Integrative array-based approach identifies MZB1 as a frequently methylated putative
tumor-suppressor in hepatocellular carcinoma

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Although hepatocellular carcinoma is one of the most common malignancies worldwide, the molecular mechanisms underlying hepatocarcinogenesis remain unclear. Epigenetic abnormalities, such as DNA methylation, may promote tumorigenesis as well as genomic alterations in hepatocarcinogenesis. To discover novel methylation-target sequences with high specificity and sensitivity in a genome-wide manner, we performed expression-array analyses as well as methylated-DNA immuno precipitation in combination with an oligonucleotide array, which allows for rapid and efficient genome-wide assessment of DNA methylation, resulting in the identification of marginal zone B and B1 cell-specific protein (MZB1) as a tumor-suppressor gene silenced by DNA hypermethylation in hepatoma. Among 162 patients with primary hepatocellular carcinoma, silencing of MZB1 protein was significantly and independently associated with a worse outcome. Moreover, restoration of MZB1 expression in hepatoma cells reduced cell proliferation in vitro and in vivo through G1-arrest. Our findings provide a novel insight into the prevention, diagnosis, and treatment of hepatocellular carcinoma.
Abstract

Purpose: The aim of the present study was the identification of novel tumor-suppressor genes silenced by DNA hypermethylation in hepatocellular carcinoma.

Experimental Design: We performed integrative array-based approach for genome-wide screening of methylation targets using a methylated DNA immunoprecipitation-CpG island microarray and expression array in three universal hepatoma cell lines and normal liver tissue. Through detailed expression and functional analyses using hepatoma cell lines and primary hepatocellular carcinoma samples, we isolated novel tumor-suppressor genes for hepatocellular carcinoma.

Results: A total of 642 genes were identified as methylated in three hepatoma cell lines but unmethylated in normal liver tissue, whereas 204 genes on autosomes were identified as genes unexpressed but restored after treatment with 5-aza-2'-deoxycytidine in these cell lines and expressed in normal tissue. Through the integration of results of the two array analyses and further validation analyses of expression and methylation status in 17 cell lines and 30 primary tumors of hepatoma, we identified MZB1, marginal zone B and B1 cell-specific protein, encoding an endoplasmic reticulum protein, as a putative tumor-suppressor gene frequently methylated within its CpG island in hepatoma. Among 162 patients with primary hepatocellular carcinoma, silencing of MZB1 protein was significantly and independently associated with a worse outcome. Restoration of MZB1 expression in hepatoma cells reduced cell proliferation in vitro and in vivo through G1-arrest.

Conclusions: These results suggest that methylation-mediated silencing of MZB1 expression leads to loss of its tumor-suppressive activity, which may be a factor in the hepatocarcinogenesis, and is a useful prognosticator in hepatocellular carcinoma.
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Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies worldwide, is associated with hepatitis virus infections, dietary aflatoxin, chronic alcohol/tobacco consumption, and cirrhosis. Genomic alterations, such as the gain or loss of chromosomal regions and specific gene mutations, have been frequently noted in hepatocarcinogenesis (1). Furthermore, epigenetic abnormalities, such as DNA methylation and chromosome remodeling, may also promote tumorigenesis (2,3).

DNA hypermethylation of promoter CpG islands leads to the inactivation of tumor-suppressor genes (TSGs) and critical cancer-related genes in human cancers including HCC (4,5). DNA methylation changes have been reported to be specific to the cancerous tissue making it possible to distinguish HCC and surrounding non-tumorous tissues (6). Indeed, abnormal DNA methylation of several TSGs, such as RASSF1A, CDKN2A, CRABP1, GSTP1, CHRNA3, DOK1, SFRP1, GAAD45a and CDKN2B, is reported to be associated with HCC, and hypermethylation of specific genes, such as CHFR and SYK is detected in advanced stages of HCC (6). However, the number of reported methylation-target genes is far fewer for HCC than for colon cancer or gastric cancer (7). Therefore, further identification of remaining targets for methylation may clarify the specific molecular events involved in HCC progression, enabling the prevention, diagnosis, and treatment of HCC to be approached as potential clinical applications of DNA methylation signature at a molecular level.

To discover novel methylation-target sequences with high specificity and sensitivity in a genome-wide manner, large-scale screening methods, which have the potential to find novel methylation targets in a whole range of cancers, have been developed. A combination of three types of pretreatments, such as enzyme digestion, affinity enrichment, or sodium bisulfite, was followed by different analytical steps, such as gel-, array-, or next-generation sequencing-based analysis (8). Among them, methylated-DNA immunoecipitation (MeDIP; Ref. 9) in combination with an oligonucleotide array or next-generation sequencing allows for rapid and efficient genome-wide assessment of DNA methylation, although these methodologies generally result in a list of several hundred candidate genes. Although an analysis of possible promoters or dense CpG islands is used to narrow down the number of candidate genes, the list is still too long.
Pharmacological unmasking expression-microarray approaches are also used widely to identify methylation targets (10), although they are also prone to give false-positive genes that are indirect methylation targets themselves and not considered to be a reliable gauge of DNA methylation at a given locus.

To identify genes that are downregulated due to DNA hypermethylation and to concentrate those genes most frequently involved in HCC, we applied the following integrative array-based approach to three hepatoma cell lines: (a) MeDIP in combination with CpG island-array (MeDIP-chip) analysis to identify genes methylated in a cancer-specific manner, (b) expression-microarray analysis to identify genes down-regulated in a cancer-specific manner and re-activated upon treatment with 5-aza-2'deoxycytidine (5-aza-dCyd), and (c) a combination of data from both approaches. Through further examination of a subset of obtained candidates, we identified marginal zone B and B1 cell-specific protein (MZB1), also known as proapoptotic caspase adaptor protein (PACAP), pERP1, or MGC29506 (11), whose inactivation is related with a worse prognosis in primary tumors as a possible TSG for HCC.
Materials and Methods

Cell lines and primary tumor samples

A total of 17 hepatoma cell lines including 15 HCC lines (cHc4, Hep3B, Hep-Kano, Hep-TABATA, HLE, HLF, HuH-7, JHH-1, JHH-4, JHH-5, JHH-7, Li-7, PLC/PRF/5 and SK-HEP-1) and 2 hepatoblastoma lines (HepG2 and HUH-6) were used (12). All 162 primary HCC samples were obtained during surgery from HCC patients treated at Tokyo Medical and Dental University (Tokyo, Japan) between 2000 and 2005. Relevant clinical and survival data were available for all patients (Table 1). The median follow-up period for the surviving patients was 19 months (ranging from 1 to 103 months). Samples from 17 of these HCC patients were immediately frozen in liquid nitrogen and stored at -80ºC until required for DNA and RNA analyses. Normal liver tissues were obtained from surgical samples in two patients (cases C20 and C40) with colon cancer metastasis. Written consent was obtained after approval by the local ethics committee.

MeDIP-chip analysis

The DNA methylation profiles were analyzed by MeDIP using anti-5-methylcytidine antibody (Eurogentec, Seraing, Belgium) followed by the Human 244K CpG island microarray (Agilent, Santa Clara, CA), which contains 237,220 probes, covering 27,800 CpG islands (97.5% of UCSC annotated CpG islands), according to the manufacturer's instructions. Image analysis with data extraction was performed using Feature Extraction Software, version 9.0 (Agilent Technologies). The analytical procedure used for the results of the MeDIP-chip assay was described in the Supplementary experimental procedure8.

Gene expression array

Gene expression was profiled using a 4x44K Human Whole Genome Ver. 2.0 gene expression array (Agilent Technologies) according to the manufacturer's instructions. Total RNAs extracted from hepatoma cell lines and from these cells treated with 10 μM 5-aza-dCyd for 5 days were used for conventional and pharmacological unmasking analyses, respectively. Two normal livers (C20
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and C40) were used as controls. All samples were analyzed twice. Image analysis with data extraction and the data analysis were performed using Feature Extraction Software, version 9.0 and GeneSpring GX10 software (Agilent Technologies), respectively.

Real-time reverse transcription-PCR (RT-PCR)

To analyze the restored expression of genes, the cell lines were cultured with 1 to 10 μM 5-aza-dCyd for 5 days. Levels of mRNA expression were measured with ABI PRISM 7500 sequence detection System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays (Hs01048042_m1 for ANGPT2, Hs00174937_m1 for CCK, Hs00405322_m1 for DERL3, Hs00417143_m1 for RADIL, Hs00191390_m1 for KCNK6, Hs00219458_m1 for L1TD1, Hs00414907_m1 for MZB1, Hs00386153_m1 for FAR1, Hs00382235_m1 for OCIAD2, Hs00257935_m1 for PBX4, and Hs00610060_m1 for SFRP1; Applied Biosystems) according to the manufacturer’s instructions. Gene expression values are given as ratios between the genes of interest and an internal reference gene (Hs99999903_m1 for ACTB; Applied Biosystems) that provides an internal normalization factor, and subsequently normalized with the value in the controls (relative expression level). The assay was performed twice for each sample.

Methylation analysis

Sodium bisulfite-treated genomic DNA was subjected to PCR using primer sets to amplify regions of interest (Supplementary Table S1). For the combined bisulfite restriction analysis (COBRA), PCR products were digested with BstU1, Taq1, or Hha1 and electrophoresed (13). The intensity of methylated alleles as a percentage on the ethidium bromide-stained gels was calculated, and a methylation density cutoff point of 20% was considered significant as described elsewhere (14). For bisulfite genomic sequencing (BGS), PCR products were subcloned and sequenced.

Immunohistochemistry

Indirect immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue sections...
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using an automated immunostainer (BenchMark XT; Ventana Medical Systems, Tucson, AZ) with heat-induced epitope retrieval, anti-MZB1 (1:50; 11454-1-AP, Protein Tech, Chicago, IL), anti-PCNA (1:1000; #2586, Cell Signaling Technology, Beverly, MA), or anti-Ki-67 antibodies (1:100; M7240, Dako, Carpinteria, CA). The slides were counterstained with Mayer's hematoxylin, and analyzed under a light microscope by two pathologists blinded to clinical characteristics and outcomes. Twenty representative fields per slide were examined, and the percentage of the total cell population that expressed MZB1 was evaluated for each case at x200 magnification. Expression of MZB1 protein was graded as either positive (≥10% of tumor cells showing immunopositivity) or negative (≤10% of tumor cells showing immunopositivity or no staining). Plasma cells and bile duct epithelial cells were used as positive and negative controls, respectively.

Western Blotting

Western blotting was performed as described elsewhere (12). Anti-FLAG-tag and anti-β-actin antibodies were purchased from Sigma (St. Louis, MO), and the anti-cleaved caspase-3 antibody (#9661) were purchased from Cell Signaling Technology.

Fluorescent immunocytochemistry (FIC)

The plasmid expressing C-terminally FLAG-tagged MZB1 (pCMV-3Tag3A-MZB1) was obtained by cloning the full coding sequence of MZB1 in-frame along with the 3xFLAG-epitope into the pCMV-3Tag3A vector (Stratagene, La Jolla, CA). Cells were fixed in 10% trichloroacetic acid, permeabilized with 0.2% Triton X-100, and treated with blocking solution (1% bovine serum albumin in PBS). After incubation with the primary antibodies (anti-MZB1, 1:100 and/or anti-Calnexin, 1:100, Santa Cruz, CA) for 1 h, the bound antibody was visualized using a Cy3-conjugated or fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:1000). After being mounted with DAPI (4′,6′-diamidino-2-phenylindole) to stain nuclei, the cells were observed under a fluorescence microscope (BZ-8100, Keyence, Osaka, Japan).
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**In vitro and in vivo growth assay**

Colony formation assays using cells transiently introduced with pCMV-3Tag3A-MZB1 or the empty vector (pCMV-3Tag3A-mock) were performed as described elsewhere (15). The expression of MZB1 protein was confirmed 48 h after transfection by western blotting and FIC.

Stable MZB1 transfectants and control counterparts were obtained by introducing pCMV-3Tag3A-MZB1 or pCMV-3Tag3A-mock into cells with G418 selection, and 2.5x10^4 cells were seeded in 24-well plates. The numbers of viable cells were assessed 24-72 h after seeding by the water-soluble tetrazolium salt assay. The cell cycle was evaluated 48 h after seeding by a fluorescence-activated cell sorting (FACS) as described elsewhere (15).

The in vivo tumor-suppressive ability of MZB1 was investigated by conducting tumor xenograft experiments. Six-week-old female severe combined immunodeficient (SCID) mice were injected subcutaneously in the lower back with MZB1-expressing or control mock-transfected cells (4x10^7). All procedures involving animals were approved by and conformed to the guidelines of our Institutional Animal Care and Use Committee. Tumor formation in SCID mice was monitored daily and the recipient mice were sacrificed for tumor weight evaluation and protein expression analyses 5-w postinjection.

**Statistical analysis**

The χ² or Fisher’s exact test was used to test for differences between groups. Kaplan-Meier method and log-rank test were used for the survival analyses. Univariate and multivariate survival analyses were performed using the likelihood ratio test of the stratified Cox proportional-hazards model. Differences between subgroups were tested with the Student’s t-test. For multiple group comparisons, ANOVA followed by Scheffé’s post-hoc test was used. Differences were assessed with a two-sided test, and considered significant at the P<0.05 level.
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Results

Screening in the MeDIP-chip analysis

For the screening of aberrantly methylated genes by MeDIP-chip analysis, we used a CpG island microarray, in which 11229 genes harbor CpG islands upstream or within, with the algorithm shown in the Supplementary experimental procedure and Supplementary Figure S1. Among 11229 genes, CpG islands of 2,476 genes were unmethylated in normal liver tissue (case C20) but methylated in at least one of three hepatoma cell lines (Hep3B, HepG2, and HuH-7). Since CpG islands of 642 of those 2,476 genes were methylated in all three cell lines (Fig. 1A), we selected them as candidates consistently hypermethylated in hepatomas.

Screening in the expression-array analysis

In expression array analysis performed in a duplicate manner, we used only reproducible probes (the coefficient of variation, CV<50%) in each set of experiments to evaluate obtained values. We focused on genes satisfying two criteria: (a) genes whose expression was observed in normal liver tissue but repressed in hepatoma cells, and (b) genes whose expression was restored after treatment with 5-aza-dCyd in hepatoma cells. Among genes expressed in normal liver tissue (C20 and C40), the expression of 1,730, 1,158, and 1,521 genes was silenced but restored by 5-aza-dCyd in Hep3B, HepG2, and HuH-7 cells, respectively. Among them, 202 genes on autosomes commonly satisfied criteria in all three lines (Fig. 1A), indicating 202 genes to be candidates consistently silenced through methylation in hepatocarcinogenesis.

Integration and validation of results in two microarray-based analyses

Based on the MeDIP-chip and expression-array analyses, 11 genes were selected as overlapping genes in two different genome-wide array-based screening methods, suggesting them to be pharmacologically unmasked, tumor-specific methylation targets in all three hepatoma cell lines (Fig. 1A and Supplementary Table S2). Since these genes seem to be silenced in a tumor-specific manner, they are also candidates for TSG in hepatocarcinogenesis.
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We then determined the status of methylation and expression for all 11 candidates through COBRA and real-time RT-PCR, respectively, in a panel of 17 hepatoma cell lines and two normal liver tissues (Fig. 1B). Among them, ANGPT2 was highly methylated in both the hepatoma cells and normal liver tissue. Among the other 10 genes, 8 genes (L1TD1, Mzb1, PBX4, FAR1, RADIL, DERL3, SFRP1, and CCK) harbored at least one hypermethylated region in >50% of hepatoma cell lines compared with normal liver tissue, whereas OCIAD2 and KCNK6 were infrequently methylated in the hepatoma lines. Among those 8 genes, only Mzb1 and FAR1 were downregulated (<0.1 relative to normal liver) in all cell lines with their hypermethylation, whereas the other 6 genes were expressed even in cell lines with their hypermethylation, suggesting Mzb1 and FAR1 to be possible methylation targets for gene silencing in hepatoma cells, although the expression of all 11 genes was more or less restored by 1-10 μM of 5-aza-dCyd treatment in each of the three cell lines (Supplementary Fig. S2).

MZB1 is frequently silenced through CpG island methylation in primary tumors

We next determined the methylation and expression status of Mzb1 and FAR1 in 17 paired tumorous and non-tumorous tissues from primary HCCs (cases L81-L104; Fig. 2A and Supplementary Fig. S3). Tumor-specific Mzb1 (region 8) and FAR1 (region 3) hypermethylation was observed in 9 (52.9%) and 8 (47.1%) cases, respectively. In those cases with hypermethylation, tumor-specific downregulation of Mzb1 and FAR1 expression was observed in 9/9 (100%) and 5/8 (62.5%) cases, respectively, suggesting Mzb1 to be the most probable candidate for a gene silenced through tumor-specific methylation. In additional 13 cases (Fig. 2A, cases L07-L79), tumor-specific Mzb1 hypermethylation was observed in 6 (46.2%) cases and all of those cases also showed tumor-specific downregulation of this gene. Among 12 cases available for immunohistochemical staining of Mzb1 protein, Mzb1 was expressed in non-tumorous tissues and the methylation status and protein expression status of Mzb1 matched in 8 tumorous tissues (Fig. 2B).

To clarify the methylation status of the CpG island of Mzb1, we performed BGS in cell lines...
and tumorous and non-tumorous tissues of HCC as well as normal liver tissue. CpG sites within the CpG island tended to be differentially methylated among the cell lines and primary samples: MZB1-non-expressing cell lines except HuH-7 and primary tumors showed a highly methylated pattern within the CpG island, whereas MZB1-expressing normal liver tissue and non-tumorous tissue of HCC case showed partially methylated (Fig. 2C). Although total %methylation of CpG sites within the CpG island of HuH-7 cells is higher than but close to those in MZB1-expressing samples, dense methylation without unmethylated allele was observed in specific regions within CpG island in HuH-7 cells. In addition, we confirmed that treatment with 5-aza-dCyd partially restored methylation within the CpG island of MZB1 in HepG2, Hep3B, and HuH-7 cells, whose expression of MZB1 was restored after the same treatment (Fig. 1B), suggesting that methylation within the CpG island upstream to MZB1 occurs in cell lines and primary tumors of hepatoma and at least partly contributes to the silencing of its expression of the mRNA and protein level. Notably, 5-aza-dCyd-treated hepatoma cells and C20 showed similar methylation level of the MZB1 CpG island (Fig. 2C), whereas 5-aza-dCyd-treated hepatoma cells showed much higher MZB1 expression level compared with C20 (Fig. 1B), suggesting that 5-aza-dCyd treatment indirectly activates transcription of MZB1 through demethylation of transcription factors/cofactors for MZB1, which might downregulated in normal hepatocytes or other mechanisms.

Immunohistochemical staining of MZB1 in primary HCCs

To determine clinicopathologic significance of the MZB1 downregulation in primary HCCs, we performed an immunohistochemical analysis of the MZB1 in 162 primary cases (Fig. 3 and Table 1). In the tumorous regions, 78 (48.1%) showed immunoreactivity to MZB1 (positive in Table 1), whereas 84 (51.9%) did not (negative in Table 1). In the non-tumorous regions, on the other hand, 135 (83.3%) showed immunoreactivity to MZB1, whereas 27 (16.7%) did not. Negative MZB1 immunoreactivity was more frequent in cases with portal invasion (P=0.0222) and in higher tumor stages (P=0.0207). However, the MZB1 protein expression in each tumor was not associated with other characteristics.
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In Kaplan-Meier survival curves, univariate analyses of overall and non-recurrent survival with log-rank tests demonstrated a significant association between negative MZB1 immunoreactivity and a poor survival rate of patients ($P=0.0031$ and 0.0044, respectively; Fig. 3B). In the Cox proportional hazard regression model (Table 2), univariate analyses demonstrated that negative MZB1 immunoreactivity, AFP, tumor size, tumor number, portal vein invasion, background liver parenchyma, and tumor stage were significantly associated with overall survival. Multivariate analysis using a stepwise Cox regression procedure revealed that MZB1 immunoreactivity, tumor size, and tumor stage were independently selected as predictive factors for overall survival in both forward and backward procedures ($P=0.0234$, 0.0319, and 0.0301, respectively).

MZB1 re-expression suppresses proliferation and tumor formation of cancer cells in vitro and in vivo

To investigate the biological significance of MZB1 in hepatocarcinogenesis, MZB1 expression was transiently or stably restored in hepatoma cells lacking MZB1 expression. We then measured the proliferation and tumor formation of those cells in comparison to the control counterparts transduced with an empty vector in vitro and in vivo.

In colony-formation assays using transiently transfected cells, the occupancy of the stained area of colonies produced by MZB1-transfected HLE cells (Fig. 4A), which show MZB1 hypermethylation pattern (Supplementary Fig. S4), and other hepatoma cells (data not shown) decreased compared with those of control counterparts. In an in vitro proliferation assay using stably transfected cells, cells expressing MZB1 protein, which was predominantly colocalized with an endoplasmic reticulum (ER) marker in the cytoplasm, grew slightly but significantly slower than the control cells (Fig. 4B). In a FACS analysis to examine the mode of action of MZB1 in the cell cycle, an accumulation of cells in G0-G1 phase and a decrease in S and G2-M phase cells but no increase in sub-G1 phase cells was observed among MZB1-transfected cells compared with mock-transfected counterparts (Fig. 4C), suggesting that MZB1 contributes to the arrest of hepatoma cells at the G1-S checkpoint without inducing apoptosis. Indeed, a similar expression
pattern of cleaved caspase 3, one of markers of apoptosis, was observed between stable MZB1 transfectants and control counterparts even after treatment with CDDP for induction of apoptosis (Fig. 4C). Subcutaneous tumor growth experiments using stable transfectants demonstrated that restored expression of MZB1 in hepatoma cells correlated with reduced tumor volume and weight in vivo probably due to a decrease in cell proliferation shown by lower PCNA and Ki-67 positivities in MZB1-transfected cells (Fig. 4D). In resected tumors, no induction of apoptosis detected by cleaved caspase 3 expression in MZB1-transfected cells was observed compared with mock-transfected counterparts in vivo (Fig. 4D).
Discussion

Epigenetic silencing of TSGs plays an important role in the carcinogenesis (16), including hepatomagenesis (5-7). Although many studies have reported aberrant hypermethylation of genes in HCC, e.g. CDH1, RASSF1A, GSTP, SOCS1, SFRP1, and PTEN identified as TSGs silenced by hypermethylation, most of these studies were limited to the analysis of a single or a few genes (5-7,17). Since the number of methylation-target TSGs identified to date is far fewer for HCC than for other cancers possibly due to fewer attempts to perform genome-wide analysis (18), there remain many genes hypermethylated in HCC. With advancements in microarray technology, the number of genes found to be hypermethylated in HCC in a cancer-specific manner is expected to increase (8,19). Although the functional consequence of promoter hypermethylation is transcriptional silencing of the associated gene, this assumption often goes untested, as few have concurrently investigated both methylation and expression (6,7). Based on these hypotheses and background, we conducted genome-wide screening of methylation-target TSGs using a combination of two microarray-based approaches: MeDIP-chip analysis in hepatoma cell lines and expression-array analysis for genes pharmacologically unmasked in the same lines. As a result of the present approach, several genes were newly identified as candidate methylation targets, and among them MZB1 was demonstrated to be the most possible TSG, which is silenced through methylation and contributes to the hepatocarcinogenesis.

Among 11 genes we identified through genome-global screening of methylation-mediated silenced genes in hepatoma cells, 10 were newly identified candidates and only SFRP1 was known as possible methylation-target TSG in HCC (20,21). The remarkable reduction in the number of candidate genes and lack of various known HCC-related methylation targets within candidates may be due to a small number of commonly methylated and/or silenced genes among the three cell lines used in this study. Indeed, several frequently methylated genes, such as SLIT2, PTGS2 (COX2), and HHIP, for which methylation data are available in all three hepatoma cell lines, showed different methylation patterns among the cell lines (22-24). These variations may come from the different backgrounds of the three cell lines, such as hepatitis B virus infection in Hep3B and no hepatitis virus
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infection in the other two lines, because hypermethylated genes in HCC tumors are known to exhibit remarkably distinct patterns depending on associated risk factors (25,26). Therefore, it is suggested that the candidate genes identified and validated in the present study may contribute to functional pathways shared among different subtypes of HCC regardless of associated risk factors, and this might be the reason why MZB1 protein expression status was not statistically associated with the status of hepatitis virus infection and background liver parenchyma in our analysis.

One of striking findings of our immunohistochemical analysis of MZB1 using a panel of primary tumor samples of HCC was that immunoreactivity to the MZB1 protein in each sample was significantly associated with a worse clinical outcome even after stratification with other clinicopathological characteristics. This result indicates that MZB1 might be useful as an independent prognosticator in patients with HCC, although MZB1 immunoreactivity in each case was significantly associated with portal invasion and in tumor stages. Since MZB1 seems to be downregulated in a cancer-specific manner in HCC and its expression is observed in most normal human tissues (11), it will be interesting whether MZB1 works as a TSG in specific tissues including liver or in various tissues.

MZB1 was first identified as a caspase 2-binding molecule through a yeast two-hybrid system using a human B lymphocyte cDNA library, which was performed to determine the mechanism of activation of casp-2 in apoptosis of B cells triggered by ligation of the antigen receptor (11). Although MZB1 was shown to bind caspase-2 and 9 in vitro and in vivo and be triggered upon the transient transfection of human kidney cells and Rat-1 fibroblasts and stable transfection of human B cell lines (11), the precise mechanisms by which it exerts proapoptotic activity remain unclear due to the absence of structural hallmarks besides a CXXC thioredoxin motif and no homology with other molecules in apoptotic pathways. Recently, it was shown that MZB1 occurs in the luminal ER and affects multiple cellular processes, such as (a) the oxidative folding and assembly and secretion of immunoglobulin in plasma cells (27,28) and (b) the regulation of Ca$^{2+}$ homeostasis and ER Ca$^{2+}$ stores, integrin-mediated adhesion, and antibody secretion in marginal zone B cells of the spleen and innate-like B cells (B1 cells, Ref. 29). Since those processes are
associated with the functional differentiation of B-cells, it is possible that the MZB1-induced assembly of several target proteins including integrin may contribute to the anti-proliferative effect of MZB1 on hepatoma cells without induction of apoptosis observed in the present study. The expression of this gene is observed not only in the B-cell lineage including plasma cells, marginal zone B cells, or B1 cells (27-29), but also in most normal human tissues except the placenta constitutively even in the absence of an apoptotic stimulus (11), suggesting MZB1 to affect various biological processes in different tissues possibly through interaction with various proteins and/or the targeting of various molecules. Indeed, MZB1 protein was reported to be downregulated in intestinal-type gastric cancer, although the clinicopathological and biological significance was not analyzed (30), suggesting MZB1 to act as a TSG at least in some tissues including stomach and liver tissue. Further examination will be required to clarify the mechanisms of the anti-proliferative effect of MZB1.

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References


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Figure legends

Figure 1

Identification of 11 genes as possible methylation targets.

A, Left upper, overview of the screening approach using MeDIP-chip analyses in three hepatoma cell lines (Hep3B, HepG2, and HuH-7) and control normal liver tissue (case C20). Right upper, overview of the screening approach using duplicate expression-array analyses in the same three hepatoma cell lines without and with 5-aza-dCyd treatment and two normal liver tissues (cases C20 and C40). Lower, by combining results obtained from two different genome-wide array-based screening methods, 11 genes overlapped between two approaches as possible genes, which are consistently silenced by tumor-specific CpG island methylation.

B, Upper, summary of DNA methylation status of CpG islands around 11 selected candidates in 17 hepatoma cell lines and normal liver tissue determined by COBRA. Each box indicates restricted status by enzymes (% methylated allele). A methylation density cutoff point of 20% was considered significant (13). Arrowheads indicate regions in which more than 50% (>9/17) of cell lines showed tumor-specific hypermethylation compared with normal liver tissue. Lower, profiles of expression of 11 candidates determined by real-time RT-PCR in 17 hepatoma cell lines. Three (Hep3B, HepG2, and HuH-7) of 17 lines were treated with 5-aza-dCyd. Ratio relative to normal liver tissue (C20) is shown by a 7-gradient pattern. ’M’s indicates that genes showed hypermethylation (see Figure 1C, upper panel) in each cell line: non-underlined and underlined ’M’ s indicate silenced (<0.01 compared with C20) and retained gene expression, respectively.

Figure 2

Correlation of methylation and expression status of MZB1 in primary HCC.

A, representative results of the methylation status of region 8 within the MZB1 CpG island determined by COBRA (upper) and the relative level of MZB1 mRNA expression determined by real-time RT-PCR (lower) in primary HCC tumors (T) and corresponding non-cancerous liver tissues (N). Arrowheads, fragments specifically restricted at sites recognized as methylated CpGs; arrow,
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undigested fragments indicating unmethylated CpGs. A methylation density of cut-off point of 20% was considered significant (14). Closed and open circles indicate samples with tumor-specific methylation (positive in tumor but negative in corresponding non-tumorous tissue) and those with the reduced mRNA expression (>50% decreased expression) in tumor tissue compared with paired non-tumorous tissue, respectively.

**B,** representative results of immunohistochemical analysis of MZB1 protein expression in primary HCC indicate that methylation status of MZB1 is inversely correlated with its mRNA and protein expression. Bars, 20 μm. Magnifications are x200.

**C,** Upper, a schematic map of the CpG-rich region around the CpG island and exon 1 of MZB1. Vertical ticks, CpG sites on the expanded axis. Gray box, CpG island. Horizontal closed arrows, the regions examined in the COBRA and BGS. Downward arrows, restriction sites (BstU1) for the COBRA. Lower, representative results of BGS of the MZB1 CpG island in three hepatoma cell lines without and with 5-aza-dCyd, two normal livers (C20 and C40), and a paired representative case (cases L87) examined in the COBRA. Open and filled squares represent unmethylated and methylated CpG sites, respectively, and each row represents a single clone. Restriction sites are indicated by downward arrows.

**Figure 3**

**A,** representative results of immunohistochemical staining of MZB1 protein in normal liver tissue, non-tumorous liver tissue and tumorous tissues in HCC. Both normal and non-tumorous hepatocytes showed MZB1 immunopositivity, whereas tumor cells showed either negative or positive immunoreactivity. Bars, 25 μm. Magnifications are x200.

**B,** Kaplan-Meier curves for overall survival (left) and recurrence-free survival (right) rates of 162 patients with primary HCC. Negative MZB1 immunoreactivity of tumor cells was significantly associated with worse overall and recurrence-free survivals (P=0.0031 and 0.0044, respectively; log-rank test).
MZB1 methylation in hepatocellular carcinoma

Figure 4

Tumor-suppressive effects of restoration of MZB1 expression in hepatoma cells in vitro and in vivo.

A, HLE cells lacking MZB1 expression were transiently transfected with pCMV-3Tag3A-MZB1 or pCMV-3Tag3A-mock, and selected with G418 for 2 weeks. MZB1 expression was confirmed by immunoblotting using 10 μg of protein extract and anti-MZB1 antibody (left). The drug-resistant colonies formed by the MZB1-transfected cells were more numerous than those formed by control counterparts (right upper). Occupancy of the stained colony area was calculated (right lower). Columns, means of three separate experiments, each performed in triplicate; bars, SD (histogram). Asterisks (*), P<0.05 versus mock control (Student’s t-test). Similar results were obtained in the Hep3B and SK-HEP-1 cell lines (data not shown).

B, stable HepG2 transfectants were established by transfection of pCMV-3Tag3A-MZB1 or pCMV-3Tag3A-mock with G418 selection. Immunofluorescence cytochemistry demonstrated almost all pCMV-3Tag3A-MZB1-transfected cells to express FLAG-tagged MZB1 predominantly with an endoplasmic reticulum- (ER-) marker (calnexin, left). After plating into 24-well plates (2.5 x 10⁴ cells per well), the proliferative activity of stably MZB1-transfected cells to be less than that of the control counterparts. Results of relative growth-ratio are shown with the mean ± SD for three separate experiments, each done in triplicate (right). Differences were analyzed by one-way ANOVA with subsequent Scheffé’s tests: a and b, P<0.05 versus mock#1 and #2, respectively.

C, Left upper, representative results of the population in each phase of the cell cycle in stable transfectants described in 4B assessed by FACS. Left lower, Columns, means of three separate clones, each performed in triplicate; bars, SD (histogram). Asterisks (*), P<0.05 versus mock control (Student’s t-test). Right, representative results of immunoblotting of MZB1 protein and cleaved caspase-3 (cCASP3), one of apoptotic markers, without (no treatment, NT) or with cis-platinum (CDDP, 20 μM) treatment for 48 h.

D, representative results of tumors formed in SCID mice following injection of stably MZB1-transfected cells. MZB1-transfected HepG2 cells or mock-transfected cells (4x10⁷) were
MZB1 methylation in hepatocellular carcinoma

injected into the right and left dorsal flanks. SCID mice were sacrificed for tumor weight evaluation 5-w postinjection (upper left). Results of tumor weight are shown with the mean ± SD for three separate experiments, each done in triplicate (upper right). Differences were analyzed as described in Figure 4B. Lower left, representative results of immunohistochemical analysis of MZB1 as well as PCNA and Ki-67, markers for cell proliferation, in resected tumors. A positive MZB1 immunoreactivity was detected in MZB1-transfetced cells but not in mock-transfected counterparts, and both PCNA and Ki-67 immunoreactivities were less frequently observed in MZB1-transfetced cells compared with mock-transfected counterparts. Bars, 25 μm. Magnifications are x200. Lower right, representative results of immunoblotting of MZB1 protein and cCASP3 in resected tumors.
MZB1 methylation in hepatocellular carcinoma

Tables

Table 1
Association between clinicopathologic characteristics and MZB1 expression.

Table 2
Cox proportional hazard regression analysis for overall survival.
MeDIP on CpG-island array

Total 24,142 genes on CpG-island array

- 1 normal liver (case C20)
- 3 hepatoma cell lines (Hep3B, HepG2, HuH-7)

Total 41,057 genes on expression array

- 2 normal liver (cases C20 and C40)
- 3 hepatoma cell lines (Hep3B, HepG2, HuH-7)

2,476 genes methylated in at least 1 cell line, but unmethylated in normal liver

7,945 genes unmethylated in normal liver

2,476 genes methylated in at least 1 cell line, but unmethylated in normal liver

204 autosomal genes commonly downregulated in hepatoma and restored after 5-aza-dCyd treatment

642 genes commonly methylated in all 3 lines, but unmethylated in normal liver

11 overlapped genes among two analyses

264 autosomal genes in hepatoma and restored after 5-aza-dCyd treatment

Overlapping genes in 2 normal liver

1,730, 1,158, and 1,521 genes restored in Hep3B, HepG2, and HuH-7, respectively (vs. normal liver, >4-fold)

7,945 genes unmethylated in normal liver

2,476 genes methylated in at least 1 cell line, but unmethylated in normal liver

2,476 genes methylated in at least 1 cell line, but unmethylated in normal liver
Figure 2

A

Primary HCC

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<th>L31</th>
<th>L34</th>
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<th>L57</th>
<th>L62</th>
<th>L67</th>
<th>L74</th>
<th>L78</th>
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<th>L97</th>
<th>L99</th>
<th>L100</th>
<th>L101</th>
<th>L102</th>
<th>L104</th>
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</thead>
</table>

BstU1

Region 8

Methylation Silencing

MZB1

Silencing 22/30 (73.3%)

Relative mRNA

Expression (n=4)

0

1

2

HepG2

HepG2 +5-aza-dCyd

Hep3B

Hep3B +5-aza-dCyd

HuH-7

HuH-7 +5-aza-dCyd

C20

C40

L87N

L87T

Bisulfite PCR
(5q31.2)

Tel.

CpG site

CpG island

Methylation (%)

Total

Region 8

93.5

95.8

33.7

31.8

87.5

100

53.0

61.4

33.1

73.5

20.5

45.8

24.1

34.8

26.7

67.4

30.4

52.7

66.6

87.9

MZB1 (5q31.2)

Exon1

200bp

Cen.

L82

L09

L25

L31

L34

L50

L57

L62

L67

L74

L78

L81

L82

L83

L84

L85

L86

L87

L88

L89

L92

L97

L99

L100

L101

L102

L104

L87N

L87T

Tumor-specific hypermethylation (T >20%, N <20%)

>50% decreased expression in tumor tissue

Scale bars: 100μm

Methylation (·) expression (+)

Methylation (-) expression (-)
Figure 3

A

Normal liver  Non-tumor  Tumor

MZB1 (-)  MZB1 (+)

083297#15  707903#2  0807302#1

B

Overall survival probability

MZB1 positive (n=78)

MZB1 negative (n=84)

Log-rank test, \(P = 0.0031\)

Recurrence-free survival probability

MZB1 positive (n=78)

MZB1 negative (n=84)

Log-rank test, \(P = 0.0044\)
Table 1  Association between clinicopathologic characteristics and MZB1 expression

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MZB1 immunoreactivity</th>
<th>P valuea</th>
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<tr>
<td></td>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
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<tr>
<td>Total</td>
<td>162</td>
<td>84 (51.9)</td>
<td>78 (48.1)</td>
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<td>Gender</td>
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<tr>
<td>Male</td>
<td>122</td>
<td>66 (54.1)</td>
<td>56 (45.9)</td>
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<tr>
<td>Female</td>
<td>40</td>
<td>18 (45.0)</td>
<td>22 (55.0)</td>
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<tr>
<td>Age (y)</td>
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<tr>
<td>Mean</td>
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<td>65.7</td>
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<td>&gt;65</td>
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<td>49 (50.0)</td>
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<tr>
<td>&lt;65</td>
<td>64</td>
<td>35 (54.7)</td>
<td>29 (45.3)</td>
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<td>Virus</td>
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<tr>
<td>HCV(+)</td>
<td>79</td>
<td>39 (49.4)</td>
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<td>HBV(*)</td>
<td>38</td>
<td>25 (65.8)</td>
<td>13 (34.2)</td>
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<tr>
<td>HCV(-), HBV(-)</td>
<td>45</td>
<td>20 (44.4)</td>
<td>25 (55.6)</td>
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<tr>
<td>AFP (ng/mL)b</td>
<td></td>
<td>5390 ± 22966</td>
<td>2819 ± 13373</td>
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<td>PIVKA-II (mAU/mL)b</td>
<td></td>
<td>7252 ± 43097</td>
<td>6767 ± 25669</td>
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<td>AST (IU/L)b</td>
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<td>48.6 ± 25.3</td>
<td>54.7 ± 34.2</td>
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<td>ALT (IU/L)b</td>
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<td>42.8 ± 32.6</td>
<td>53.7 ± 34.8</td>
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<td>PT (%)b</td>
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<td>84.1 ± 13.1</td>
<td>85.9 ± 13.6</td>
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<tr>
<td>Total bilirubin (mg/dL)b</td>
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<td>0.86 ± 0.44</td>
<td>0.86 ± 0.40</td>
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<td>Albumin (g/dL)b</td>
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<td>3.9 ± 0.5</td>
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<td>Child-Pugh scoreb</td>
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<td>5.4 ± 0.6</td>
<td>5.3 ± 0.7</td>
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<td>Tumor size (cm)b</td>
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<td>4.6 ± 2.8</td>
<td>4.5 ± 3.1</td>
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<td>29</td>
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<td>12 (41.4)</td>
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<td>Absent</td>
<td>92</td>
<td>40 (43.5)</td>
<td>52 (56.5)</td>
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<tr>
<td>Present</td>
<td>70</td>
<td>44 (62.9)</td>
<td>26 (37.1)</td>
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<tr>
<td>Surgical margin (cm)</td>
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<tr>
<td>Absent</td>
<td>139</td>
<td>73 (52.5)</td>
<td>66 (47.5)</td>
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<td>Present</td>
<td>23</td>
<td>11 (47.6)</td>
<td>12 (52.2)</td>
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<td>Background liver parenchyma</td>
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<td>Normal liver (NL)</td>
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<td>8 (80.0)</td>
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<td>Chronic hepatitis (CH)</td>
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<td>39 (50.0)</td>
<td>39 (50.0)</td>
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<td>Liver chrosis (LC)</td>
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<td>43 (58.1)</td>
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<tr>
<td>I</td>
<td>13</td>
<td>3 (23.1)</td>
<td>10 (76.9)</td>
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<tr>
<td>II</td>
<td>57</td>
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<td>32 (56.1)</td>
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<tr>
<td>III</td>
<td>58</td>
<td>33 (56.9)</td>
<td>25 (43.1)</td>
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<td>IVA</td>
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<td>23 (67.6)</td>
<td>11 (32.4)</td>
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NOTE: Statistically significant values are in boldface type.

aP values are from χ² or Fisher’s exact test and were statistically significant at < 0.05.
bmean ± SD.
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<th>Factor</th>
<th>Univariate</th>
<th>Multivariate&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
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<td>Hazard ratio</td>
<td>95% confidence interval</td>
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<td>Gender Male versus Female</td>
<td>0.990</td>
<td>(0.509-1.925)</td>
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<td>Age (y) &lt;65 versus &gt;65</td>
<td>0.957</td>
<td>(0.527-1.738)</td>
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<td>AFP &gt;200 ng/ml versus &lt;200 ng/ml</td>
<td>2.202</td>
<td>(1.214-3.995)</td>
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<td>(1.496-8.401)</td>
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<td>Tumor number Multiple versus Single</td>
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<td>(1.406-4.554)</td>
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<td>Histopathological Grading Poor-moderate versus Well</td>
<td>2.312</td>
<td>(1.074-4.975)</td>
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<td>Portal vein invasion Present versus Absent</td>
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<td>(1.136-3.716)</td>
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<td>Surgical margin Present versus Absent</td>
<td>1.948</td>
<td>(0.963-3.940)</td>
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<td>Background liver parenchyma&lt;sup&gt;c&lt;/sup&gt; LC versus CH+NL</td>
<td>1.825</td>
<td>(1.009-3.300)</td>
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<td>Stage III+IVA versus I+II</td>
<td>3.466</td>
<td>(1.710-7.024)</td>
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<td>MZB1 expression&lt;sup&gt;d&lt;/sup&gt; Negative versus Positive</td>
<td>2.532</td>
<td>(1.338-4.791)</td>
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NOTE. Statistically significant values are in **boldface** type.

* P-values are from two-sided tests and were statistically significant at <0.05.

<sup>a</sup> Forward- and backward-stepwise analyses were used for multivariate analysis.

<sup>b</sup> LC, liver cirrhosis; CH, chronic hepatitis; NL, normal liver

<sup>c</sup> MZB1 expression was evaluated by immunohistochemical analysis as described in Materials and Methods.
Integrative array-based approach identifies MZB1 as a frequently methylated putative tumor-suppressor in hepatocellular carcinoma

Satoshi Matsumura, Issei Imoto, Ken-ichi Kozaki, et al.

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