

Dynamin 3: a new candidate tumor suppressor gene in hepatocellular carcinoma detected by triple combination array analysis

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Background: To identify genes associated with hepatocellular carcinoma (HCC) pathogenesis, we developed a triple combination array strategy comprising methylation, gene expression, and single nucleotide polymorphism (SNP) array analysis.

Methods: Surgical specimens obtained from a 68-year-old female HCC patient were analyzed by triple combination array, and identified *Dynamin 3* (*DNM3*) as a candidate tumor suppressor gene in HCC. Subsequently, samples from 48 HCC patients were evaluated for *DNM3* methylation and expression status using methylation specific polymerase chain reaction (PCR; MSP) and semi-quantitative reverse transcriptase (RT)-PCR, respectively. The relationship between clinicopathological factors and *DNM3* methylation status was also investigated.

Results: *DNM3* was shown to be hypermethylated (methylation value 0.879, range 0–1.0) in cancer tissue compared with adjacent normal tissue (0.213) by methylation array in the 68-year-old female patient. Expression arrays revealed decreased expression of *DNM3* in cancerous tissue. SNP arrays revealed that the copy number of chromosome 1q24.3, in which *DNM3* resides, was normal. MSP revealed hypermethylation of the *DNM3* promoter region in 33 of 48 tumor samples. A trend toward decreased *DNM3* expression was observed in patients with *DNM3* promoter methylation ($P = 0.189$). Furthermore, patients with reduced expression of *DNM3* in tumor tissues exhibited worse prognosis with decreased disease specific survival compared to patients without decreased expression ($P = 0.014$).

Conclusion: The present study indicates that a triple combination array strategy is an effective method to detect novel genes related to HCC. We propose that *DNM3* is a tumor suppressor gene in HCC.

Keywords: *DNM3*, hepatocellular carcinoma, methylation, triple combination array

Introduction

Hepatocellular carcinoma (HCC) is currently the sixth most prevalent neoplasm and the third most frequent cause of cancer death worldwide,¹ and its overall incidence is steadily rising. Although several therapeutic options for the treatment of HCC currently exist,^{2–5} the prognosis of patients with advanced HCC remains poor.^{6,7} Therefore, further research to clarify the mechanisms underlying hepatocarcinogenesis is urgently required.⁸

We recently developed a double combination array analysis consisting of gene expression and single nucleotide polymorphism (SNP) array analysis, and reported a number of tumor suppressor genes in HCC.^{9–14} In these studies, we hypothesized that deoxyribonucleic acid (DNA) methylation of the promoter region of these genes led to downregulation of gene expression, causing HCC progression. In addition to

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this double combination array analysis, we also performed methylation array analysis of the same specimens, thus allowing a more conclusive association between gene expression and DNA methylation. This triple combination array analysis represents an efficient procedure for the detection of tumor suppressor genes of HCC.^{15,16}

Dynamin 3 (DNM3) is a candidate tumor suppressor gene detected by this triple combination array analysis. This gene encodes a member of the dynamin family, which possess mechanochemical properties used to tubulate and sever membranes.¹⁷ However, to date, there are few reports describing the relationship between *DNM3* and any malignant disease.^{18,19} Furthermore, no previous studies have investigated the role of *DNM3* in HCC. In this study, we aimed to evaluate the expression and methylation status of *DNM3* in HCC.

Materials and methods

Sample collection and DNA preparation

Nine HCC cell lines (HepG2, Hep3B, HLE, HLF, HuH1, HuH2, HuH7, PLC/PRF/5 and SK-Hep1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum and incubated in 5% CO₂ at 37°C.

A 68-year-old woman with chronic hepatitis C was diagnosed with HCC in the right lobe and biochemical examination showed that des-gamma-carboxy (DCP) prothrombin level was as high as 7029 mAU/mL whereas alpha-fetoprotein (AFP) level was 9 ng/mL. She underwent partial liver resection. Specimens of her tumor and adjacent non-tumorous tissues were excised. The tumor was pathologically confirmed as moderately differentiated HCC and the surrounding parenchymal tissue showed liver cirrhosis.

HCC tissue (HT) and normal tissue (NT) samples were obtained from 48 patients (43 males, five females, aged between 39–77 years, mean \pm standard deviation [SD], 62.4 ± 7.9 years) who underwent liver resection at Nagoya University Hospital, Nagoya, Japan between 1994 and 2001. Thirty-eight patients had hepatitis C and seven had hepatitis B. The median duration of follow-up was 80.7 months (range 15.2–213.1 months). All tissues were reviewed pathologically to confirm the diagnosis of HCC. Written informed consent, as required by the institutional review board, was obtained from all patients. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until required. Genomic DNA was obtained from tissue samples by proteinase K digestion, followed by phenol/chloroform extraction.

Ribonucleic acid isolation, microarray, and gene chip affymetrix procedures

Gene expression and SNP arrays were performed as previously described,^{9–14} using total ribonucleic acid (RNA) and DNA extracted from tissue samples taken from the 68-year-old female patient described above. Total RNA and DNA were extracted from an area consisting of $>80\%$ cancerous cells. RNA was isolated from each of the frozen samples with the RNeasy mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. Total RNA was processed for expression array analysis by Affymetrix HGU133A and HGU133B Gene Chips (Affymetrix, Santa Clara, CA, USA). Genomic DNA was used for SNP-Chip array analysis by Affymetrix GeneChip Mapping 500 K arrays (Affymetrix).

Methylation array platform

Methylation arrays were performed using DNA extracted from tissue samples taken from the 68-year-old female patient described above. Bisulfite-converted DNA (500 ng to 1 μg) was used for DNA methylation analysis using Illumina Infinium Human Methylation 27 BeadChip arrays (Illumina, San Diego, CA, USA).²⁰ Of the ~ 28 million CpG sites identified throughout the haploid human genome, Illumina initially designed Infinium methylation probes for 27,578 CpG sites located in promoter regions (up to 1 kb upstream or 500 bp downstream of the transcription start sites). Of these, 27,324 CpG sites relate to 14,475 consensus coding sequences, including approximately 1,000 cancer-associated genes, and 254 CpG sites relate to approximately 100 microRNA genes. The probes were preferentially selected to occur within CpG islands using the National Center for Biotechnology Information (NCBI) "relaxed" definition of a CpG island: CpG islands identified bioinformatically with a CpG content of $>50\%$ and an observed/expected ratio of >0.6 .²¹

Bisulfite-converted DNA was whole-genome amplified, enzymatically fragmented, and hybridized to arrays. During hybridization, the bisulfite-converted DNA anneals to methylation-specific probes on the chip. Each CpG locus is represented by two bead types, one of which is specific to the methylated state and the other specific to the unmethylated state, which is directly related to the underlying sequence change catalyzed during bisulfite conversion. Therefore, for each CpG site, a possible C/T variant can be assayed through the single-base extension step, which is possible because of the ability to hybridize to either the "protected" methylated cytosine or the converted (unmethylated) thymine.

After hybridization, a single-base extension step was performed using a multi-layer staining process, as described below. The BeadChip was then scanned on the Illumina iScan and the resulting “idat” files were analyzed using BeadStudio software (Illumina, San Diego, CA, USA). The output of the BeadStudio analysis is a β -value for each CpG site. This is a continuous value between 0 and 1, where 0 = 0% methylation and 1 = 100% methylation at a given CpG site. Therefore, this assay enables quantitative analysis of methylation at individual CpG sites.

Reverse transcription-polymerase chain reaction

DNM3 messenger (m)RNA expression was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR. Total RNA (10 μ g) isolated from nine HCC cell lines, primary HTs and NTs were used to generate complementary DNA (cDNA). The resulting cDNAs were then amplified by PCR using primers for *DNM3* (sense, 5'-ACC CCA CAC CTG CAG AAG GT-3' in exon 6); antisense 5'-TGG AGA GCA ACT GTC CCT GTA-3' in exon 7), which amplified a 100 bp product. PCR was performed using the following parameters: initial denaturation at 94°C for 5 minutes followed by 35 cycles of 94°C for 10 seconds, 60°C for 8 seconds and 72°C for 4 seconds. RT-PCR of β -actin was performed to confirm equal amounts of cDNA template in each amplification. Each PCR product was loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under ultraviolet (UV) illumination.

Real-time quantitative RT-PCR analysis

PCR was performed using SYBR Green PCR Core Reagents (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) under the following conditions: 1 cycle at 95°C for 10 seconds, followed by 48 cycles at 95°C for 10 seconds and at 60°C for 30 seconds. SYBR Green emission was detected in real-time with an ABI prism 7000 Sequence Detector (Perkin-Elmer Applied Biosystems). The primers used for PCR were the same as those described above for RT-PCR. Quantitative RT-PCR was performed at least three times, including negative controls without template. The expression of *DNM3* was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in each sample.

Methylation-specific PCR

DNA from HCC cell lines, HTs and NTs were subject to bisulfite treatment. Briefly, 2 μ g of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples

were then purified using the Wizard purification resin (Promega Corporation, Madison, WI, USA), treated with NaOH, precipitated with ethanol and resuspended in water. Primer pairs were used to detect methylation (sense, 5'-GAG GTC GTG GTA TTT GGT CG-3'; antisense, 5'-AAC GCG CCT ACG CGA ACG AA-3'; 149-bp product) and non-methylation (sense, 5'-GGG GTG TTG TGG AGA TAG GT-3'; antisense, 5'-CCA CCA ACC CAC CAC TTA AC-3'; 90-bp product) of the *DNM3* promoter region near exon 1. Methylation-specific PCR (MSP) amplification was performed as follows: denaturation at 94°C for 5 minutes followed by 44 cycles at 94°C for 15 seconds, 60°C for 10 seconds and 72°C for 9 seconds. Unmethylated-Specific PCR (UNMSP) amplification was performed as follows: denaturation at 94°C for 5 minutes followed by 36 cycles at 94°C for 15 seconds, 60°C for 10 seconds and 72°C for 8 seconds. PCR products were loaded directly onto 3% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Sequence analysis

Bisulfite-treated genomic DNA obtained from HCC cell lines was sequenced and PCR was performed in all cases. Semi-nested PCR was performed to gain adequate products for TA cloning. PCR amplification was performed as follows: denaturation at 94°C for 3 minutes followed by 37 cycles of 94°C for 15 seconds, 54°C for 15 seconds, and 72°C for 20 seconds with primer pairs (sense 5'-TTG GGT TTT GGT TTG GTA GT-3'; antisense, 5'-TCA ACC AAC CAA ATC CTA AC-3'; 342-bp product). PCR products were used as templates for subsequent PCR using the same antisense primer and a different sense primer (sense, 5'-GGT TTT TGA GTT TTG GTT ATA G-3'; 253-bp product). The PCR products were subcloned into a TA cloning vector (Invitrogen, Carlsbad, CA, USA). Six clones were selected from two HCC cell lines (HuH2 and PLC/PRF/5). Each DNA clone was mixed with 3 μ L of M13 specific primer and 4 μ L of Cycle Sequence Mix (ABI PRISM Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA). Samples were then subjected to the following cycling conditions: 95°C for 30 seconds followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes, and purified by ethanol precipitation. Sequence analysis was performed using an Applied Biosystems ABI310 and sequence electropherograms were generated using ABI Sequence Analysis software version 3.0.

5-aza-2'-deoxycytidine treatment

HCC cell lines were treated with 1 μ M 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, St Louis, MO, USA) to inhibit

DNA methylation. Cells (1.5×10^6) were cultured for 6 days with medium changes on days 1, 3, and 5. On day 6, cells were harvested, RNA was extracted and RT-PCR was performed as described above.

Statistical analysis

Continuous variables are expressed as medians (range) and comparisons were made using the Mann–Whitney U test. Categorical variables were compared using χ^2 tests or Fisher's exact tests, where appropriate. Disease specific survival rates were analyzed by Kaplan–Meier and log-rank tests. All statistical analyses were performed using JMP software version 9.0.2 (SAS International Inc., Cary, NC, USA). The level of statistical significance was set at $P < 0.05$.

Results

Results of expression, SNP, and methylation-arrays

To identify novel tumor-related genes in HCC, we first searched for genes with decreased expression in HCC samples compared with corresponding normal tissue. According to the expression array results, *DNM3* was strongly down-regulated in HCC tissue (log2 ratio -1.0 , 2-fold decrease in expression; Table 1). Decreased expression of *DNM3* mRNA was subsequently confirmed by semi-quantitative RT-PCR in tumor tissue from the same patient (Figure 1A).

SNP array analysis of DNA from the same patient revealed deletions in chromosomes 3q, 8p, 11q, 12p, 12q, 16p, 17p, 19p, and X, and chromosomal gains in 1q, 3q, 11q, 12p, and 12q. Although we observed amplifications affecting chromosome 1, which contain the *DNM3* gene, no deletions were observed (Figure 1B). A detailed analysis of the SNP array at the *DNM3* gene locus (1q24.3) identified 61 SNPs. Of these 61 SNPs, three exhibited a heterozygous AB allele in both the non-cancerous and cancerous samples (Table 2). These results suggest that the *DNM3* gene locus retained biallelically, and the expression of *DNM3* was diminished without chromosomal deletion.

Table 1 Expression array analysis of surgical samples from a 68-year-old female patient with HCC

| Probe set ID | Gene symbol | Log2 ratio | Sample | Signal | Detection |
|--------------|-------------|------------|--------|--------|-----------|
| 209839_at | <i>DNM3</i> | -1.0 | Normal | 43.2 | p |
| | | | Tumor | 10.9 | p |

Abbreviations: *DNM3*, dynamin 3; HCC, hepatocellular carcinoma; p, positive; ID, identification.

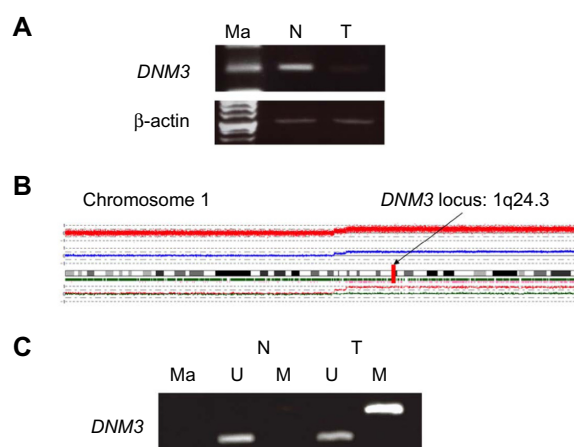


Figure 1 Analysis of specimens from a 68-year-old female with HCC.

Notes: (A) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) revealed down-regulation of *DNM3* in tumor compared with corresponding normal tissue. (B) Copy number analysis of chromosome 1 using single nucleotide polymorphism arrays in HCC specimens revealed amplification of 1q24.3, containing the *DNM3* locus, but no deletions. (C) Methylation specific PCR revealed *DNM3* promoter hypermethylation in tumor samples only.

Abbreviations: *DNM3*, dynamin 3; HCC, hepatocellular carcinoma; M, methylated; Ma, marker; N, normal; PCR, polymerase chain reaction; U, unmethylated; T, tumor.

We subsequently performed methylation array analysis using DNA from the same 68-year-old female HCC patient. The continuous β -values were 0.879 for tumor tissue versus 0.213 for normal tissue, indicating high methylation in the HCC sample (Table 3). Using MSP, we confirmed hypermethylation of the *DNM3* promoter in tumor tissue obtained from the same HCC patient (Figure 1C). The *DNM3* gene was particularly notable, since expression in cancer tissue was decreased despite amplification of the copy number of chromosome 1 containing this gene. Taken together, these results suggest that decreased expression of *DNM3* in the absence of loss of heterozygosity (LOH) may be due to promoter hypermethylation.

Effect of inhibiting methylation on *DNM3* expression in HCC cell lines

To confirm that silencing of *DNM3* expression was due to promoter hypermethylation, we analyzed *DNM3* mRNA expression in nine HCC cell lines before and after treatment with 5-aza-dC. Expression of *DNM3* was clearly reactivated following 5-aza-dC treatment in three cell lines, Hep3B, HLE, and HuH2, as shown by semi-quantitative RT-PCR (Figure 2A).

MSP and UNMSP of HCC cell lines and one primary HCC specimen

We next investigated the methylation status of *DNM3* in nine HCC cell lines using primers for (MSP) and (UNMSP).

Table 2 Results of SNP signals at the *DNM3* gene locus

| Probe Set ID | Chromosome | Physical position | Normal call | Confidence | Tumor call | Confidence |
|---------------|------------|-------------------|-------------|------------|------------|------------|
| SNP A-4301010 | I | 171831910 | AB | 0.056641 | AB | 0.008301 |
| SNP A-2062980 | I | 171880994 | AB | 0.007813 | AB | 0.039063 |
| SNP A-4198760 | I | 172062792 | AB | 0.007813 | AB | 0.007813 |

Abbreviations: *DNM3*, dynamin 3; SNP, single nucleotide polymorphism; ID, identification.

For MSP, bands of the appropriate size were observed for HepG2, Hep3B, HLE, HLF, HuH1, HuH2, HuH7, and SK-Hep1 cell lines (Figure 2B). For UNMSP, appropriate bands were identified in all cell lines, except HuH2 (Figure 2B). These results indicate complete methylation of *DNM3* in HuH2, partial methylation in HepG2, Hep3B, HLE, HLF, HuH1, HuH7, and SK-Hep1 and no methylation in PLC/PRF/5 cells.

Sequence analysis

To confirm the results obtained by MSP, we next performed sequence analysis of the *DNM3* promoter region in HuH2 and PLC/PRF/5 cells. Almost all CpG dinucleotides in HuH2 were methylated, while those of PLC/PRF/5 were unmethylated (Figure 3), thus confirming the accuracy of MSP and UNMSP.

MSP and UNMSP of normal and tumor tissues from HCC patients

We next assessed *DNM3* promoter hypermethylation in tumor and normal tissues from 48 HCC patients. Overall, 68.7% (33/48) of tumor samples displayed *DNM3* promoter hypermethylation, compared to 27% (13/48) of normal samples (Figure 4A). Thus, hypermethylation of *DNM3* was significantly more frequent in tumor tissues ($P < 0.001$). Four representative cases of MSP and UNMSP status are shown in Figure 4B.

Analysis of *DNM3* expression in normal and tumor tissues from HCC patients

We next examined the expression of *DNM3* mRNA in the 48 HCC patients by real-time quantitative RT-PCR. The *DNM3* expression index was calculated as the value of

the tumor tissue expression level divided by the expression level of adjacent normal tissue. Analysis of the association between methylation status and the expression index revealed that hypermethylated cancerous tissues tended to have a lower *DNM3* expression index than other tissues examined, however this was not statistically significant ($P = 0.189$; Figure 5).

Correlation between *DNM3* gene expression and clinicopathological characteristics in HCC patients

We next analyzed the correlation between *DNM3* expression and clinicopathological features of the 48 HCC patients. Growth status (expansive growth or infiltrative growth) was the only variable associated with *DNM3* expression (Table 4). We also observed that patients with decreased *DNM3* expression had a worse prognosis compared to patients with increased *DNM3* expression ($P = 0.014$; Figure 6). Univariate analysis identified liver damage, cirrhosis and decreased expression of *DNM3* as prognostic factors of disease specific survival, however these were not significant in multivariate analysis (Table 5).

Discussion

DNA methylation in the promoter region of several genes in HCC has been previously investigated.^{22–25} To detect new cancer-related genes in HCC, we developed a double combination array analysis,^{9–14} and triple combination array analysis including gene expression, SNP and methylation array analysis.^{15,16} In the current study, we used this triple combination array analysis to identify *DNM3* as a candidate tumor suppressor gene in HCC.

Table 3 Methylation array analysis of surgical samples from a 68-year-old female patient with HCC

| Probe ID | Gene symbol | Sample | Methylation value (0–1) | Status | | | Confidence | Chromosomal location |
|-------------|-------------|--------|-------------------------|--------|--------------|------------|------------|----------------------|
| | | | | Total | Unmethylated | Methylated | | |
| cg 23391785 | <i>DNM3</i> | Normal | 0.213 | 2315 | 1799 | 516 | 3.68E-38 | Chr1q24.3 |
| | | Tumor | 0.879 | 5019 | 519 | 4500 | 3.68E-38 | |

Abbreviations: *DNM3*, dynamin 3; HCC, hepatocellular carcinoma; ID, identification.

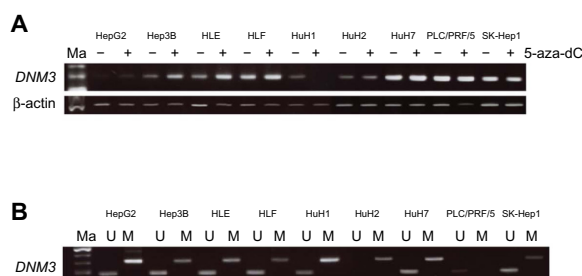


Figure 2 (A) Semi-quantitative RT-PCR revealed reactivation of *DNM3* expression in three (Hep3B, HLE, and HuH2) of nine HCC cell lines. **(B)** MSP revealed complete methylation of *DNM3* in HuH2, partial methylation in HepG2, Hep3B, HLE, HLF, HuH1, HuH7, and SK-Hep1 and no methylation in PLC/PRF/5 cells.

Abbreviations: HCC, hepatocellular carcinoma; *DNM3*, dynamin 3; Ma, marker; 5-aza-dC, 5-aza-2'-deoxycytidine; M, methylated; U, unmethylated; MSP, methylation specific PCR. PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction.

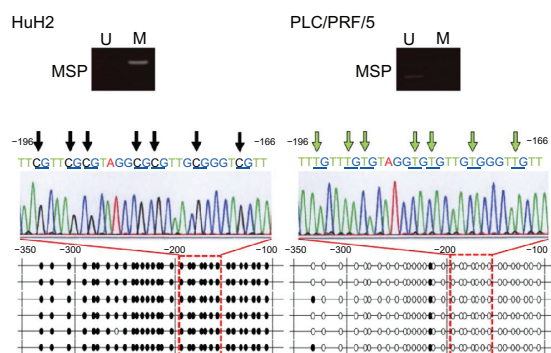


Figure 3 Sequence analysis of bisulfite-treated DNA in the *DNM3* promoter region. **Notes:** The methylation status of 31 CpG islands in six clones between -350 and -100 from the transcription initiation site of *DNM3* exon I is shown. Closed circles represent methylated CpG islands; open circles indicate unmethylated CpG islands. The CpG islands in the *DNM3* promoter region in HuH2 cells were abundantly methylated, whereas CpG islands in PLC/PRF/5 cells were predominantly unmethylated. Sequence analysis of CpG islands between -196 and -166 (boxed regions) is shown, where C (black arrows) indicates methylated CpG islands. Bisulfite treatment converts cytosine residues to Ts (green arrows), and indicate unmethylated CpG islands. These results validated the accuracy of MSP and UNMSP in upper region in this figure.

Abbreviations: DNA, deoxyribonucleic acid; *DNM3*, dynamin 3; Ma, marker; 5-aza-dC, 5-aza-2'-deoxycytidine; M, methylated; U, unmethylated; MSP, methylation specific PCR; UNMSP, Unmethylated-Specific PCR; PCR, polymerase chain reaction.

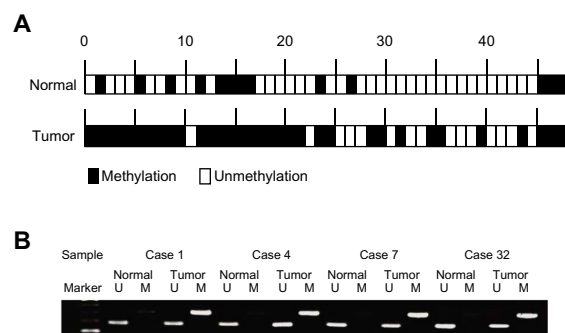


Figure 4 (A) Methylation status of *DNM3* in 48 primary HCC samples. Thirty-three of 48 (68.7%) cancer tissues exhibited hypermethylation of *DNM3*, compared to 13 of 48 (27.0%) cases in adjacent, normal tissues. **(B)** Four representative cases showing hypermethylation of the promoter region of *DNM3* in tumor tissues and no methylation in normal tissues.

Abbreviations: HCC, hepatocellular carcinoma; M, methylated; U, unmethylated; *DNM3*, dynamin 3.

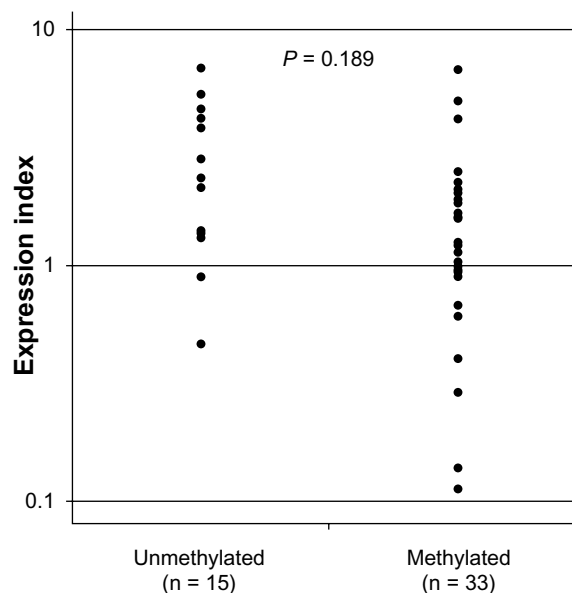


Figure 5 Expression levels of *DNM3* mRNA in specimens from 48 patients with HCC. The expression index $[(DNM3\text{-tumor}) \times (GAPDH\text{-normal}) / (DNM3\text{-normal}) \times (GAPDH\text{-tumor})]$ was calculated for all 48 cases. A trend towards a lower expression index in methylated cases compared to unmethylated cases was observed, however this was not statistically significant ($P = 0.189$).

Abbreviation: HCC, hepatocellular carcinoma; *DNM3*, dynamin 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

DNM3 is a member of the dynamin family of genes that are essential for endocytosis, and possess mechanochemical properties utilized to tabulate and sever membranes.^{17,26} To date, few studies have investigated the impact of dysregulated *DNM3* expression in malignant disease.^{18,19} Indeed, no studies have yet been conducted to investigate the relationship between HCC and *DNM3* expression. A study by Shen et al, reporting genome-wide DNA methylation profiles in 62 HCC patients,²⁷ identified *DNM3* in a subset of genes methylated in tumor tissues compared to adjacent normal tissue. However, this study did not evaluate the expression levels of *DNM3* or its impact on prognostic outcome. Our current study revealed that methylation of *DNM3* downregulates gene expression and is correlated with worse prognosis, suggesting that this gene may represent a novel tumor suppressor gene of HCC.

DNM2 is also a member of the dynamin family. Lee et al reported that low *DNM2* expression was associated with tumor invasion and metastasis in invasive squamous cell carcinoma of the cervix.²⁸ This group also demonstrated that inhibition of *DNM2* enhanced the expression of metalloproteinase-2 (*MMP2*), a type IV collagenase,²⁹ whose elevated expression is associated with various carcinomas, including HCC.³⁰⁻³² *MMP2* encodes an enzyme which degrades type IV collagen, the major structured component of the basement membrane. Overexpression of *MMP2* may allow cancer cells to cross the epithelial basement membrane, invade surrounding stroma,

Table 4 Correlation between *DNM3* expression and clinico-pathological characteristics in 48 HCC patients

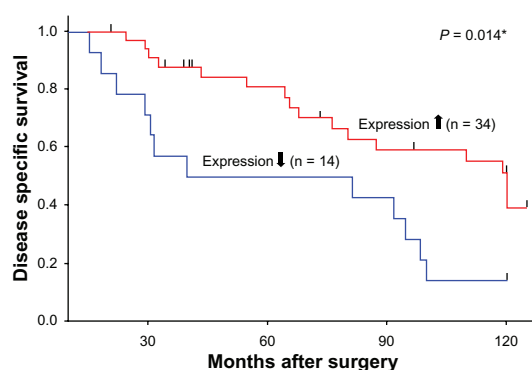
| Variables | Expression ↑ (n = 14) | Expression ↓ (n = 34) | P-value |
|-----------------|--------------------------|--------------------------|---------|
| Age | | | 0.411 |
| ≥65 years | 8 | 15 | |
| <65 years | 6 | 19 | |
| Sex | | | 0.583 |
| Male | 12 | 31 | |
| Female | 2 | 3 | |
| Liver damage | | | 0.112 |
| A | 6 | 23 | |
| B | 8 | 11 | |
| Cirrhosis | | | 0.411 |
| Present | 8 | 15 | |
| Absent | 6 | 19 | |
| Virus | | | 0.986 |
| HBV | 2 | 5 | |
| HCV | 11 | 27 | |
| Negative | 1 | 2 | |
| Tumor | | | 0.882 |
| ≥50 mm | 4 | 9 | |
| <50 mm | 10 | 25 | |
| Tumor number | | | 0.411 |
| Solitary | 7 | 24 | |
| Multiple | 7 | 10 | |
| Fc | | | 0.939 |
| Present | 12 | 28 | |
| Absent | 2 | 5 | |
| Fc-inf | | | 0.820 |
| Present | 11 | 27 | |
| Absent | 2 | 6 | |
| Eg or Ig | | | 0.010 |
| Eg | 9 | 32 | |
| Ig | 4 | 1 | |
| Vessel invasion | | | 0.115 |
| Present | 5 | 5 | |
| Absent | 9 | 29 | |
| AFP | | | 0.075 |
| ≥20 | 11 | 17 | |
| <20 | 3 | 16 | |
| Japan-Stage | | | 0.121 |
| I + II | 7 | 25 | |
| III + IVa | 7 | 9 | |

Abbreviations: AFP, alpha-fetoprotein; *DNM3*, *dynamin 3*; Eg, expansive growth; Fc, formation of capsule; Fc-inf, infiltration to capsule; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Ig, infiltrative growth.

Table 5 Prognostic factors for disease specific survival for 48 HCC patients in univariate and multivariate analysis

| | Univariate analysis | | | Multivariate analysis | | |
|------------------------|---------------------|-----------|---------|-----------------------|-----------|---------|
| | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Liver damage | 2.61 | 1.22–5.58 | 0.014 | 1.98 | 0.85–4.26 | 0.114 |
| Cirrhosis | 2.30 | 1.09–4.91 | 0.027 | 2.00 | 0.93–4.38 | 0.075 |
| <i>DNM3</i> expression | 2.50 | 1.15–5.30 | 0.021 | 2.09 | 0.93–4.58 | 0.070 |

Abbreviations: CI, confidence interval; *DNM3*, *dynamin 3*; HCC, hepatocellular carcinoma; HR, hazard ratio.

**Figure 6** Disease specific survival stratified by *DNM3* (*dynamin 3*) expression status. Patients with lower *DNM3* expression exhibited poorer prognosis with decreased disease specific survival, compared to patients with higher *DNM3* expression ($P = 0.014$).

Note: *, statistically significant.

enter blood vessels or lymphatics, extravasate and establish new proliferating colonies.³¹ Therefore, decreased expression of *DNM3* by promoter methylation may increase the expression of *MMP2*, thereby contributing to the poor prognosis of these patients.

Using our triple array analysis, we have extracted an interesting molecule, *DNM3*, and verified an association between gene expression and carcinogenesis or tumor progression, using a panel of 48 primary human specimens. It may be pointed out that the efficiency of the extraction of interesting molecules will be increased if we use multiple cases for arrays. Although we had to check data from many genes for extracting the molecule from array data of one HCC patient, it seems that the results of triple combination array analysis were reliable because we have reported our results after investigation of as many as 48 samples of HCC cases.

In clinical practice, *DNM3* expression may be used as a prognostic biomarker for resected HCC patients. The methylation of certain genes, for example *p15* in myelodysplastic syndrome (MDS) patients is well documented,³³ and hypomethylating agents such as 5-aza-2'-deoxycytidine (decitabine) have already been introduced in the clinical setting for MDS patients.³⁴ In the future, decitabine may also be used in patients with aberrant methylation of *DNM3*.

In conclusion, we identify *DNM3* as a new candidate tumor suppressor gene in HCC by triple combination array analysis. Further studies are required to confirm the role of this gene as a tumor suppressor in carcinogenesis.

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Disclosure

The authors report no conflicts of interest in this work.

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