BN HCC. This should result in lower *Mat1A* mRNA decay and *Mat2A* mRNA stabilization in BN tumors, thus explaining the observed low changes in *Mat1A* and *Mat2A* expression with respect to normal liver.

A remarkable aspect of our results is the observation that genes responsible for genetic susceptibility to hepatocarcinogenesis control *Mat1A* and *Mat2A* methylation and posttranscriptional regulation, demonstrating, for the first time, that the regulation of CpG methylation of *Mat1A* and *Mat2A* promoter and posttranscriptional regulation of MATs can be connected to the genetic susceptibility of rats to HCC. This indicates that MATs epigenetic regulation is under genetic control and contributes to create a phenotype susceptible/resistant to hepatocarcinogenesis. In accordance with this assumption, our observations strongly suggest that *MAT1A:MAT2A* and MATI/III:MATII ratios are prognostic markers for HCC. Indeed: (1) the *MAT1A:MAT2A* expression ratio and the MATI/III:MATII activity ratio were directly correlated with proliferation rate and inversely correlated with apoptosis of HCC cells; (2) the direct correlation with global DNA methylation and genomic instability links these ratios to genomic alterations that are known to facilitate tumor progression[41]; (3) MATI/III:MATII ratio is strongly predictive of patient survival. Notably, according to our results, the MATI/III:MATII ratio influences the proliferation rate and global DNA methylation even in SL, suggesting that in human, as in rodent, early deregulation of the MATI/III:MATII ratio could influence the evolution of preneoplastic lesions to cancer.

The mechanisms involved in modulation of hepatocarcinogenesis by the *MATI/III:MATII* ratio are still incompletely known. *Mat1A* knockout (KO) mice undergo spontaneous oxidative stress and HCC development.[15] Conversely, HCC cells transfected with *MAT1A* exhibit lower proliferation and higher apoptosis and tumorigenesis in nude mice than untransfected cells[16,20] (and present results). *MAT1A* expression and the *MAT1A:MAT2A* ratio are major determinants of SAM content in rat and human hepatic cells that largely influences liver cell proliferation and tumorigenesis.[14,16] The decrease in expression of *IKK* in transfected cells, probably consequent to the increase in SAM level,[35] leads to *NF-κB* down-regulation, which could result in inhibition of the G_1 phase of cell cycle and G_1-S transition through restriction of Cyclin D1 and c-MYC expression, leading to *E2F1* down-regulation,[42] which was indeed found in *MAT1A* transfected cells. Furthermore, down-regulation of antiapoptotic *BCL2* and *XIAP* genes, and up-regulation of proapoptotic *BAX* and *BAK* genes, targeted by NF-κB,[43] may contribute to increased apoptosis. Interestingly, G_1/S transition and expression of NF-κB and its targets are significantly lower in preneoplastic and neoplastic lesions from BN rats and human HCCB than in F344 rats and HCCP.[2,7,8]

Various other mechanisms may be involved in the antiproliferative and proapoptotic effects of a high *MAT1A:MAT2A* ratio and SAM level. SAM has an antioxidant effect[15] and inhibits the activity of different growth-related genes and proteins,[16] including

iNos expression[44] and ERK1/2 activation.[16] Notably, SAM stabilizes APEX1 protein,[45] which should result in more efficient DNA repair and reduced GI. Thus, low MATI/III activity and SAM content in precancerous liver and HCC of susceptible rats should result in increased oxidative and nitrative stress, GI, overexpression of NF-κB and its targets, including c-*Myc, Cyclin D1, iNos*, and *VegfA*. All of these changes have indeed been observed in F344 HCC and HCCP, whereas they are low/absent in BN HCC and HCCB.[41]

In conclusion, our data indicate, for the first time, a posttranscriptional regulation of *MAT1A* and *MAT2A* by AUF1 and HuR in HCC. Notably, a low *MAT1A: MAT2A* ratio is a prognostic marker that contributes to determine a phenotype susceptible to HCC and patient survival. Finally, interference with cell cycle progression, through inhibition of IKK/NF-κB signaling, Cyclin D1, and E2F1, might represent one of the multiple mechanisms mediating the antiproliferative and proapoptotic effect of high SAM levels.

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Cancer Letters 322 (2012) 204–212

Contents lists available at SciVerse ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Development of a novel approach, the epigenome-based outlier approach, to identify tumor-suppressor genes silenced by aberrant DNA methylation

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ARTICLE INFO

Article history:
Received 4 January 2012
Received in revised form 10 March 2012
Accepted 12 March 2012

Keywords:
Epigenetics
Aberrant DNA methylation
Tumor-suppressor gene

ABSTRACT

Identification of tumor-suppressor genes (TSGs) silenced by aberrant methylation of promoter CpG islands (CGIs) is important, but hampered by a large number of genes methylated as passengers of carcinogenesis. To overcome this issue, we here took advantage of the fact that the vast majority of genes methylated in cancers lack, in normal cells, RNA polymerase II (Pol II) and have trimethylation of histone H3 lysine 27 (H3K27me3) in their promoter CGIs. First, we demonstrated that three of six known TSGs in breast cancer and two of three in colon cancer had Pol II and lacked H3K27me3 in normal cells, being outliers to the general rule. *BRCA1*, *HOXA5*, *MLH1*, and *RASSF1A* had high Pol II, but were expressed only at low levels in normal cells, and were unlikely to be identified as outliers by their expression statuses in normal cells. Then, using epigenome statuses (Pol II binding and H3K27me3) in normal cells, we made a genome-wide search for outliers in breast cancers, and identified 14 outlier promoter CGIs. Among these, *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* were confirmed to be methylated in primary breast cancer samples. Knockdown of *DZIP1* in breast cancer cell lines led to increases of their growth, suggesting it to be a novel TSG. The outliers based on their epigenome statuses contained unique TSGs, including *DZIP1*, compared with those identified by the expression microarray data. These results showed that the epigenome-based outlier approach is capable of identifying a different set of TSGs, compared to the expression-based outlier approach.

1. Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs), especially that of nucleosome-free regions (NFRs), causes silencing of their downstream genes [1], and is known as an alternative mechanism to point mutations and chromosomal deletion for inactivation of tumor-suppressor genes (TSGs) [2–6]. Now, TSGs involved in various biological pathways such as cell proliferation, cell adhesion, and DNA repair are known to be silenced by aberrant DNA methylation in human cancers [7,8]. To identify novel TSGs silenced by aberrant DNA methylation, two major approaches for genome-wide screening have been reported, namely (1) genome-wide screening of methylated genes and (2) screening of genes whose expression is induced by a DNA demethylating agent (chemical genomic screening) [9–13].

A genome-wide screening of methylated genes usually isolates several hundreds to one thousand genes whose promoter CGIs are methylated in cancers [12,14]. Unfortunately, most of these genes are not expressed or expressed at only low levels in normal cells [14]. Since genes with low transcription tend to be methylated [15], most of the genes identified by genome-wide screening of methylated genes are considered to be methylated as a consequence of carcinogenesis. In chemical genomic screening, cell lines are treated with a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), and genes whose expression is induced are screened by expression microarray analysis. However, since 5-aza-dC forms DNA adducts [16] and induces double-strand breaks [17], treatment with 5-aza-dC leads to up-regulation of not only methylation-silenced genes but also numerous genes involved in responses to DNA damage, including p53-activated genes [18–21].

It was recently shown that the vast majority of genes aberrantly methylated in cancers have specific epigenetic statuses in normal cells, namely the lack of RNA polymerase II (Pol II) and the presence of trimethylation of histone H3 lysine 27 (H3K27me3) [14,22]. H3K27me3 is known to be most influential on the susceptibility of genes to become methylated among various histone

Abbreviations: CGIs, CpG islands; ChIP, chromatin immunoprecipitation; FHC, normal fetal human colon epithelial cell line; HMECs, normal human mammary epithelial cells; H3K27me3, trimethylation of histone H3 lysine 27; NFR, nucleosome-free region; Pol II, RNA polymerase II; TSG, tumor-suppressor gene.

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http://dx.doi.org/10.1016/j.canlet.2012.03.016

modifications [14]. At the same time, approximately 5% of the genes methylated in cancers do not follow the general rule [14], constituting a group of "outliers". Since a TSG is functioning in normal cells, it is expected to have Pol II in normal cells and belong to the outliers. Even if silencing of a TSG is a very rare event due to the Pol II binding, a cell with its silencing will become dominant due to the resultant growth advantage, and the TSG appears as if it is susceptible to methylation. Indeed, by searching outliers with high expression in normal cells, TSGs have been successfully identified [11,23,24]. However, some TSGs, including *CDKN1A*, are known to have no or low expression in normal cells and are activated by extracellular stimuli [25,26].

In this study, we aimed to show that a significant fraction of known TSGs silenced by aberrant DNA methylation are outliers. Then, we will show that a different set of TSGs could be identified by searching for outliers with high Pol II and without H3K27me3 (epigenome-based outlier approach), compared to those identified by searching for outliers with high expression (expression-based outlier approach).

2. Materials and methods

2.1. Cell lines and primary breast cancer samples

Human breast cancer cell lines (BT-20, BT-474, HCC38, HCC1428, HCC1937, Hs 578T, MCF7, MDA-MB-231, MDA-MB-436, MDA-MB-468, SK-BR-3, T-47D, and ZR-75-1), normal fetal human colon epithelial cell line (FHC), and 293TN cell line were purchased from the American Type Culture Collection (Rockville, MD). Normal human mammary epithelial cells (HMECs) were purchased from Cambrex (East Rutherford, NL). Forty primary breast cancer samples were obtained from patients who underwent mastectomy or breast conserving surgery with informed consent. Resected primary samples were frozen and stored at −80 °C until the extraction of genomic DNA. The analysis was approved by the Institutional Review Boards.

2.2. Chromatin immunoprecipitation (ChIP)

Eight μg of chromatin extracted from HMECs was incubated with 0.7 μg of antibodies against Pol II (ab5095; Abcam, Cambridge, UK) or H3K27me3 (07-449; Millipore, Billerica, MA) overnight at 4 °C. Then the immunocomplex was collected with Dynabeads Protein A (Invitrogen Dynal AS, Oslo, Norway). Recovered DNA was dissolved in 33 μl of 1 × TE (10 mM Tris-HCl and 1 mM EDTA) [14], and one μl of DNA was used for ChIP-quantitative PCR (ChIP-qPCR) using primer sets listed in Supplementary Table 1. The specificity of the ChIP assay was assessed by using primers for control regions whose histone modification statuses were already known [14] (Supplementary Fig. 1).

2.3. Analyses of microarray data

DNA methylation data of 9838 promoter CGIs in HMECs, BT-474, MCF7, MDA-MB-231, MDA-MB-468, and ZR-75-1 were obtained from our previous study using methylated DNA immunoprecipitation (MeDIP)-CGI microarray analysis [27]. Pol II binding and H3K27me3 data of 9838 promoter CGIs in HMECs were obtained from our previous study using ChIP-CGI microarray analysis [14]. Pol II binding and H3K27me3 levels of each CGI were evaluated using the average of the signal ratio [Cy5 signal (bound)/Cy3 signal (input)] of the probes within a NFR. CGIs with a mean signal ratio larger than that at the 80th percentile of the total probes were considered as CGIs with high Pol II binding or H3K27me3, and those with an average signal ratio smaller than that at the 20th percentile of the total probes were considered as CGIs with low Pol II binding or H3K27me3. Other CGIs were considered as genes with intermediate Pol II binding or H3K27me3 levels. Gene expression data in HMECs were obtained from our previous study using the GeneChip Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA), and genes were classified into those with high, moderate, and low expression levels, as described previously [14].

2.4. Quantitative methylation-specific PCR (qMSP)

Genomic DNA was digested with *Bam*HI, and one μg of digested DNA was used for bisulfite modification [28]. qMSP was performed using primer sets specific to methylated target loci and to the *Alu* repeat sequence (Supplementary Table 2). Modified DNA was dissolved in 40 μl of 1 × TE, and one μl was used for qMSP. The number of DNA molecules in a sample was obtained by comparing its amplification curve with those of standard samples with known numbers of DNA molecules. DNA methylation levels were calculated as the percentage of the methylation reference (PMR) = [(number of molecules methylated at a target CGI in a sample)/(number of *Alu* repeat sequences in the sample)]/[(number of molecules methylated at the target CGI in a fully methylated DNA)/(number of *Alu* repeat sequences in the fully methylated DNA)] × 100 [29,30]. Genomic DNA treated with *SssI* methylase (New England Biolabs, Beverly, MA) was used as a fully methylated DNA. Since the copy number of the target CGI was normalized to the copy number of the *Alu* repeat sequence, PMR could reach more than 100% when the locus containing the target CGI had an increased copy number.

2.5. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was synthesized from one μg of total RNA using SuperScript III reverse transcriptase and an oligo $(dT)_{12-18}$ primer (Life Technologies, Carlsbad, CA). qRT-PCR was performed using primer sets listed in Supplementary Table 3, as described previously [31]. The number of cDNA molecules in a sample was obtained by comparing its amplification curve with those of standard samples with known numbers of cDNA molecules, and the number of target cDNA molecules was normalized to those of human *GAPDH* cDNA molecules.

2.6. 5-Aza-dC and trichostatin A (TSA) treatment

T-47D cells were seeded on day 0, and exposed to a designated dose of freshly prepared 5-aza-dC (SIGMA-ALDRICH, St. Louis, MO) for 24 h on days 1 and 3, and treated with TSA (SIGMA-ALDRICH) on day 4. The cells were harvested on day 5, and total RNA was extracted.

2.7. Knockdown of DZIP1 and cell growth assay

DZIP1 was knocked down by two different short hairpin RNAs (shRNA1 and shRNA2; Supplementary Table 4) designed by using siDirect version 2.0 [32], and shRNA for firefly luciferase was used as the control. The sense and antisense oligonucleotides containing each shRNA sequence were annealed, and then ligated into *BamHI*/*EcoRI* sites of pGreenPuro™ shRNA Cloning and Expression Lentivector (System Biosciences, Mountain View, CA). The shRNA expression construct was co-transfected with pPACK Packaging Plasmid Mix (System Biosciences) into 293TN cells using Lipofectamine™ with Plus™ Reagent (Life Technologies). Medium containing pseudovirus was collected 48 h after transfection, and stored at −80 °C until use. HCC1937 and MDA-MB-436 cells were infected with pseudovirus according to the manufacturer's protocol.

Cell growth was analyzed by seeding cells at a density of 5×10^4 cells/6 cm plate on day 0, and counting the cell numbers on days 1, 2, 3, 4, 5, and 8 using TC™ Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA).

3. Results

3.1. Confirmation of known TSGs as outliers

To confirm that TSGs are outliers to the general rule of genes methylated in cancer cells, we first analyzed six known TSGs silenced in breast cancer by aberrant DNA methylation, *BRCA1*, *CDKN2A* (*p16*), *HOXA5*, *MASPIN*, *RASSF1A*, and *RBP1* [33–38]. The Pol II binding and H3K27me3 levels of these six genes were analyzed in HMECs (Fig. 1A). Among the six TSGs, *BRCA1*, *HOXA5*, and *MASPIN* had high Pol II binding and almost undetectable levels of H3K27me3 in the HMECs, demonstrating that these genes are outliers. In contrast, *RASSF1A* had low Pol II binding and high H3K27me3 levels, showing that *RASSF1A* was methylated according to the rule. *CDKN2A* and *RBP1* had intermediate levels of Pol II binding and H3K27me3. We also analyzed expression levels of the six TSGs in normal cells. Among the three outliers, *MASPIN* was expressed at high levels (active Pol II), and *BRCA1* and *HOXA5* were expressed at low levels (stalled Pol II) (Fig. 1C). In the case of three TSGs known to be methylation-silenced in colon cancer, *MLH1* and *RASSF1A* [7] had high levels of Pol II binding and low expression levels (stalled Pol II) (Fig. 1B and D). These results indicated that a significant fraction of TSGs are "outliers" to the general rule of genes methylated in cancer cells, and that the outliers could be classified into those with active Pol II and with stalled Pol II.

3.2. Screening of outliers in breast cancer based on their epigenome statuses

Confirming that three of six known TSGs in breast cancer and two of three known TSGs in colon cancer are outliers, we pro-

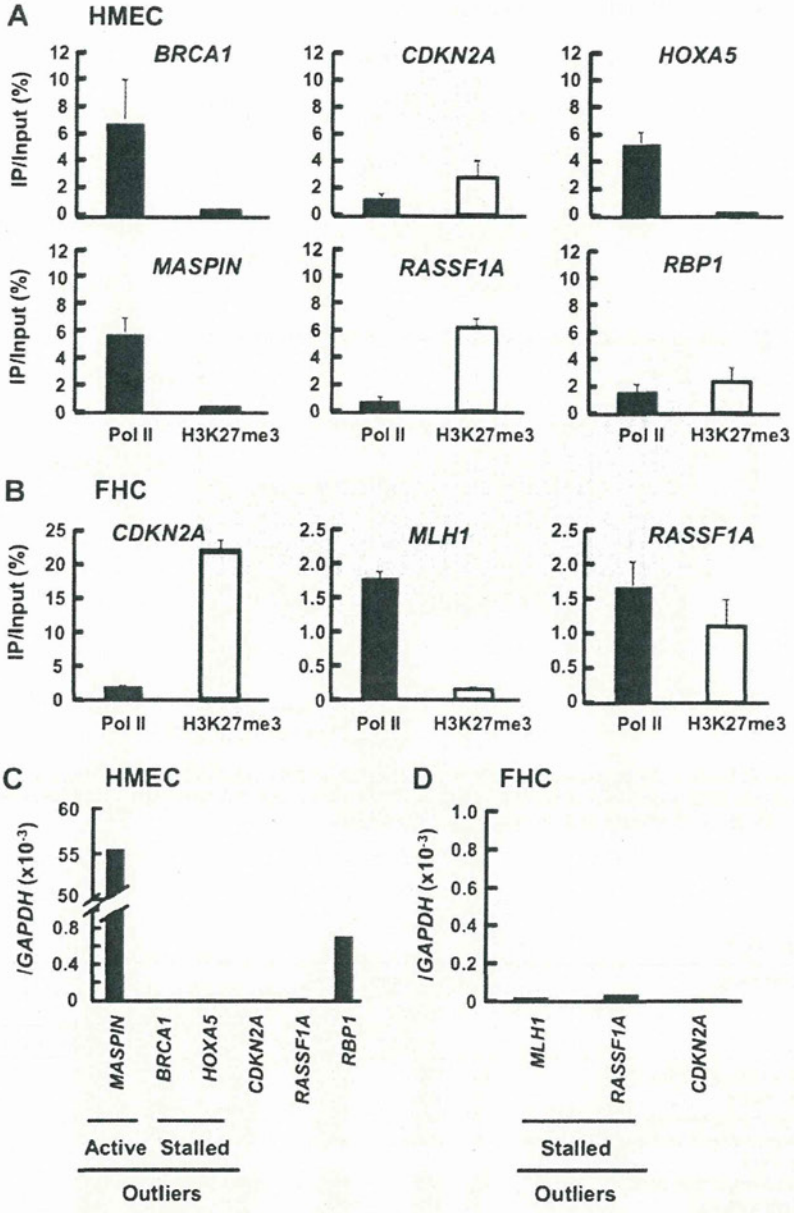

Fig. 1. Confirmation of known TSGs as outliers to the general rule. (A) Pol II binding and H3K27me3 levels in HMECs of the six TSGs in breast cancer analyzed by ChIP-qPCR. Three of the six TSGs showed relatively high Pol II binding levels and low H3K27me3 levels in the HMECs, showing that they are outliers to the general rule. The mean ± SE values of three independent experiments are shown. (B) Pol II binding and H3K27me3 levels in FHC of the three TSGs in colon cancer analyzed by ChIP-qPCR. Two of the three TSGs showed high Pol II binding and low H3K27me3 levels, being outliers. (C) and (D) Expression levels of the known TSGs in normal cells. Expression levels of TSGs in HMECs (C) and FHC (D) were analyzed by qRT-PCR. Among the five outliers in breast and colon cancers, only *MASPIN* was expressed at high levels in HMECs, and the other four outliers were expressed at low levels. Active; active Pol II, Stalled; stalled Pol II.

ceeded to identify novel TSGs in breast cancer by making a genome-wide search for outliers. Based on DNA methylation statuses of two different lots of normal breast epithelial cells (HMECs) and five breast cancer cell lines obtained by MeDIP-CGI microarray analyses, we previously identified 280 promoter CGIs unmethylated in both lots of the HMECs and methylated in two or more of the breast cancer cell lines as methylation-susceptible promoter CGIs in breast cancer [27]. To identify outliers based on the epigenome statuses, Pol II binding and H3K27me3 statuses in the HMECs [14] were utilized. Among the 280 promoter CGIs susceptible to methylation induction during breast carcinogenesis, 37 CGIs had high Pol II binding (Fig. 2A), and 14 of the 37 CGIs had low H3K27me3 levels in the HMECs (Fig. 2B). Since two promoter CGIs were shared by two genes, respectively, 16 genes downstream of these 14 promoter CGIs were considered as outliers in breast cancers (Table 1).

3.3. DNA methylation of outlier promoter CGIs in primary breast cancer samples

DNA methylation levels were quantified by qMSP for 13 of the 14 promoter CGIs (except for a CGI upstream of two core histone genes) in 13 breast cancer cell lines, including the five breast cancer cell lines used for identification of methylation-susceptible promoter CGIs, and HMECs (Fig. 3A and Supplementary Fig. 2). All the 13 outlier promoter CGIs showed low methylation in the HMECs, and five (*DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3*) of them showed

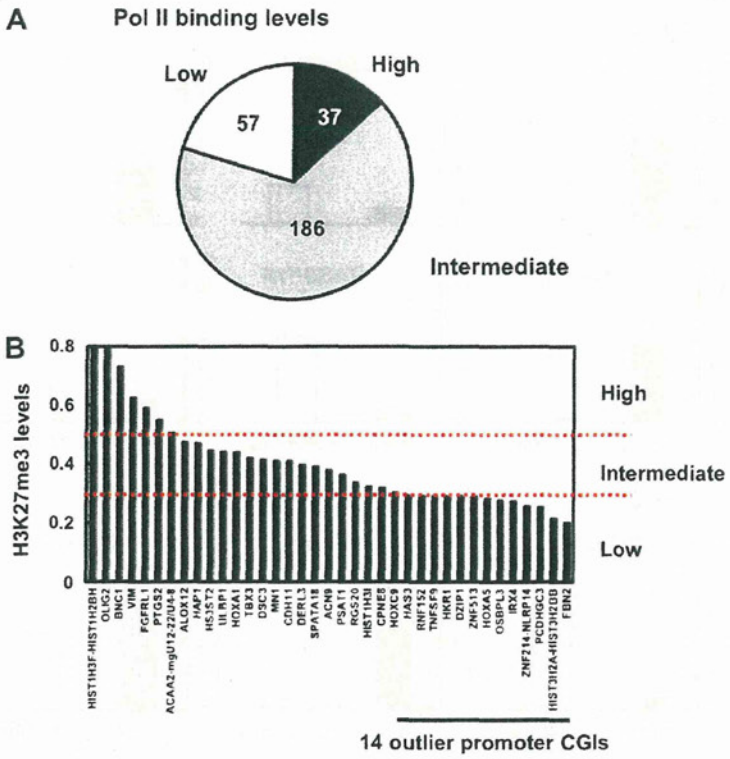

Fig. 2. Genome-wide screening of outliers based on the epigenome. (A) Pol II binding levels of the 280 methylation-susceptible promoter CGIs in the HMECs. Thirty-seven CGIs (13% of susceptible CGIs) showed high Pol II binding levels in the HMECs. (B) H3K27me3 levels of the 37 CGIs with high Pol II binding in the HMECs. Fourteen promoter CGIs with high Pol II binding levels showed low H3K27me3 levels in the HMECs, being outliers.

Table 1
List of outlier promoter CGIs in breast cancer.

	Gene symbol	Gene name	Chr	DNA methylation						
				Normal		Cancer				
				HMEC#1	HMEC#2	BT-474	MCF7	MDA-MB-231	MDA-MB-468	ZR-75-1
1	*DZIP1*	DAZ interacting protein 1	13	UM	UM	UM	HM	UM	HM	MM
2	*FBN2*[a]	Fibrillin 2	5	UM	UM	UM	MM	HM	HM	UM
3	*HAS3*	Hyaluronan synthase 3	16	UM	UM	MM	HM	MM	MM	HM
4	*HIST3H2A-HIST3H2BB*[b]	Histone cluster 3, H2a/histone cluster 3, H2bb	1	UM	UM	UM	UM	HM	HM	UM
5	*HKR1*	GLI-Kruppel zinc family member	19	UM	UM	UM	UM	HM	UM	HM
6	*HOXA5*[a]	Homeobox A5	7	UM	UM	UM	UM	HM	HM	HM
7	*HOXC9*	Homeobox C9	12	UM	UM	UM	UM	HM	HM	UM
8	*IRX4*	Iroquois homeobox 4	5	UM	UM	UM	UM	HM	HM	UM
9	*OSBPL3*	Oxysterol binding protein-like 3	7	UM	UM	HM	HM	UM	UM	UM
10	*PCDHGC3*	Protocadherin gamma subfamily C, 3	5	UM	UM	UM	UM	HM	HM	UM
11	*RNF152*	Ring finger protein 152	18	UM	UM	UM	UM	HM	HM	UM
12	*TNFSF9*	Tumor necrosis factor (ligand) superfamily, member 9	19	UM	UM	HM	UM	UM	HM	UM
13	*ZNF214-NLRP14*[b]	Zinc finger protein 214/NLR family, pyrin domain containing 14	11	UM	UM	UM	HM	UM	HM	UM
14	*ZNF513*	Zinc finger protein 513	2	UM	UM	UM	HM	HM	UM	HM

UM, unmethylated; MM, moderately methylated; HM, highly methylated; chr, chromosome number.
[a] Known tumor-suppressor gene.
[b] Promoter CGIs shared by two genes.

high methylation levels (PMR higher than 100%) in at least one cancer cell line (Fig. 3A).

Then, using 40 primary breast cancer samples, the methylation levels of these five outlier promoter CGIs were analyzed. Methylation of *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* was present in primary breast cancer samples, while that of *OSBPL3* was not observed (Fig. 3B). These results indicated that *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* are outliers methylated not only in cell lines but also in primary breast cancers. Among the four outliers, *HOXA5* and *FBN2* were known as TSGs silenced by aberrant DNA methylation in

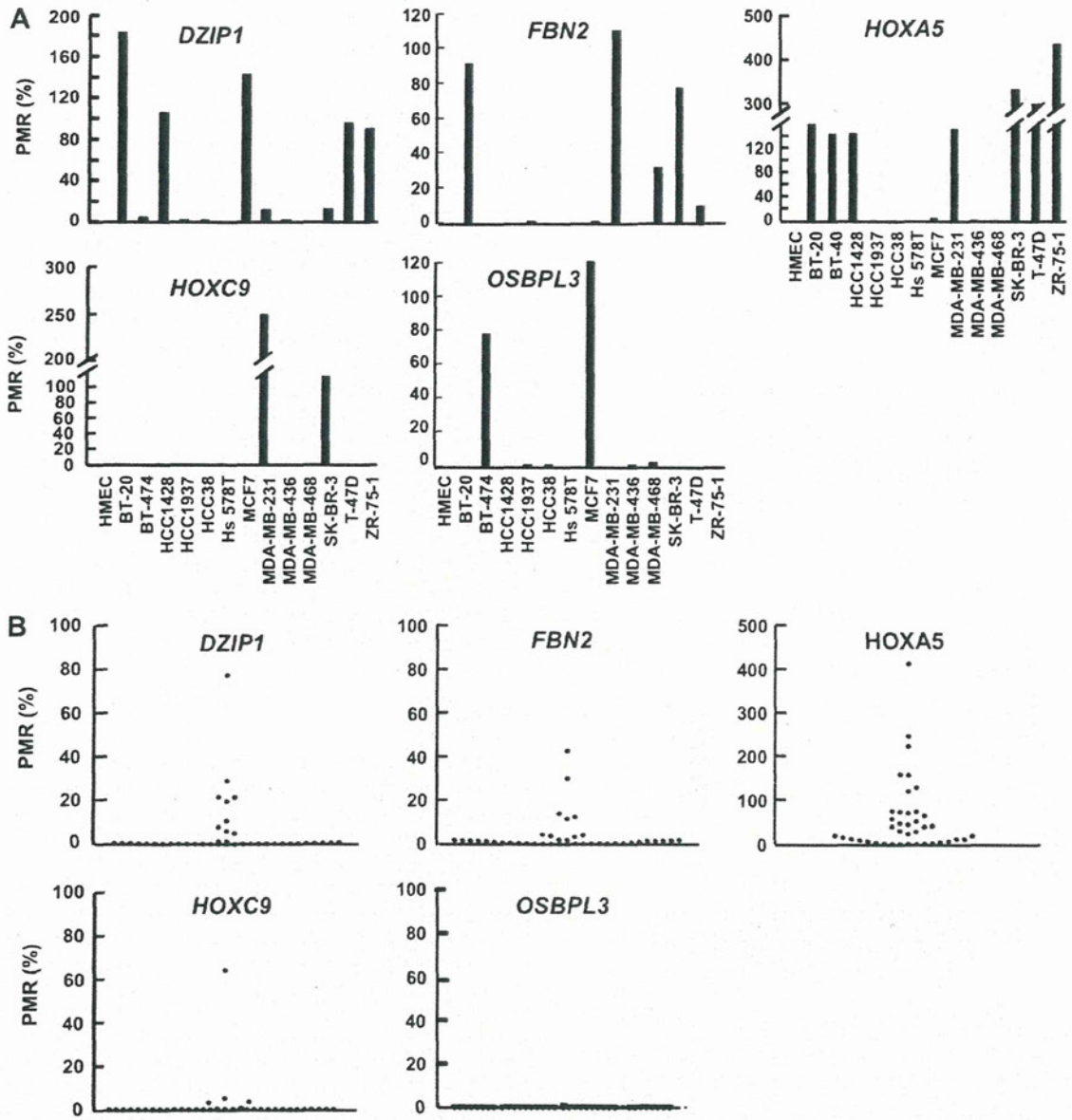

Fig. 3. DNA methylation levels of the outlier promoter CGIs in breast cancer cell lines and primary breast cancer samples. (A) Methylation levels of the outlier promoter CGIs in the HMECs and 13 breast cancer cell lines analyzed by qMSP. Five outliers, *DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3*, showed high methylation levels (PMR higher than 100%) in one or more breast cancer cell line. Since the copy number of the target CGI was normalized to the copy number of the *Alu* repeat sequence, it was possible that PMR reached more than 100% when the locus containing the target CGI had an increased copy number. (B) DNA methylation levels of *DZIP1*, *FBN2*, *HOXA5*, *HOXC9*, and *OSBPL3* in primary breast cancer samples. Methylation of each CGI was analyzed in 40 primary breast cancer samples by qMSP. *DZIP1*, *FBN2*, *HOXA5*, and *HOXC9* showed aberrant methylation in primary breast cancer samples.

breast cancer and renal cell carcinoma, respectively [37,39]. These results indicated that searching for outliers might be an efficient way to identify TSGs.

3.4. Silencing of DZIP1 by aberrant methylation of its promoter CGI

Excluding known TSGs, *HOXA5* and *FBN2*, we focused on *DZIP1* and *HOXC9*. First, an association between aberrant DNA methylation and loss of expression was analyzed in normal cells and cancer cell lines (Fig. 4A). *DZIP1* was expressed both in normal cells and in cancer cell lines without its aberrant methylation, except for BT-474 and SK-BR-3, and was not expressed in cancer cell lines with its aberrant methylation. In contrast, *HOXC9* was not expressed or had only very low expression levels both in normal cells and in all cancer cell lines, regardless of their methylation levels. Since a knockdown experiment could not be performed for *HOXC9*, it was excluded from further analyses.

Methylation-silencing of *DZIP1* was confirmed by observing its re-expression after treatment with 5-aza-dC and TSA using T-47D cells that had methylated promoter CGIs among the 13 breast cancer cell lines and did not express *DZIP1*. First, T-47D cells were treated with 0, 10, and 20 μM of 5-aza-dC to obtain a dose that inhibited cell growth to 50–70% of non-treated cells. The growth of T-47D cells was inhibited to 50% at 20 μM (Supplementary Fig. 3). The presence of demethylated DNA molecules was confirmed at this dose. A combination of 5-aza-dC with TSA induced *DZIP1* re-expression while TSA only did not (Fig. 4B). These results indicated that *DZIP1* was silenced by aberrant DNA methylation.

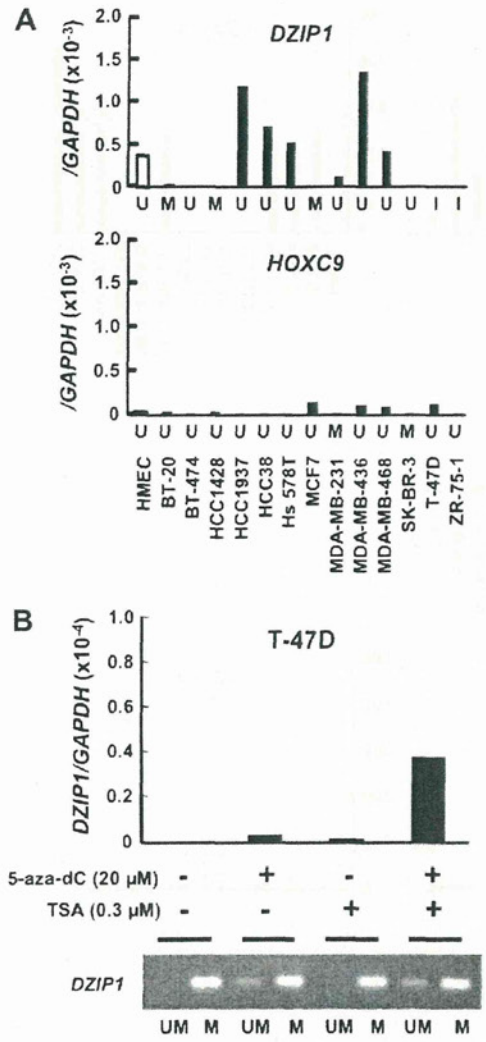

Fig. 4. Evaluation of methylation-silencing of two outliers, *DZIP1* and *HOXC9*. (A) An association between DNA methylation and loss of expression (analyzed by qRT-PCR). *DZIP1* was expressed in cell lines without its aberrant methylation and was silenced in cell lines with its aberrant methylation. In contrast, *HOXC9* was not expressed in any cell lines regardless of its low methylation. M, PMR > 100%; I, PMR 20–100%; U, PMR < 20%. (B) Re-expression of *DZIP1* by 5-aza-dC and TSA treatment. Expression levels were analyzed by qRT-PCR, and methylation statuses were analyzed by MSP. Demethylation of *DZIP1* was induced in T-47D cells by treatment with 20 μM of 5-aza-dC, and re-expression of *DZIP1* was observed after 5-aza-dC and TSA treatment. UM, unmethylated DNA; M, methylated DNA.

3.5. Tumor-suppressive function of DZIP1

To analyze whether *DZIP1* has tumor-suppressive function in breast cancer cells, *DZIP1* was knocked down by two different shRNAs (*DZIP1* shRNA1 and shRNA2) in two breast cancer cell lines that had the highest expression levels of *DZIP1*. Treatment of HCC1937 cells with shRNA1 and shRNA2 reduced the expression level of *DZIP1* mRNA to 20% and 60%, respectively, of cells with control shRNA. Treatment of MDA-MB-436 cells with shRNA1 and shRNA2 reduced the *DZIP1* expression levels to 40% of cells with control shRNA (Fig. 5A). HCC1937 cells with shRNA1 showed approximately 2-fold higher cell growth rate than HCC1937 cells with control shRNA. Likewise, MDA-MB-436 cells with shRNA1 or shRNA2 showed 1.5 to 2-fold higher cell growth rates than MDA-MB-436 cells with control shRNA (Fig. 5B). These results showed that *DZIP1* is a candidate novel TSG in breast cancer.

3.6. Comparison between epigenome-based and expression-based outlier approaches

To analyze the difference between the epigenome-based outlier approach and the expression-based outlier approach, we analyzed whether or not promoter CGIs identified by these approaches overlapped. First, to identify outliers expressed at high levels in normal cells, the expression status in the HMECs [14] was utilized. Among the 280 promoter CGIs susceptible to methylation induction during breast carcinogenesis, 21 promoter CGIs had downstream genes expressed at high levels in the HMECs (Supplementary Table 5).

Their overlap with the promoter CGIs identified by the epigenome-based outlier approach was then analyzed. Since two of the 14 promoter CGIs identified by the epigenome-based outlier approach were shared by two genes (Table 1), the remaining 12 promoter CGIs (and thus genes) were used for the analysis. Among the 12 promoter CGIs identified by the epigenome-based approach, only four promoter CGIs overlapped with those identified by the expression-based outlier approach (Fig. 6). Three and six TSGs were included in the 12 and 21 promoter CGIs, respectively, identified by the epigenome-based approach and the expression-based approach, respectively. While one TSG overlapped between the two approaches, *DZIP1* could be identified only by the epigenome-based approach. These results indicated that the epigenome-based approach could identify a different set of TSGs from the expression-based approach.

4. Discussion

We here showed (i) that a significant fraction of known TSGs silenced by aberrant DNA methylation in breast and colon cancer were outliers, and could be classified into TSGs with active Pol II and those with stalled Pol II, and (ii) that a different set of TSGs could be identified by an epigenome-based outlier approach or by an expression-based outlier approach. The epigenome-based outlier approach is established for the first time in this study. It is also applicable to obtain information on individual methylated genes whether they are TSGs or not. Epigenome information in normal cells is now being compiled by an international collaborative effort [40], and the epigenome-based outlier approach is expected to become more useful when this effort is completed.

Most genes identified by the epigenome-based and/or expression-based outlier approaches were unique. Among the five known TSGs that were outliers, only *MASPIN* had high expression, and the other four genes (*BRCA1*, *HOXA5*, *MLH1*, and *RASSF1A*) had stalled Pol II, indicating that they can be identified only by the epigenome-based approach. Among the 29 outliers identified by the genome-wide analyses, eight and 17 were uniquely identified by the epigenome-based and expression-based approaches, respectively. Genes identified only by the epigenome-based approach had stalled Pol II and thus low expression levels. Genes identified only by the expression-based approach were considered to have high levels of Pol II in gene bodies but not in promoter regions, which we analyzed in this study. It is reported that genes with active Pol II tends to have lower Pol II levels in promoter regions than those with stalled Pol II [41].

Using the epigenome-based outlier approach, *DZIP1* was identified as a candidate novel TSG. *DZIP1* is known to be involved in the regulation of hedgehog (Hh) signaling pathway [42,43]. Hh signaling pathway is activated in several types of human cancers, such as breast, esophageal, gastric, pancreatic, lung, and prostate cancers [44–51]. Although it is unknown how *DZIP1* is involved in the dysregulation of the Hh signaling pathway in mammals, in zebrafish embryo, a loss-of-function mutation of *Dzip1* is known to lead to ectopic expression of the downstream genes of the Hh signaling

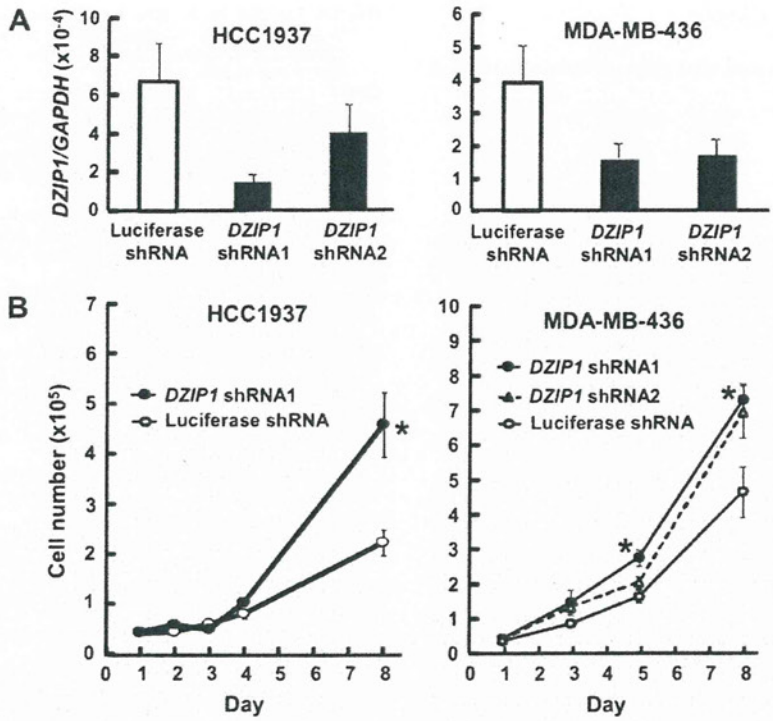

Fig. 5. Suppression of cell growth by *DZIP1*. (A) Knockdown of *DZIP1* in HCC1937 and MDA-MB-436 cells. The expression level of *DZIP1* mRNA in HCC1937 cells was reduced to about 20% and 60% of that of HCC1937 cells with control shRNA by shRNA1 and shRNA2, respectively. The expression level in MDA-MB-436 cells was reduced to about 40% of that in MDA-MB-436 cells with control shRNA by both shRNA1 and shRNA2. The mean ± SE values of three independent experiments of *DZIP1* expression levels are shown. (B) Increased growth of the HCC1937 and MDA-MB-436 cells by *DZIP1* knockdown. Cell numbers were counted at time points designated in the panels, and the mean ± SE values of three independent experiments of cell number are shown. Differences of cell growth were tested by the Student's *t* test. $^*p < 0.05$.

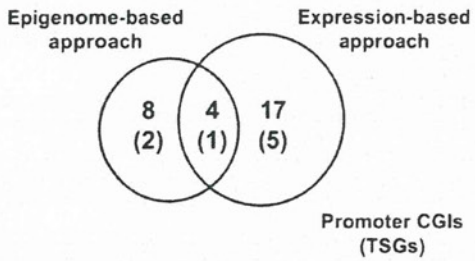

Fig. 6. The overlap of promoter CGIs identified by the epigenome-based outlier approach and those by the expression-based outlier approach. Among the 12 promoter CGIs with unique downstream genes identified by the epigenome-based approach, four promoter CGIs overlapped with those identified by the expression-based approach. *DZIP1* could be identified only by the epigenome-based approach.

pathway [42,52]. Therefore, there is a possibility that methylation-silencing of *DZIP1* induces abnormal expression of the downstream genes of the Hh signaling pathway during human carcinogenesis. Further investigations into *DZIP1* functions are necessary. *DZIP1* was also repressed in breast cancer cell lines such as BT-474 and SK-BR-3 that had unmethylated promoter CGIs of *DZIP1*. As possible causes of this repression, involvement of repressive histone modifications and defects in signaling pathways that regulate *DZIP1* expression were considered.

Among the seven known TSGs initially analyzed, *BRCA1*, *HOXA5*, and *MASPIN* were outliers in breast cancer, and *MLH1* and *RASSF1A* were in colon cancer. In contrast, *CDKN2A*, *RASSF1A*, *RBP1* in breast cancer and *CDKN2A* in colon cancer were not outliers. Especially, *RASSF1A* was an outlier in colon cancer, but not in breast cancer. This difference might explain the different incidence of aberrant DNA methylation of *RASSF1A* between breast cancers that show 50–60% incidence [44,53] and colon cancers that show 20–45% incidence [54,55].

Among the TSGs confirmed as outliers in the initial analysis, *HOXA5* was identified by the following genome-wide screening, but *BRCA1* and *MASPIN* were not. This was because the microarray used in this study did not have probes in the NFRs of *BRCA1* and *MASPIN*, and these genes cannot be identified as frequently methylated genes using the microarray used here.

Cancer cell lines were used to obtain DNA methylation-susceptible genes in this study. However, some TSGs, such as *BRCA1*, are reported to be frequently methylated in primary breast cancer tissues [56–58], but infrequently in cancer cell lines [59]. By use of primary cancer samples, identification of such types of TSGs will be facilitated.

In summary, we showed that a significant fraction of TSGs are outliers to the general rule of genes methylated in cancer cells, and that a different set of TSGs could be identified by the epigenome-based outlier approach compared to the expression-based outlier approach.

Acknowledgment

M.K. is a recipient of Research Resident Fellowships from the Foundation for Promotion of Cancer Research. This work was supported by Grants-in-Aid for the Third-Term Comprehensive Cancer Control Strategy from the Ministry of Health, Labour and Welfare, Japan; for Young Scientists (B) from Japan Society for the Promotion of Science (JSPS); and a grant from the Foundation for Promotion of Cancer Research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.03.016.

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