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

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
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.\textsuperscript{15,16}

\textit{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 mechnanochemical properties used to tabulate and sever membranes.\textsuperscript{17} However, to date, there are few reports describing the relationship between \textit{DNM3} and any malignant disease.\textsuperscript{18,19} Furthermore, no previous studies have investigated the role of \textit{DNM3} in HCC. In this study, we aimed to evaluate the expression and methylation status of \textit{DNM3} in HCC.

\section*{Materials and methods}

\subsection*{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% CO\textsubscript{2} 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. During hybridization, the bisulfite-converted DNA anneals with a CpG content of $\sim$28 million 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 $>0.6$.\textsuperscript{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.

\subsection*{Ribonucleic acid isolation, microarray, and gene chip affymetrix procedures}

Gene expression and SNP arrays were performed as previously described,\textsuperscript{8–14} 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).

\subsection*{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).\textsuperscript{20} Of the $\sim$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 $>0.6$.\textsuperscript{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 \( \beta \)-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 \( \mu \)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 \( \beta \)-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 bisulfitation treatment. Briefly, 2 \( \mu \)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

Bisulfité-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 GTG TTT TGT TTG GTA GT-3′; antisense, 5′-TCA ACC AAC CAA ATC TTA 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 GTA AT-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 \( \mu \)L of M13 specific primer and 4 \( \mu \)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 \( \mu \)M 5-aza-2′-deoxycytidine (5-aza-dC) (Sigma-Aldrich, St Louis, MO, USA) to inhibit
DNA methylation. Cells (1.5 × 10⁶) 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 χ² 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.

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

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

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 2C). 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 | 1          | 171831910         | AB          | 0.056641   | AB         | 0.008301   |
| SNP A-2062980 | 1          | 171880994         | AB          | 0.007813   | AB         | 0.039063   |
| SNP A-4198760 | 1          | 172062792         | AB          | 0.007813   | AB         | 0.007813   |

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

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 | Unmethylated | Methylated | Confidence | Chromosomal location |
|----------|-------------|--------|-------------------------|--------|--------------|------------|------------|---------------------|
| cg 23391785 | DNM3       | 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.

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.
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; RT-PCR, reverse transcription-polymerase chain reaction.

**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 1 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 arrow) 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-tumor) × (GAPDH-normal)/(DNM3-normal) × (GAPDH-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).

**Abbreviations:** 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 mechanochanical 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 metalloprotease-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

| Variables         | 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 |
| DNM3 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.

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.

Acknowledgments

This work was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant-in-Aid for Scientific Research (C) Number 22591427.
Disclosure

The authors report no conflicts of interest in this work.

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127(12):2893–2917.
2. Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. Lancet. 2012;379(9822):1245–1255.
3. Livraghi T, Goldberg SN, Lazzaroni S, Meloni F, Solbiati L, Gazelle GS. Small hepatocellular carcinoma: treatment with radio-frequency ablation versus ethanol injection. Radiology. 1999;210(3):655–661.
4. Takayasu K, Arii S, Ikai I, et al; Liver Cancer Study Group of Japan. Prospective cohort study of transarterial chemoembolization for unresectable hepatocellular carcinoma in 8510 patients. Gastroenterology. 2006;131(2):461–469.
5. Llovet JM, Ricci S, Mazzaferro V, et al; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008;359(4):378–390.
6. Yu MC, Yuan JM. Environmental factors and risk for hepatocellular carcinoma. Gastroenterology. 2004;127(5 Suppl 1):S72–S87.
7. Cusnir M, Patt YZ. Novel systemic therapy options for hepatocellular carcinoma. Cancer. 2004;10(2):97–103.
8. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology. 2007;132(7):2557–2576.
9. Kanda M, Nomoto S, Okamura Y, et al. Detection of metallothionein 1G as a methylated tumor suppressor gene in hepatocellular carcinoma using a novel method of double combination array analysis. Int J Oncol. 2009;35(3):477–483.
10. Nomoto S, Kanda M, Okamura Y, et al. Epidermal growth factor-containing fibulin-like extracellular matrix protein 1, EIFEMP1, a novel tumor-suppressor gene detected in hepatocellular carcinoma using double combination array analysis. Ann Surg Oncol. 2010;17(3):923–932.
11. Okamura Y, Nomoto S, Kanda M, et al. Leukemia inhibitory factor receptor (LIFR) is detected as a novel suppressor gene of hepatocellular carcinoma using double-combination array. Cancer Lett. 2010;289(2):170–177.
12. Okamura Y, Nomoto S, Kanda M, et al. Reduced expression of reelin (RELN) gene is associated with high recurrence rate of hepatocellular carcinoma. Ann Surg Oncol. 2011;18(2):572–579.
13. Kanda M, Nomoto S, Okamura Y, et al. Promoter hypermethylation of fibulin 1 gene is associated with tumor progression in hepatocellular carcinoma. Mol Cancrcog. 2011;50(8):571–579.
14. Hayashi M, Nomoto S, Kanda M, et al. Identification of the A kinase anchor protein 12 (AKAP12) gene as a candidate tumor suppressor of hepatocellular carcinoma. J Surg Oncol. 2012;105(4):381–386.
15. Okamura Y, Nomoto S, Hayashi M, et al. Identification of the bcl2mycin hydrolase gene as a methylated tumor suppressor gene in hepatocellular carcinoma using a novel triple-combination array method. Cancer Lett. 2011;312(2):150–157.
16. Hishida M, Nomoto S, Inokawa Y, et al. Estrogen receptor 1 gene as a tumor suppressor gene in hepatocellular carcinoma detected by triple-combination array analysis. Int J Oncol. 2013;43(1):88–94.
17. Orth JD, McNiven MA. Dynamin at the actin-membrane interface. Curr Opin Cell Biol. 2003;15(1):31–39.
18. Booken N, Gratschv A, Utikal J, et al. Sézary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CD01 and DNM3. Leukemia. 2008;22(2):393–399.
19. Teicher BA. Searching for molecular targets in sarcoma. Biochem Pharmacol. 2012;84(1):1–10.
20. Bibikova M, Fan JB. GoldenGate assay for DNA methylation profiling. Methods Mol Biol. 2009;507:149–163.
21. Takai D, Jones PA. The CpG island searcher: a new WWW resource. In Silico Bio (Gedrakt). 2003;3(3):235–240.
22. Yoshikawa H, Matsubara K, Qian GS, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet. 2001;28(1):29–35.
23. Wong IH, Lo YM, Zhang J, et al. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res. 1999;59(1):71–73.
24. Yang B, Guo M, Herman JG, Clark DP. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am J Pathol. 2003;163(3):1101–1107.
25. Lee S, Lee HI, Kim JH, Lee HS, Jang JJ, Kang GH. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. Am J Pathol. 2003;163(4):1371–1378.
26. Sever S. Dynamin and endocytosis. Curr Opin Cell Biol. 2002;14(4):463–467.
27. Shen J, Wang S, Zhang YJ, et al. Genome-wide DNA methylation profiles in hepatocellular carcinoma. Hepatology. 2012;55(6):1799–1808.
28. Lee YY, Do IG, Park YA, et al. Low dynamin 2 expression is associated with tumor invasion and metastasis in invasive squamous cell carcinoma of cervix. Cancer Biol Ther. 2010;10(4):329–335.
29. Devarajan P, Johnston JJ, Ginsberg SS, Van Wart HE, Berliner N. Structure and expression of neutrophil gelatinase cDNA. Identity with type IV collagenase from HT1080 cells. J Biol Chem. 1992;267(35):25228–25232.
30. Gao J, Ding F, Liu Q, Yao Y. Knockdown of MACC1 expression suppressed hepatocellular carcinoma cell migration and invasion and inhibited expression of MMP2 and MMP9. Mol Cell Biochem. 2013;376(1–2):21–32.
31. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2(3):161–174.
32. Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev. 2006;25(1):9–34.
33. Daskalakis M, Nguyen TT, Nguyen C, et al. Demethylation of a novel tumor-suppressor gene detected in hepatocellular carcinoma detected by triple-combination array analysis. Int J Oncol. 2001;18(2):572–579.
34. Kantarjian H, Issa JP, Rosenfeld CS, et al. Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study. Cancer. 2006;106(8):1794–1803.