sequencing of *MICA* promoter region from 50 randomly selected HCV-HCC patients.
(TIF)

Table S1 Characteristics of samples and methods used in this study.
(DOCX)

Table S2 The sequences of each oligo used in the EMSA and ChIP assay.
(DOCX)

Table S3 Copy number variation between HCV-HCC and control samples.
(DOCX)

Author Contributions

Conceived and designed the experiments: PHYL YN KM. Performed the experiments: PHYL YU VK. Analyzed the data: PHYL YU CT. Contributed reagents/materials/analysis tools: KK NK DM KC MK. Wrote the paper: PHYL KM.

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MicroRNA-140 Acts as a Liver Tumor Suppressor by Controlling NF-κB Activity by Directly Targeting DNa Methyltransferase 1 (Dnmt1) Expression

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MicroRNAs (miRNAs) are small RNAs that regulate the expression of specific target genes. While deregulated miRNA expression levels have been detected in many tumors, whether miRNA functional impairment is also involved in carcinogenesis remains unknown. We investigated whether deregulation of miRNA machinery components and subsequent functional impairment of miRNAs are involved in hepatocarcinogenesis. Among miRNA-containing ribonucleoprotein complex components, reduced expression of DDX20 was frequently observed in human hepatocellular carcinomas, in which enhanced nuclear factor-κB (NF-κB) activity is believed to be closely linked to carcinogenesis. Because DDX20 normally suppresses NF-κB activity by preferentially regulating the function of the NF-κB-suppressing miRNA-140, we hypothesized that impairment of miRNA-140 function may be involved in hepatocarcinogenesis. DNA methyltransferase 1 (Dnmt1) was identified as a direct target of miRNA-140, and increased Dnmt1 expression in DDX20-deficient cells hypermethylated the promoters of metallothionein genes, resulting in decreased metallothionein expression leading to enhanced NF-κB activity. MiRNA-140-knockout mice were prone to hepatocarcinogenesis and had a phenotype similar to that of DDX20 deficiency, suggesting that miRNA-140 plays a central role in DDX20 deficiency-related pathogenesis. ***Conclusion*****: These results indicate that miRNA-140 acts as a liver tumor suppressor, and that impairment of miRNA-140 function due to a deficiency of DDX20, a miRNA machinery component, could lead to hepatocarcinogenesis.** (HEPATOLOGY 2013;57:162-170)

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide.[1] Although multiple major risk factors have been identified, such as infection with hepatitis viruses B or C, the molecular mechanisms underlying HCC development remain poorly understood, hindering the development of novel therapeutic approaches. Therefore, a better understanding of the molecular pathways involved in hepatocarcinogenesis is critical for the development of new therapeutic options.

Nuclear factor-κB (NF-κB) is one of the best-characterized intracellular signaling pathways. Its activation is a common feature of human HCC.[2-4] It acts as an inhibitor of apoptosis and as a tumor promoter[4,5] and is associated with the acquisition of a transformed phenotype during hepatocarcinogenesis.[6] In fact, studies using patient samples suggest that NF-κB activation in the liver leads to the development of HCC.[7] Although there are conflicting reports,[8] activation of the NF-κB pathway in the liver is crucial for the initiation and promotion of HCC.[4]

Abbreviations: DEN, diethylnitrosamine; Dnmt1, DNA methyltransferase 1; HCC, hepatocellular carcinoma; miRNA; microRNA; miRNP, miRNA-containing ribonucleoprotein; MT, metallothionein; NF-κB, nuclear factor-κB; RT-PCR, reverse-transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; UTR, untranslated region.

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MicroRNAs (miRNAs) are small RNA molecules that regulate the expression of target genes and are involved in various biological functions.[9-12] Although specific miRNAs can function as either suppressors or oncogenes in tumor development, a general reduction in miRNA expression is commonly observed in human cancers.[13-22] In this context, it can be hypothesized that deregulation of the machinery components involved in miRNA function may be related to the functional impairment of miRNAs and the pathogenesis of carcinogenesis.

In this study, we show that the expression of DDX20, an miRNA-containing ribonucleoprotein (miRNP) component, is frequently decreased in human HCC. Because DDX20 is required for both the preferential loading of miRNA-140 into the RNA-induced silencing complex and its function,[23] we hypothesized that DDX20 deficiency would lead to hepatocarcinogenesis via impaired miRNA-140 function. MiRNA-140 knockout mice were indeed more prone to hepatocarcinogenesis, and we identified a possible molecular pathway from DDX20 deficiency to liver cancer.

Materials and Methods

Mouse and Liver Tumor Induction. MiRNA-140$^{-/-}$ mice have been described.[24] Recombinant murine tumor necrosis factor-α (TNF-α) (25 μg/kg; Wako, Osaka, Japan) was injected into the tail vein, and the mice were sacrificed 1 hour later. To induce liver tumors, 15-day-old mice received an intraperitoneal injection of diethylnitrosamine (DEN) (25 mg/kg body weight), and were sacrificed 32 weeks later. All animal experiments were performed in compliance with the regulations of the Animal Use Committee of the University of Tokyo and the Institute for Adult Disease, Asahi Life Foundation.

Plasmids. FLAG-tagged human DDX20-expressing plasmids were as described.[23] The pGL3-based reporter plasmid containing Dnmt1 3′ untranslated region (UTR) sequences was provided by G. Marucucci.[25]

Detailed Materials and Methods. The detailed experimental procedures of clinical samples, cells, plasmids, reporter assays, reverse-transcription polymerase chain reaction (RT-PCR) analysis, antibodies, western blotting, cell assays, immunohistochemistry, microarray analysis, methylation analysis, and electrophoretic mobility-shift assay are described in the Supporting Information.

Statistical Analysis. Statistically significant differences between groups were determined using a Wilcoxon rank-sum test. A Wilcoxon signed-rank test was used for statistical comparisons of protein expression levels between HCC and surrounding noncancerous tissues.

Table 1. Cases with Differential Expression Levels of miRNP Components in HCC (n = 10)

Gene ID	Gene Symbol	Decreased	Increased	No Change
23405	Dicer1	2	1	7
27161	EIF2C2 (AGO2)	1	1	8
6895	TARBP2(TRBP2)	2	0	8
11218	DDX20 (GEMIN3)	8	0	2
50628	GEMIN4	1	0	9

The expression levels of each miRNP component were determined via immunohistochemistry.

The numbers indicate the number of cases that had the differential expression levels (decreased, increased, or no change) in HCC tissues compared with those in surrounding liver tissues.

Results

DDX20 Expression Is Frequently Decreased in HCC. The expression levels of proteins reported to be miRNP components (Dicer, Ago2, TRBP2, DDX20 [also known as Gemin3], and Gemin4)[26] were initially determined via immunohistochemistry in HCC and noncancerous background liver tissues from 10 patients. DDX20 expression was lower in HCC tissue compared with the surrounding noncancerous tissue in 8 of 10 cases, whereas expression of the other genes was unchanged (Table 1 and Supporting Fig. 1). Therefore, and because DDX20 was recently identified as a possible liver tumor suppressor in mice,[27] we determined its role as a human HCC suppressor.

DDX20 protein expression was lower in several HCC cell lines, such as Huh7 and Hep3B (Fig. 1A), compared with normal hepatocytes. DDX20 protein levels were also lower in human HCC needle biopsy specimens than in surrounding noncancerous liver tissue (Fig. 1B). Immunohistochemical analysis

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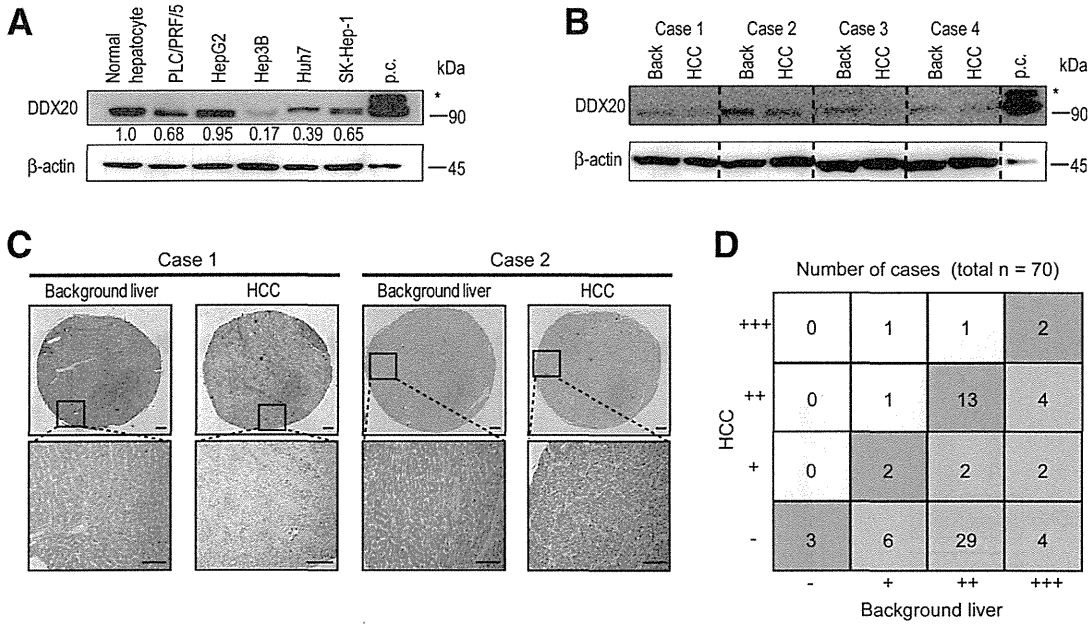

Fig. 1. Reduced DDX20 expression levels in hepatocellular carcinoma. (A) DDX20 protein expression in HCC cell lines. Numbers between the panels indicate DDX20 protein levels normalized to β-actin levels. Lysates of 293T cells transiently transfected with a FLAG-tagged DDX20-expressing plasmid yielded two DDX20 bands corresponding to the endogenous DDX20 protein and the transfected FLAG-tagged DDX20 protein (*) as a positive control (p.c.; far right lane). Data represent the results of three independent determinations. (B) DDX20 protein expression in four HCC needle biopsy specimens and in the surrounding noncancerous background liver tissue (Back). *Positive control. (C) Immunohistochemical analysis of DDX20 protein expression in HCC and surrounding tissues (background liver). Two representative cases are shown. Scale bars, 500 μm. The lower panels display magnified images of the boxed areas in the upper panels. (D) Grid summarizing DDX20 immunohistochemical staining data from 70 cases. In 47 cases (pink shading), DDX20 protein levels were lower in the HCC tissues than in the surrounding tissues ($P < 0.05$; Wilcoxon signed-rank test).

confirmed that DDX20 expression was frequently lower in HCC than in surrounding noncancerous liver tissue (Fig. 1C,D). Specifically, 47 of 70 cases examined showed reduced DDX20 protein expression in HCC versus background noncancerous liver tissue (Fig. 1D and Supporting Table 1). These results indicate that the expression of DDX20, an miRNP component, is frequently reduced in human HCC, and suggest that this reduced DDX20 expression might be involved in the pathogenesis of a subset of HCC cases.

NF-κB Activity Is Enhanced by DDX20 Deficiency. Because DDX20 knockout mice are embryonic-lethal,[28] DDX20 has been suggested to have important biological roles. DDX20, a DEAD-box protein,[29] was originally found to interact with survival motor neuron protein.[30] Later, it was identified as a major component of miRNPs,[31] which may mediate miRNA function. As we have reported, DDX20 is preferentially involved in miRNA-140-3p function,[23] acting as a suppressor of NF-κB activity in the liver.[32] DDX20-knockdown PLC/PRF/5 cells exhibit enhanced NF-κB activity[23] (Fig. 2A). Whereas the proliferation rates of DDX20-knockdown cells and control cells were comparable (Fig. 2B), apoptotic cell death after stimulation with TNF-related apoptosis-inducing ligand (TRAIL), which induces both cell apoptosis and NF-κB activation,[33] was significantly reduced in DDX20-knockdown cells (Fig. 2C). Similar results were obtained using DDX20-knockdown HepG2 cells (Supporting Fig. 2A-D). Conversely, NF-κB activity was reduced, but cell proliferation remained unchanged, in Hep3B cells stably overexpressing DDX20 (Fig. 2D,E). Sensitivity to TRAIL-induced apoptosis was restored in these cells (Fig. 2F). Similar results were also obtained using Huh7 cells (Supporting Fig. 2E-H). These data confirm a previous report that DDX20 deficiency enhances NF-κB activity and the downstream events of this pathway.

Metallothionein Expression Is Decreased by DDX20 Deficiency. Next, to investigate the biological consequences of DDX20 deficiency, we examined the changes in transcript levels in DDX20-knockdown cells using microarrays (GEO accession number: GSE28088). The expression of genes driven by NF-κB that are related to carcinogenesis, such as FASLG, IRAK1, CARD9, and Galectin-1, were enhanced significantly in DDX20-knockdown cells, as expected (Table 2). To determine the mechanism underlying the enhanced NF-κB activation in DDX20-deficient cells, we searched for candidate genes and noticed that the

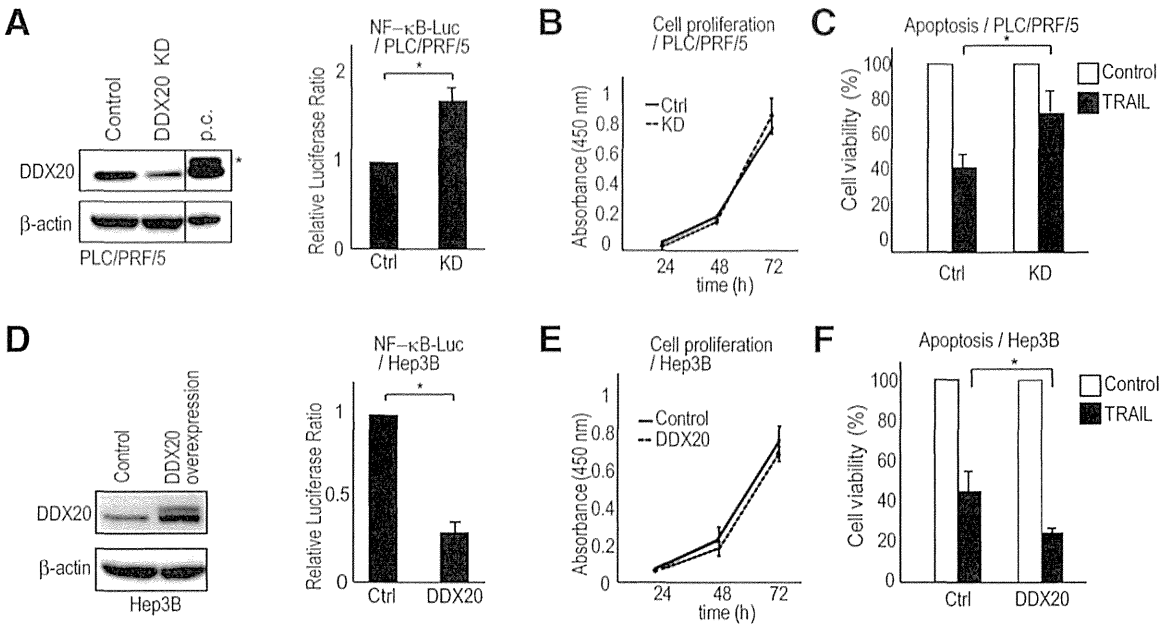

Fig. 2. Modulation of downstream events of the nuclear factor-κB pathway by DDX20. (A) Left: Establishment of stable DDX20-knockdown (DDX20 KD) PLC/PRF/5 cells. *Positive control (p.c.). Right: DDX20 deficiency enhances TNF-α–induced NF-κB activity. NF-κB reporter plasmids were transiently transfected into control (Ctrl) or DDX20-knockdown (KD) PLC/PRF/5 cells. The cells were then treated with TNF-α (5 ng/mL) or vehicle for 6 hours. $*P < 0.05$. Data are presented as the mean ± SD of three independent determinations. (B) Cell proliferation rates were comparable for control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells. Data are presented as the mean ± SD of three determinations. (C) DDX20 deficiency reduces TRAIL-induced apoptotic cell death. Control (Ctrl) and DDX20-knockdown (KD) PLC/PRF/5 cells were incubated with 25 ng/mL TRAIL. Data represent cell viability after TRAIL stimulation (gray bars) relative to the number of vehicle-treated cells (white bars). $*P < 0.05$. Data are presented as the mean ± SD of triplicate determinations. (D) Left: Establishment of stable DDX20-overexpressing cells. Hep3B cells were infected with control or FLAG-tagged DDX20-overexpressing lentiviruses and selected on puromycin. Western blot analysis confirmed increased expression of DDX20 protein. Right: DDX20 overexpression suppresses TNF-α–induced NF-κB activity. NF-κB reporter plasmids were transiently transfected into Hep3B control (Ctrl) and DDX20-overexpressing (DDX20) cells treated with TNF-α for 6 hours. Data are presented as the mean ± SD of three independent determinations. $*P < 0.05$. (E) Proliferation of control (Ctrl) and DDX20-overexpressing (DDX20) Hep3B cells was measured as described in (B). (F) DDX20 overexpression reduces TRAIL-induced apoptotic cell death. Data for control (Ctrl) and DDX20-overexpressing (DDX20) Hep3B cells are shown. $*P < 0.05$.

Table 2. Increased Expression of NF-κB–Related Genes in DDX20-Knockdown HepG2 Cells Compared with Wild-Type Cells

RefSeq ID	Symbol	Description	Ratio	Representative Gene Function
NM_000639	FASLG	Fas ligand	3.5	NF-κB target, apoptosis
NM_052813	C9orf151	CARD9	2.5	NF-κB cascade, NF-κB target
NM_014959	CARD8	Tumor up-regulated CARD-containing antagonist of CASP9 (TUCAN)	2.2	NF-κB target
NM_131917	FAF1	FAS-associated factor 1 (hFAF1)	1.9	Cytoplasmic sequestering of NF-κB, NF-κB target
NM_020644	TMEM9B	Transmembrane protein 9B precursor	1.9	Positive regulation of NF-κB transcription factor activity
NM_017544	NKRF	ITBA4 protein	1.9	Negative regulation of transcription
NM_006247	PPP5C	Protein phosphatase T	1.8	Positive regulation of NF-κB cascade
NM_020345	NKIRAS1	KappaB-Ras1	1.8	NF-κB cascade
NM_001569	IRAK1	IRAK-1	1.7	Positive regulation of NF-κB transcription factor activity
NM_177951	PPM1A	Protein phosphatase 1A	1.7	Positive regulation of NF-κB cascade
NM_018098	ECT2	Epithelial cell-transforming sequence 2 oncogene	1.6	Positive regulation of NF-κB cascade
NM_002305	LGALS1	Galectin-1 (putative MAPK-activating protein MP12)	1.6	Positive regulation of NF-κB cascade
NM_015093	TAB2	TAK1-binding protein 2	1.6	Positive regulation of NF-κB cascade
NM_004180	TANK	TRAF-interacting protein	1.5	NF-κB cascade
NM_014976	PDCD11	Programmed cell death protein 11	1.5	rRNA processing
NM_015336	ZDHHC17	Putative NF-κB–activating protein 205	1.5	Positive regulation of NF-κB cascade
NM_002503	NFKBIB	IKB-β	1.5	Cytoplasmic sequestering of NF-κB
NM_138330	ZNF675	Zinc finger protein 675	1.5	Negative regulation of NF-κB transcription factor activity

The genes were identified as NF-κB–related based on the Gene Ontology and the GeneCodis Databases.

Table 3. Decreased Expression Levels of MT Genes in DDX20 Knockdown HepG2 Cells Compared with Wild-Type Cells

Symbol	Description	Ratio
MT1E	Metallothionein-1E	**0.12**
MT1F	Metallothionein-1F	**0.36**
MT1H	Metallothionein-1H	**0.16**
MT1G	Metallothionein-1G	**0.06**
MT1M	Metallothionein-1M	**0.24**
MT1X	Metallothionein-1X	**0.27**
MT2A	Metallothionein-2	**0.28**
MT3	Metallothionein-3	0.84
MTL5	Metallothionein-like 5 (Tesmin)	1.12

Numbers in boldface type indicate values <0.5.

expression levels of a group of metallothioneins (MTs), such as MT1E, MT1F, MT1G, MT1M, MT1X, and MT2A, were all significantly decreased when DDX20 was deficient (Table 3). The decreased expression of MTs in DDX20-knockdown HepG2 and PLC/PRF/5 cells was confirmed via quantitative RT-PCR (Fig. 3a and Supporting Fig. 3). Expression of MT-3, which was not altered in the microarray analysis, was similarly unaltered in quantitative RT-PCR analysis. Notably, it was already known that MTs are frequently silenced in human primary liver cancers.[34-36] In addition, MT knockout mice have enhanced NF-κB activity, likely due to reactive oxygen species, and these mice are more prone to hepatocarcinogenesis.[37] These results suggest that DDX20 deficiency enhances NF-κB activity by decreasing the expression of MTs, which could facilitate the development of liver cancer.

MiRNA-140 Directly Targets Dnmt1. Because MT expression is regulated principally by CpG island methylation in their promoter regions,[38,39] we examined the quantitative methylation status of MT promoters in DDX20-knockdown cells. The CpG islands of the MT1E, MT1G, MT1M, MT1X, and MT2A promoters, and the CpG shores of the MT1F promoters, were significantly more highly methylated under DDX20-deficient conditions, as determined by the comprehensive Illumina Quantitative Methylation BeadChip method (Table 4, Supporting Table 2, and GSE 37633). A crucial step in DNA methylation involves DNA methyltransferase (Dnmt), which catalyzes the methylation of CpG dinucleotides in genomic DNA.[40] The methylation status of MT promoters is mediated specifically by Dnmt1.[41] Because Dnmt1 contains a predicted miRNA-140-3p target site in its 3′ UTR, with a perfect match to its seed sequences (Fig. 3B), and because the effects of miRNA-140-3p activity were impaired in DDX20-knockdown cells,[23] it was hypothesized that whereas miRNA-140 normally targets and suppresses Dnmt1 protein expression, miRNA-140-3p dysfunction due to DDX20 deficiency results in enhanced Dnmt1 expression, leading to hypermethylation of MT promoters. Consistent with this hypothesis, Dnmt1 expression was increased significantly in DDX20-knockdown cells (Fig. 3C). miRNA-140 precursor overexpression suppressed activity of the Dnmt1 3′ UTR reporter construct, the effect of which was lost when two mutations were introduced into its seed sequences (Fig. 3D). MiRNA-140 precursor overexpression suppressed Dnmt1 protein expression (Fig. 3E). These results indicate that miRNA-140 directly targets Dnmt1 and suppresses its expression in the normal state. Consistently, decreased DDX20, increased Dnmt1, and decreased MT expression were detected together in human clinical HCC samples, as determined via immunohistochemistry (Fig. 3F). By contrast, miRNA-140 precursor-overexpressing Huh7 cells showed increased expression of MTs and reduced NF-κB activity *in vitro* (Supporting Fig. 4A,B). Moreover, the increase in the number of spheres formed from PLC/PRF/5 cells due to DDX20 knockdown was antagonized by treatment with an NF-κB inhibitor or a demethylating agent (Supporting Fig. 5). Taken together, these results suggest that the up-regulated Dnmt1 protein expression caused by functional impairment of miRNA-140-3p due to DDX20 deficiency results in decreased expression of MTs *via* enhanced methylation at the CpG sites in their promoters. This may lead to enhanced NF-κB activity and cellular transformation at least *in vitro*.

MiRNA-140 Is a Liver Tumor Suppressor. To further examine the biological consequences of functional impairment of miRNA-140 due to DDX20 deficiency, we determined the phenotypes of miRNA-140 knockout (miRNA-140$^{-/-}$) mice (Fig. 4A). Similar to the *in vitro* DDX20 knockdown results, Dnmt1 expression was increased and MT levels decreased in the liver tissue of these mice (Fig. 4B). NF-κB–DNA binding activity was enhanced in the livers of miRNA-140$^{-/-}$ mice after tail-vein injection of TNF-α, a crucial cytokine that induces NF-κB activity and hepatocarcinogenesis (Fig. 4C). As was found in MT knockout mice, phosphorylation of p65 at serine 276, which is critical for p65 activation, was significantly increased in the livers of miRNA-140$^{-/-}$ mice after DEN exposure, which induces NF-κB activation and liver tumors[37] (Fig. 4D). Notably, the size and number of liver tumors that developed 8 months after DEN exposure were markedly elevated in miRNA-140$^{-/-}$ mice compared with control mice (Fig. 4E,F). These results indicate that miRNA-140$^{-/-}$ mice are indeed

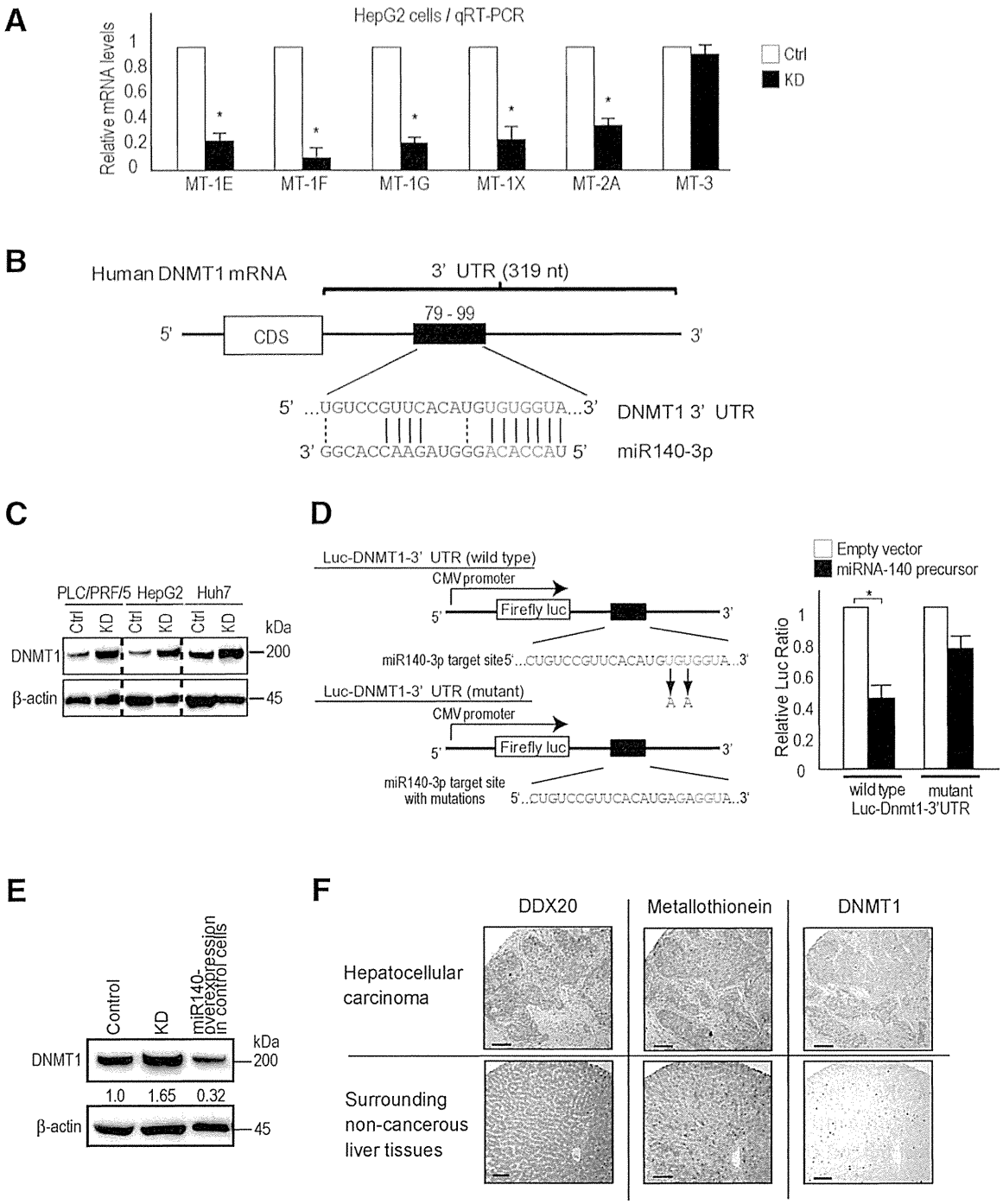

Fig. 3. Targeting of Dnmt1 by miRNA-140-3p and reduced MT expression. (A) The expression levels of MTs were determined using quantitative reverse-transcriptase polymerase chain reaction. The relative expression ratios of the MTs in control (white bars) and DDX20-knockdown (black bars) HepG2 cells were calculated by normalizing control cell values to 1.0. The data represent the mean ± SD of three independent determinations. $*P < 0.05$. (B) Putative miRNA-140-3p target sites in the 3′ UTR of human Dnmt1. Seed sequences are indicated in red. (C) Dnmt1 expression was increased in DDX20-knockdown cells. Ctrl, control cells; KD, DDX20-knockdown cells. (D) Left: Schematic diagrams of wild-type (upper) and mutant (lower) luciferase reporter constructs (Luc-Dnmt1-3′ UTRs) carrying the Dnmt1 3′ UTR region harboring the putative miRNA-140-3p target site. The mutant seed sequence contained two nucleotide substitutions. Right: The Dnmt1 3′ UTR is targeted directly by miRNA-140-3p. Cells were cotransfected with Luc-Dnmt1-3′ UTR (wild-type or mutant) plus either an empty vector (white bars) or a plasmid expressing the miRNA-140 precursor (black bars). Data are the mean ± SD of three independent determinations. (E) Overexpression of miRNA-140 reduces Dnmt1 expression in control cells. Values between the panels indicate Dnmt1 protein levels normalized to those of β-actin. KD, DDX20 knockdown cells. (F) Representative histochemical images showing expression of DDX20, Dnmt1, and MT proteins in HCC (upper three panels) and surrounding tissue (lower panels). Compared with adjacent noncancerous liver tissue, HCCs exhibited decreased DDX20 and MT expression and increased Dnmt1 expression. Note that adjacent sections were stained for each protein. Scale bar, 50 μm.

Table 4. Methylation Levels in CpG Islands of the MT Genes in DDX20-Knockdown HepG2 Cells Compared with Control Cells

Symbol	CpG Island Methylation Ratio	Target ID
MT1E	**1.14**	cg00178359
	1.29	cg06463589
	3.65	cg02512505
	1.02	cg15134649
MT1G	**2.14**	cg16452857
	1.03	cg27367960
	1.00	cg03566142
	0.99	cg07791866
MT1M	**1.16**	cg02132560
	0.98	cg02160530
	1.03	cg04994964
MT1X	**1.24**	cg05596720
	1.05	cg26802333
	1.06	cg09147880
	1.01	cg08872713
MT2A	**2.06**	cg07395075
	0.94	cg20430434

Values were determined using the quantitative Illumina Human Methylation BeadsChip. Boldface values indicate increased methylation levels in DDX20 knockdown cells.

more prone to liver cancer development and suggest that miRNA-140 acts as a liver tumor suppressor, probably by suppressing NF-κB activity, although we cannot completely exclude other molecular mechanisms. Nonetheless, these results also suggest that the impairment of miRNA-140 function due to DDX20 deficiency may lead to hepatocarcinogenesis in humans, as we have observed in miRNA-140$^{-/-}$ mice (Supporting Figs. 6 and 7).

Discussion

Here, we report that miRNA-140$^{-/-}$ mice have increased NF-κB activity and are more prone to HCC development. In addition, we show that DDX20, an miRNP component, is frequently decreased in human HCC tissues. Because DDX20 deficiency preferentially causes impaired miRNA-140 function,[23] the functional impairment of miRNA-140 may result in phenotypes similar to those of miRNA-140$^{-/-}$ mice and may lead to hepatocarcinogenesis. In support of the hypothesis that DDX20 dysfunction is involved in hepatocarcinogenesis, DDX20 is located at 1p21.1-p13.2, a frequently deleted chromosomal region in human HCC,[27] and DDX20 was recently identified as a possible liver tumor suppressor in a functional screen in mice.[27] Although the possibility that intracellular signaling pathways other than miRNA-140 may also be involved in the biological consequences of DDX20 deficiency cannot be denied, we believe that functional impairment of miRNA-140 plays a major role in the phenotypes induced by DDX20 deficiency, based on the phenotypic similarities.

Changes in miRNA expression levels have been reported in various tumors.[7,12,42] However, in this study, we found that reduced expression of an miRNA machinery component might lead to carcinogenesis, at least in part, through functional impairment of miRNAs. Recent studies have shown that components of the RNA interference machinery are associated with the outcome of ovarian cancer patients,[43] and that single-nucleotide polymorphisms in miRNA machinery genes can be used as diagnostic risk markers.[44,45] Therefore, the impairment of miRNA function caused by deregulated miRNA machinery components may also be involved in carcinogenesis.

Our study identified Dnmt1 as a critical target of miRNA-140. The decreased MT expression due to the CpG promoter methylation induced by Dnmt1 resulted in enhanced NF-κB activity. This finding was consistent with the results obtained using MT gene knockout mice, in which enhanced NF-κB activation promoted hepatocarcinogenesis.[37] The decrease in MT expression that results from increased Dnmt1 expression caused by functional impairment of miRNA-140, together with increased NF-κB activation and hepatocarcinogenesis in MT knockout mice,[37] supports the concept that the DDX20/miRNA-140/Dnmt1/MT/NF-κB pathway may play a crucial role in hepatocarcinogenesis. However, we cannot fully exclude the possibility that other intracellular signaling pathways are also involved in the induction of hepatocarcinogenesis by miRNA-140 or DDX20 deficiency, because the precise role of NF-κB in hepatocarcinogenesis has not been clearly defined,[8] although constitutive activation of NF-κB signaling has been frequently detected in human HCCs.[46] The mechanisms by which DDX20 expression is initially decreased and the reason its locus is frequently deleted in HCC remain to be elucidated. However, because DDX20 expression is also regulated by methylation of its CpG promoter,[47] once this pathway is deregulated, decreased DDX20 expression could be maintained by a positive feedback mechanism, even without deletion of its locus.[27]

In conclusion, this study shows that miRNA-140 acts as a liver tumor suppressor. We show that DDX20, an miRNP component, is frequently decreased in human HCC, which may induce hepatocarcinogenesis via impairment of miRNA-140 function. These results suggest the importance of investigations of not only aberrant miRNA expression levels,[12,14,17,48] but also deregulation of miRNP

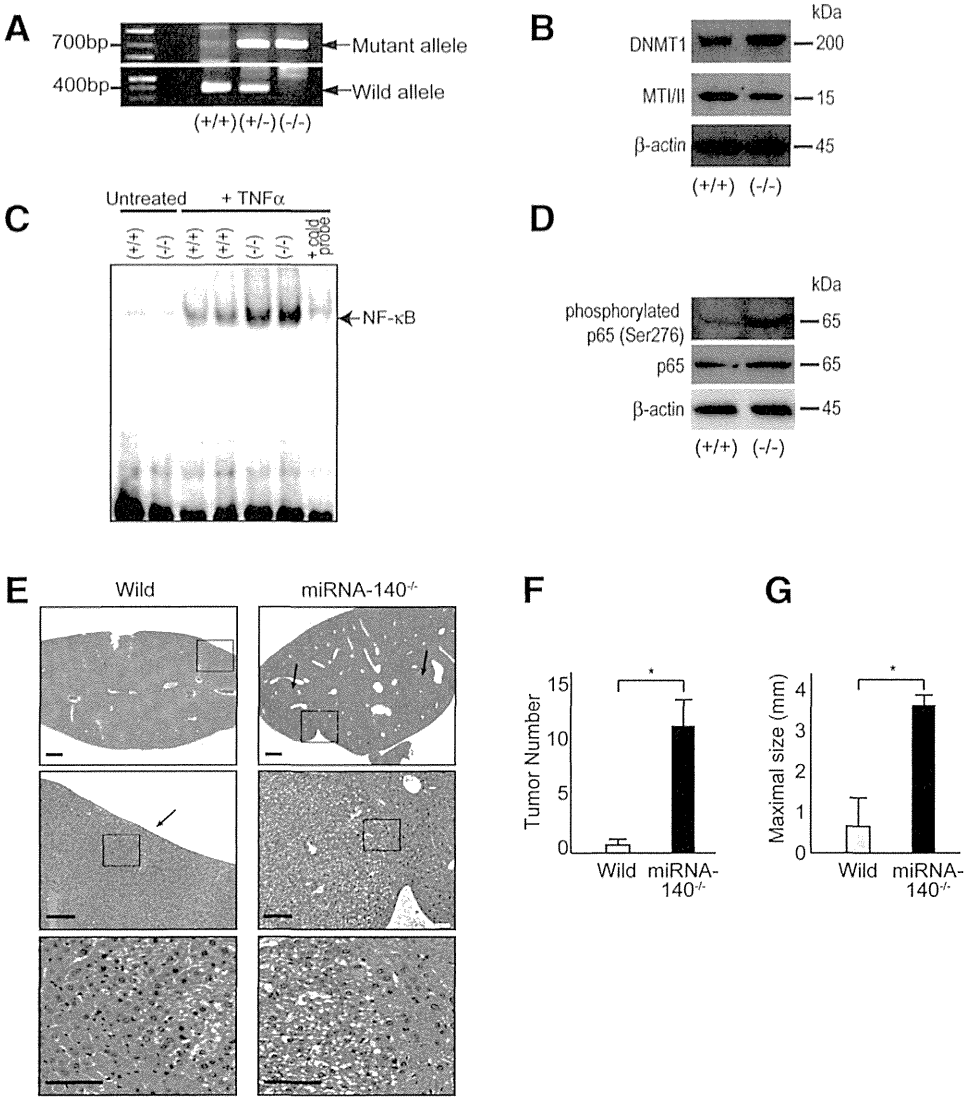

Fig. 4. miRNA-140$^{-/-}$ mice are prone to hepatocarcinogenesis. (A) Representative genotyping of mice with wild-type or mutant alleles. PCR genotyping was performed for miRNA-140 wild-type (419 bp; Wild) and knockout (734 bp; Mutant) alleles. (+/+), wild-type; (+/−), heterozygous; (−/−), knockout. (B) Increased Dnmt1 expression and decreased MTI/II expression in the liver tissues of miRNA-140$^{-/-}$ mice compared with wild-type mice. Western blotting was performed using antibodies against the indicated proteins. (+/+), wild-type; (−/−), miRNA-140$^{-/-}$. The image shown is representative of four independent experiments. (C) NF-κB-DNA binding was assessed via gel-shift assay using equal amounts of liver nuclear extracts from untreated and TNF-α-injected wild-type and miRNA-140$^{-/-}$ mice. (+/+), wild-type; (−/−), miRNA-140$^{-/-}$. Cold probe was added to TNF-α-injected knockout mouse nuclear extract to test assay specificity. A result representative of four independent experiments is shown. (D) Western blotting for phosphorylated p65 expression in the liver at 32 weeks after DEN treatment in miRNA-140$^{-/-}$ mice compared with wild-type mice. A result representative of four independent experiments is shown. (E) Representative histological images of mouse liver at 32 weeks after DEN treatment. Arrows indicate tumors. Higher-magnification images of the highlighted areas in the upper panels are shown in the lower panels. Scale bar, 500 μm. (F) The number (left panel) and size (right panel) of tumors (five random sections per mouse treated with DEN) are presented as the mean ± SD (wild-type mice, n = 8; miRNA-140$^{-/-}$ mice, n = 8). *$P < 0.05$.

components,[22] with subsequent impairment of miRNA function as molecular pathways and possible therapeutic targets for carcinogenesis and other diseases.

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CLINICAL STUDIES

Percutaneous ethanol injection for hepatocellular carcinoma: 20-year outcome and prognostic factors

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Keywords
ablation – hepatocellular carcinoma – percutaneous ethanol injection – prognostic factor – recurrence – survival – treatment outcome

Abbreviations
AFP-L3, lectin-reactive AFP; AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CI, confidence intervals; CT, computed tomography; DCP, des-γ-carboxy-prothrombin; HBs-Ag, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

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Abstract

Background: Ethanol injection is the best-known image-guided percutaneous ablation for hepatocellular carcinoma (HCC) and a well-tolerated, inexpensive procedure with few adverse effects. However, there have been few reports on its long-term results. *Aims:* We report a 20-year consecutive case series at a tertiary referral centre. *Methods:* We performed 2147 ethanol injection treatments on 685 primary HCC patients and analysed a collected database. *Results:* Final computed tomography demonstrated complete ablation of treated tumours in 2108 (98.2%) of the 2147 treatments. With a median follow-up of 51.6 months, 5-, 10- and 20-year survival rates were 49.0% [95% confidence interval (CI) = 45.3–53.0%], 17.9% (95% CI = 15.0–21.2%) and 7.2% (95% CI = 4..5–11.5%) respectively. Multivariate analysis demonstrated that age, Child–Pugh class, tumour size, tumour number and serum alpha-fetoprotein level were significant prognostic factors for survival. Five-, 10- and 20-year local tumour progression rates were 18.2% (95% CI = 15.0–21.4%), 18.4% (95% CI = 15.2–21.6%) and 18.4% (95% CI = 15.2–21.6%) respectively. Five-, 10- and 20-year distant recurrence rates were 53.5% (95% CI = 49.4–57.7%), 60.4 (95% CI = 56.3–64.5%) and 60.8% (95% CI = 56.7–64.9%) respectively. There were 45 complications (2.1%) and two deaths (0.09%). *Conclusions:* Ethanol injection was potentially curative for HCC, resulting in survival for more than 20 years. This study suggests that new ablation therapies will achieve similar or even better long-term results in HCC.

Hepatocellular carcinoma (HCC) is the fifth most common malignant neoplasm in the world. Only 20% of HCC patients are candidates for resection (1). Furthermore, recurrence is frequent even after curative resection. Liver transplantation is restricted by donor shortage. Thus, various non-surgical therapies have been introduced (2). Among these, image-guided percutaneous ablation is considered best for early-stage HCC.

The most studied percutaneous ablation is ethanol injection. Ethanol injection is a well-tolerated, inexpensive procedure with few adverse effects and has been considered the standard against which any new ablation therapy should be compared (2). Although ethanol injection was introduced into clinical practice in the 1980s (3, 4), few reports of its long-term results have been published (5–8). We report here a 20-year consecutive case series at a tertiary referral centre. This study documents the largest number of ethanol injection treatments at a single institution. Findings in this 20-year experience may be extrapolated to other ablation therapies, such as radiofrequency ablation, in which such long-term outcomes are not yet available (9).

Patients and methods

Indications for ethanol injection

Ethanol injection was performed in patients satisfying the following criteria: (i) ineligible for resection or transplantation, or had refused surgery; (ii) no extrahepatic metastasis or vascular invasion. Exclusion criteria were as follows: (i) tumour was not visualized

by ultrasonography or not accessible percutaneously; (ii) total bilirubin level ≥ 3.0 mg/dl; (iii) platelet count $<40 \times 10^9$/L; (iv) prothrombin activity <35%; (v) refractory ascites. In general, we performed ethanol injection on patients with Child–Pugh class A or B, with 3 or fewer tumours ≤ 3 cm in diameter. We performed ethanol injection on patients beyond these conditions, however, who were likely to benefit from the procedure for possible cure or prolongation of life. No patients were excluded solely because of tumour location (10). Informed consent was obtained from each patient. This study was conducted according with the Helsinki Declaration of 1975 and approved by the Institutional Review Board.

Patients

In this cohort study, we analysed a prospectively collected computerized database. Between 1985 and 2005, 2735 HCC patients were admitted to the Department of Gastroenterology, University of Tokyo (Fig. 1). At initial hospitalization, 1615 had primary HCC and the remaining 1120 had recurrent HCC. The recurrent HCC patients had undergone therapies other than ethanol injection for primary HCC.

Of the 1615 patients with primary HCC, 1459 (90.3%) underwent percutaneous ablation as the initial treatment, including ethanol injection. The remaining 156 patients received other therapies: transarterial chemoembolization for 123 patients with multinodular or large tumours that could not be treated by ablation therapies; hepatic resection for 18 with good liver function who consented to an operation; chemotherapy for four with vascular invasion or extrahepatic metastasis; and best supportive care for 11 with decompensated cirrhosis or poor general condition.

Of the 1459 patients treated by percutaneous ablation, 685 underwent ethanol injection, 122 underwent microwave ablation, and the remaining 652 radiofrequency ablation. The type of percutaneous ablation performed varied with the date of treatment. We started ethanol injection in December 1985, microwave ablation in October 1995 and radiofrequency ablation in February 1999 (11). Between October 1995 and February 1999, both ethanol injection and microwave ablation were performed. Microwave ablation was chosen for patients who had better liver function and whose tumour was located in a position where the electrode could be inserted and held safely. Since February 1999, both ethanol injection and radiofrequency ablation have been performed. Between April 1999 and January 2001, 232 patients with three or fewer tumours, each ≤ 3 cm in diameter, and Child–Pugh class A or B were entered into a randomized controlled trial (12). Patients outside these inclusion criteria were mostly treated by radiofrequency ablation. After this trial, radiofrequency

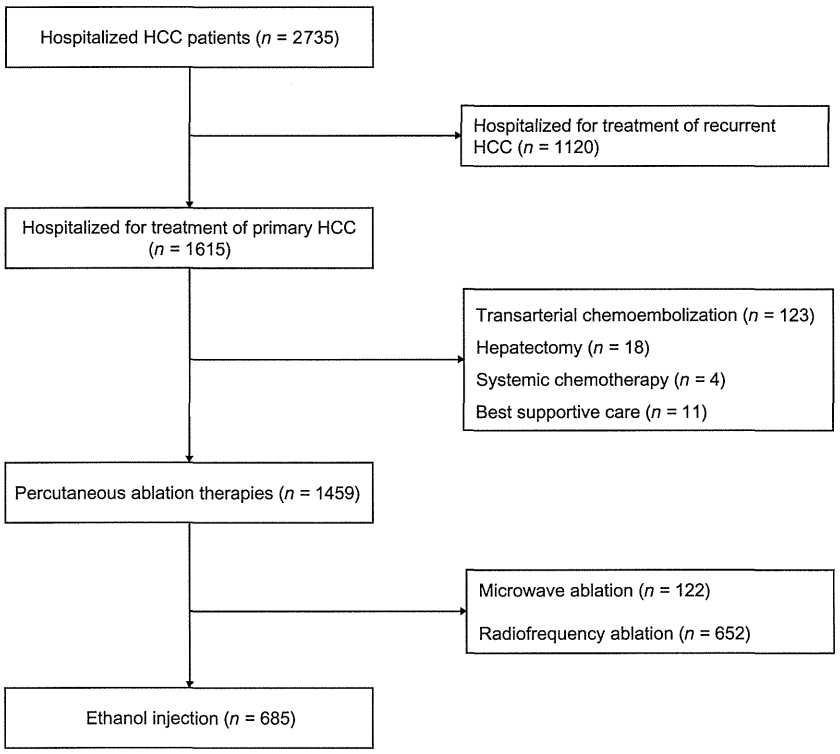

Fig. 1. Flow of patients in this study. HCC, hepatocellular carcinoma

ablation was generally the treatment of choice, and ethanol injection was used only in those unsuitable for radiofrequency ablation: those with either enterobiliary reflux or tumour adhesion to the gastrointestinal tract.

Hepatocellular carcinoma was diagnosed based on typical imaging findings of early phase enhancement and late phase contrast washout on computed tomography (CT) (13). HCC diagnosis was also confirmed by biopsy in 630 (92.0%) of the 685 patients with primary HCC treated by ethanol injection. A total of 587 (85.7%) were diagnosed as having cirrhosis.

In general, chemoembolization was combined with ethanol injection in patients with either ≥ 4 tumours or those with two or three tumours at least one of which is >3.0 cm in diameter. The combination of chemoembolization with ethanol injection was performed in 186 patients.

Treatment methods

Preoperative planning including ultrasound examination and evaluation of all imaging findings was performed to identify the tumours and to determine the access route. The procedure was performed according to an institutional protocol and under the supervision of experienced physicians who had performed this treatment more than 200 times. The precise techniques of ethanol injection are described elsewhere (12). Briefly, all procedures were performed percutaneously under ultrasound guidance. Artificial pleural effusion or artificial ascites method is much less frequently used in ethanol injection compared with radiofrequency ablation, because the procedure is necessary to be repeated several times. Since 1990, we have used two or three needles to inject ethanol into several sites in one procedure (12). Ethanol injection was performed twice per week. The procedure was repeated until ethanol appeared to have been injected throughout the tumour. To judge a timing to stop repetition of injecting ethanol and to order a CT scan, we considered total volume of injected ethanol and change of echogenicity. The general guideline for the necessary volume of injected ethanol was calculated according to the following numerical expression, $V = (4/3)\ \pi\ (r + 0.5)^3$, where V (in millilitres) is the volume of ethanol and r (in centimetres) is the radius of the tumour; 0.5 is added to provide a safety margin, which is based on the concept that some surrounding liver parenchyma all around the tumour as well as the tumour itself must be ablated (5).

A CT scan was then performed 1–3 days after the procedure to evaluate technique effectiveness (14). Complete ablation was defined as hypoattenuation of the entire tumour. When the presence of unablated tumour portions was suspected, a few more procedures were performed. We did not predefine the number of procedures in a treatment. The ethanol injection treatment was generally continued until CT demonstrated the entire tumour necrosis.

Follow-up

Follow-up investigations consisted of CT, ultrasonography and measurement of serum α-fetoprotein (AFP), des-γ-carboxy-prothrombin (DCP) (since April 1993) levels and lectin-reactive AFP (AFP-L3) (since July 1997) every 4 months. Local tumour progression was defined as appearance of viable tumour touching the original tumour (14) and distant recurrence as emergence of tumour(s) separate from the primary site. Ethanol injection was used for recurrence if the patient still met the indication criteria. If multiple recurrences were not treatable with ethanol injection, chemoembolization was generally performed.

Statistical analyses

This study is a report of a consecutive case series. All ethanol injection treatments performed on primary HCC patients at the Department of Gastroenterology, University of Tokyo between 1985 and 2005 were included. Data are presented as mean ± SD for quantitative variables, and as absolute frequencies for qualitative variables.

A 'procedure' was defined as a single intervention episode that consisted of one or more ablations performed on tumours, and a 'treatment' as the completed effort to ablate tumours. A treatment consisted of several procedures (14). 'Technique effectiveness' rate was defined as the percentage of successfully eradicated macroscopic tumours as evidenced at CT scan after the last procedure (14). In cases in which there was Lipiodol deposit inside the tumour because of the combination of chemoembolization with ethanol injection, we judged that the tumour had been successfully eradicated if it was surrounded with completely non-enhanced tissue in final CT.

Overall survival was calculated in the 685 primary HCC patients. Survival curves were generated using the Kaplan–Meier method. In addition to overall survival, subgroup analyses were performed with clinical characteristics including tumour size, tumour number and Child–Pugh class. Recurrence was evaluated in 591 patients in whom ethanol injection was performed with curative intent. All tumours were treated by ethanol injection in those patients. The remaining 94 patients were excluded from the recurrence analysis because some small tumours had been left untreated by ethanol injection on account of detection failure by ultrasonography. Recurrence rates were calculated using the Gaynor method (15). All time estimates were made from the date of the first ethanol injection. The follow-up was finalized at either death or the last visit to the outpatient clinic before December 31 2010. Transplanted patients were censored from this study at the date of transplantation.

The prognostic relevance of baseline variables (Table 1), the combination of chemoembolization,

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Table 1. Baseline characteristics of the 685 Patients undergoing percutaneous ethanol injection for primary hepatocellular carcinoma

Variable	
Age (years)	64.0 ± 8.9
Males, *n* (%)	502 (73.3)
Viral infection*	
HBs-Ag positive, *n/N* (%)	64/685 (9.3)
Anti-HCV positive, *n/N* (%)	570/673 (84.7)
Both positive, *n/N* (%)	11/673 (1.6)
Both negative, *n/N* (%)	52/673 (7.7)
Alcohol consumption >80 g/day, *n* (%)	143 (20.9)
Ascites, *n* (%)	122 (17.9)
Encephalopathy, *n* (%)	44 (6.5)
Albumin (g/dl)	3.55 ± 0.50
Total bilirubin (mg/dl)	0.96 ± 0.536
Prothrombin time (%)	71.6 ± 15.9
Platelet count ($\times 10^4/mm^3$)	10.3 ± 4.6
AST (IU/L)	80.6 ± 48.2
ALT (IU/L)	79.2 ± 61.9
Child–Pugh class, *n* (%)	
A	425 (62.1)
B	228 (33.3)
C	32 (4.6)
Tumour size (cm)	2.83 ± 1.47
Tumour number	2.0 ± 1.7
Serum AFP (ng/ml), *n* (%)	
≤100	525 (76.6)
101–400	95 (13.9)
>400	65 (9.5)
Serum DCP (mA U/ml), *n* (%)†	
≤100	428 (82.8)
101–400	49 (9.5)
>400	40 (7.7)
Serum AFP-L3 (%), *n* (%)‡	
≤15	193 (86.2)
15.1–40	16 (7.1)
>40	15 (6.7)

*Anti-HCV was not tested in 12 patients.

†Serum DCP level was not measured in 168 patients.

‡Serum AFP-L3 level was not measured in 461 patients.

HBs-Ag, hepatitis B surface antigen; HCV, hepatitis C virus; AFP, α-fetoprotein; DCP, des-gamma-carboxy-prothrombin; AFP-L3, lectin-reactive α-fetoprotein.

Data are expressed as mean ± standard deviation.

HCC recurrence and the number of ethanol injection sessions to survival was analysed by univariate and multivariate models. The prognostic relevance of baseline variables (Table 1), the combination of chemoembolization and the number of ethanol injection sessions to local tumour progression and distant recurrence was also analysed by univariate and multivariate models. In multivariate analysis, we evaluated models including Child–Pugh class and excluding its components to avoid multicollinearity. Serum DCP and AFP-L3 levels were excluded from the multivariate model because of absence of data from 168 and 461 patients respectively. Some continuous variables in which log-linearity could not be assumed were transformed into categorical variables. Variables with a P value <0.05 determined by univariate comparison were subjected to multivariate analysis. A stepwise variable selection was performed with Akaike Information Criteria in multivariate analysis. Results were expressed as hazard ratios with corresponding 95% confidence intervals (CI), with P values from the Wald test. All significance tests were two-tailed, and differences with a P value <0.05 were considered statistically significant.

Complications were defined according to the guidelines of the Society of Interventional Radiology (16).

Results

Antitumour effect

We performed 2147 ethanol injection treatments, comprising 13 526 procedures. Thus, procedure number per treatment was 6.3 ± 2.6. The total volume of injected ethanol per treatment was 40.9 ± 16.3 ml. Many patients received iterative ethanol injection treatments for recurrence. A total of 108 patients underwent ethanol injection treatment once, 118 patients twice, 196 patients 3 times, 153 patients 4 times, 71 patients 5 times, 28 patients 6 times, 8 patients 7 times and 3 patients 8 times.

Technique effectiveness rate was 98.2% (2108/2147 treatments). It was similar between the initial ethanol injection treatments and the other ethanol injection treatments for recurrence ($P = 0.397$). Complete ablation of the tumour was achieved in 675 (98.5%) of the 685 initial treatments and in 1433 (98.0%) of the 1462 other treatments. However, technique effectiveness rate significantly differed with tumour size ($P = 0.002$). No apparent viable portions remained in 758 (99.0%) of 766 treatments for tumours ≤2.0 cm in diameter, in 704 (98.4%) of 717 treatments for tumours 2.1–3.0 cm, in 570 (97.9%) of 582 treatments for tumours 3.1–5.0 cm and in 76 (92.7%) of 82 treatments for tumours >5.0 cm.

Survival

Table 1 shows clinical characteristics of the 685 patients. A total of 136 patients (19.9%) were older than 75 years. In all, 180 patients had tumours ≤2.0 cm in diameter, 274 had tumours 2.1–3.0 cm, 192 had tumours 3.1–5.0 cm and 39 had tumours >5.0 cm. A total of 367 patients had one tumour, 238 patients had 2 or 3 tumours and 80 had 4 or more tumours.

As of December 2010 (with a median follow-up of 51.6 months), 70 patients (10.2%) remained alive, 52 (7.6%) were lost to follow-up and 563 (82.2%) had died. Of the 685 patients, two were transplanted. The number of patients who survived longer than 5, 10 and 20 years after the first ethanol injection treatment was 305, 97 and 3 respectively. The cause of death was HCC

in 297 patients (52.8%), liver failure in 129 (22.9%), upper gastrointestinal bleeding in 30 (5.3%), complications related to the procedure in 2 (0.4%), liver-unrelated diseases in 84 (14.9%) and undetermined in 21 (3.7%).

The 1-, 3-, 5-, 10-, 15- and 20-year survival rates of all 685 patients were 91.0% (95% CI = 88.9–93.2%), 67.6% (95% CI = 64.1–71.3%), 49.0% (95% CI = 45.3–53.0%), 17.9% (95% CI = 15.0–21.2%), 8.6% (95% CI = 6.4–11.7%) and 7.2% (95% CI = 4.5–11.5%) respectively (Fig. 2; Table 2). Survival rates significantly differed with tumour number ($P = 0.0001$), tumour size ($P = 0.0001$) and Child–Pugh class ($P = 0.0001$). In patients with 1–3 tumours, all ≤ 3 cm, and in Child–Pugh class A or B, the 5-year survival rate was 59.5% (95% CI: 54.7–64.7%).

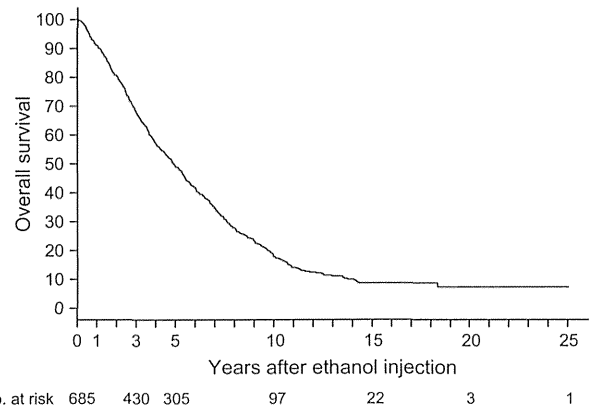

Fig. 2. Overall survival in 685 primary hepatocellular carcinoma patients who underwent ethanol injection.

Univariate analysis indicated that 13 of the 22 variables were relevant to survival. In multivariate analysis, a model that contained age, antibody to hepatitis C virus (anti-HCV), Child–Pugh class, tumour size, tumour number and serum AFP level was selected (Table 3).

Survival rates significantly differed with the time period in which the first ethanol injection was performed ($P < 0.0001$; Fig. 3). In 109 patients who underwent ethanol injection between 1985 and 1991, the 5-year survival rate was 30.3% (95% CI = 22.7–40.5%), whereas it was 51.2% (95% CI = 46.8–55.9%) in 476 patients between 1992 and 1998, and 61.1% (95% CI = 51.3–72.8%) in 100 patients between 1999 and 2005.

Recurrence

Recurrence developed in 449 patients. Local tumour progression alone was found in 61 patients, local tumour progression with distant recurrence in 44 and distant recurrence alone in 344. Of these 344 patients, eight had recurrence in extrahepatic sites: five had lymph node metastasis, one had lung metastasis, one had bone metastasis and the remainder had both lymph node and lung metastasis. Of the 449 patients, the first recurrence was treated by iterative ethanol injection in

Table 2. Survival of patients undergoing ethanol injection, based on tumour number, tumour size and Child–Pugh class

Grading	*n*	Survival (%) 3-Year	5-Year	10-Year	15-Year	20-Yar	Median (years)	*P* value
Overall survival	685	67.6	49.0	17.9	8.6	7.2	4.9	–
Tumour number								
Solitary	367	72.0	56.5	24.6	12.1	9.7	5.8	0.0001
2–3	232	71.5	46.3	12.9	5.9	–	4.7	
≥4	86	37.6	23.8	2.5	1.3	–	2.6	
Tumour size								
≤2.0 cm	240	83.6	63.8	27.6	12.3	6.1	6.9	0.0001
2.1–3.0 cm	221	68.0	47.9	15.0	10.7	10.7	4.8	
>3.0 cm	224	50.2	34.4	10.1	3.5	3.5	3.1	
Child–Pugh class								
A	425	77.3	58.7	24.4	12.5	10.4	6.2	0.0001
B	228	53.9	35.5	8.1	3.0	–	3.5	
C	32	37.5	18.8	3.1	–	–	1.9	
Combination of tumour number, tumour size, and Child–Pugh class								
Solitary, ≤3 cm	275	77.5	62.2	28.8	14.5	10.8	6.8	–
Solitary, ≤3 cm, Child–Pugh A	185	84.9	69.2	36.7	20.2	15.1	7.6	–
1–3 tumours, ≤3 cm	419	78.6	58.0	23.5	12.2	9.1	6.1	–
1–3 tumours, ≤3 cm, Child–Pugh A/B	402	80.5	59.5	24.3	12.8	9.6	6.2	–
Satisfied the indication criteria of surgical resection proposed in the BCLC protocol*	121	86.3	72.8	31.1	14.8	–	7.2	–

*Child–Pugh class A with a normal level of bilirubin, no significant portal hypertension and a single HCC.
BCLC, Barcelona Clinic Liver Cancer; HCC, hepatocellular carcinoma.

Table 3. Multivariate analysis of variables relevant to survival, local tumour progression and distant recurrence

Variable	Multivariate analysis Hazard ratio (95% CI)	*P* value
Survival		
Age (per year)	1.03 (1.02–1.04)	<0.0001
Anti-HCV-positive	0.81 (0.69–0.94)	0.006
Child–Pugh class		
A	1	
B	2.01 (1.66–2.44)	<0.0001
C	3.11 (2.08–4.65)	<0.0001
Tumour size (cm)		
≤2.0	1	
2.1–3.0	1.26 (1.00–1.58)	0.051
3.1–5.0	1.51 (1.18–1.93)	0.001
>5.0	2.31 (1.61–3.31)	<0.0001
Tumour number		
solitary	1	
2–3	1.10 (0.90–1.35)	0.34
≥4	2.11 (1.59–2.78)	<0.0001
Serum AFP (ng/dl)		
≤100	1	
101–400	1.47 (1.14–1.90)	0.003
>400	2.16 (1.57–2.97)	<0.0001
Local tumour progression		
Tumour size (cm)		
≤2.0	1	
2.1–3.0	1.47 (1.15–1.88)	0.002
3.1–5.0 vs. ≤2.0	1.30 (0.97–1.75)	0.08
>5.0 vs. ≤2.0	2.81 (1.64-4.82)	0.0002
Distant recurrence		
Tumour size (cm)		
≤2.0	1	
2.1–3.0	1.42 (1.11–1.82)	0.006
3.1–5.0	1.28 (0.95–1.72)	0.10
>5.0	2.48 (1.43–4.28)	0.001
Tumour number		
solitary	1	
2–3	1.47 (1.16–1.85)	0.001
≥4	2.12 (1.36–3.28)	0.0008

AFP, α-fetoprotein; CI, confidence interval; HCV, hepatitis C virus.

399 (88.8%), chemoembolization in 44 (9.8%), systemic chemotherapy in three (0.7%) and best supportive care in three (0.7%).

The 1-, 3-, 5-, 10-, 15- and 20-year rates of local tumour progression with or without distant recurrence were 7.9% (95% CI = 5.7–10.0%), 15.6% (95% CI = 12.6–18.6%), 18.2% (95% CI = 15.0–21.4%), 18.4% (95% CI = 15.2–21.6%), 18.4% (95% CI = 15.2–21.6%) and 18.4% (95% CI = 15.2–21.6%) respectively (Fig. 4). Univariate analysis demonstrated that three variables were relevant to local tumour progression, whereas multivariate analysis indicated that only tumour size was significantly related to local tumour progression (Table 3).

The 1-, 3-, 5-, 10-, 15- and 20-year rates of distant recurrence without local tumour progression were 17.1% (95% CI = 14.0–20.1%), 42.6% (95% CI = 38.6–46.7%), 53.5% (95% CI = 49.4–57.7%), 60.4% (95% CI = 56.3–64.5%), 60.8% (95% CI = 56.7–64.9%) and 60.8% (95% CI = 56.7–64.9%) respectively. Univariate analysis demonstrated that five variables were relevant to distant recurrence, whereas multivariate analysis indicated that tumour size and tumour number were significantly related to distant recurrence without local recurrence (Table 3).

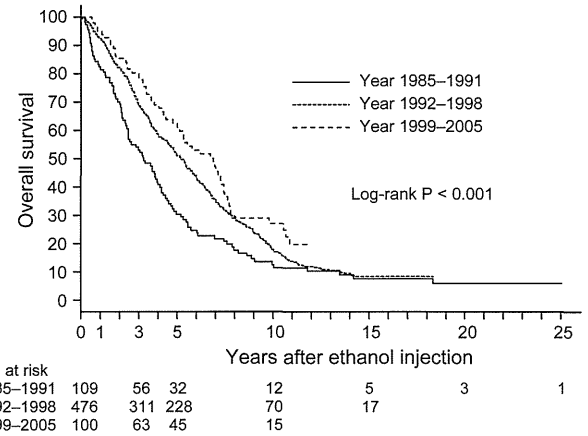

Fig. 3. Survival according to the time period in which the first ethanol injection was performed (1985–1991 vs. 1992–1998 vs. 1999–2005)

Complications

Table 4 shows complications encountered. The incidence rates per treatment and per procedure were 2.1% (45 of 2147) and 0.33% (45 of 13 526) respectively. A patient died of multiple organ dysfunction syndrome caused by procedure-related hemoperitoneum. The tumour was not on the surface but inside the liver. The patient did not have marked bleeding tendency. The other developed myocardial infarction, resulting in death during the procedure. The treatment mortality rate was 0.06%.

Discussion

This study describes a 20-year experience with ethanol injection at a high-volume centre. We performed 2147 ethanol injection treatments on the 685 primary HCC patients, showing that ethanol injection has a high antitumour effect. Tumours were judged to have been completely ablated by final CT imaging in 98.2% of the treatments. The complete response rate may be higher in this study than others (17, 18), probably because we did not predefine the number of procedures in a treatment. We generally repeated the procedure until CT demonstrated complete tumour necrosis. Many other studies limited the procedure number of ethanol injection. Complete tumour ablation has been reported to relate to improved survival (19).

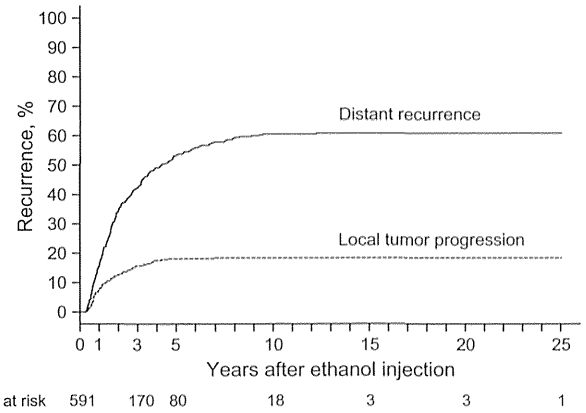

Fig. 4. Local tumour progression or distant recurrence in patients who underwent ethanol injection.

Table 4. Complications in 2147 treatments of ethanol injection for hepatocellular carcinoma

Complication	Number
Neoplastic seeding	9
Hemoperitoneum	9
Hemobilia	6
Liver abscess	6
Symptomatic pleural effusion	3
Massive hepatic infarction	3
Biliary cast	2
Hemothorax	2
Abnormal decrease in blood coagulation factor VIII	2
Biloma	1
Biliary bronchial fistula	1
Myocardial infarction	1

This study showed that ethanol injection could achieve long-term survival over 20 years. Ninety-seven patients survived for more than 10 years and three for more than 20 years. Both tumour factors and liver function were relevant to survival. In addition, age was among the prognostic factors. In this study, 19.9% were older than75 years, which may have resulted in the higher percentage (14.9%) of liver-unrelated deaths compared with other studies. Ant-HCV positivity was a good prognostic factor in this study.

Survival in ethanol injection appears to have improved with times. This is probably because of advances in imaging techniques, such as ultrasound and CT, more refined skills and greater experience in ablation and innovations in the treatment of underlying liver diseases.

Hepatocellular carcinoma frequently recurred after ethanol injection. Most recurrences were, however, not local tumour progression but distant recurrence. Frequent recurrence is not specific to ethanol injection. After hepatic resection, the tumour recurrence rate exceeds 70% at 5 years (20, 21). In this study, periodic follow-up detected most recurrence at limited stage. Ethanol injection was performed again for first recurrence in 88.8% of the cases. In hepatic resection, the rate of repeat resection for first recurrence has been reported to range from 10.4 to 30.6% (21, 22). As ethanol injection is less invasive than hepatic resection, iterative ethanol injection can be performed for recurrence more easily.

Ethanol injection was a safe procedure, although many patients in this study were at risk for surgical treatment because of advanced cirrhosis or other comorbidities. Only 121 (17.7%) of the 685 patients satisfied the indication criteria of surgical resection proposed in the BCLC (Barcelona Clinic Liver Cancer) protocol (23) and were, thus, considered good candidates for surgical resection. Other investigators also reported low complication rates of 0–3.2% (6–8, 24). For hepatic resection, morbidity rates have been reported to be 38–47% even in recent studies (25–27).

Radiofrequency ablation has steadily replaced ethanol injection (11). At our institution, radiofrequency ablation is currently the first option for percutaneous ablation (28). Several randomized controlled trials including ours (12, 18, 29, 30) demonstrated more reliable local antitumour effect and higher survival. Our 10-year outcome of radiofrequency ablation (28) appears superior to this 20-year outcome of ethanol injection. In addition, radiofrequency ablation requires fewer treatment sessions and shorter hospitalization.

A meta-analysis showed, however, that ethanol injection did not differ from radiofrequency ablation for tumours ≤ 2 cm in diameter (31). A recent randomized controlled trial also demonstrated similar 5-year survival between the two ablations (32). Ethanol injection is at least more feasible and cheaper than radiofrequency ablation.

Surgical resection has been considered the treatment of first choice for HCC. Our first option for resectable tumours was also surgery. However, most patients who came to our department declined surgical resection. Thus, some patients in this study underwent ethanol injection not because of unresectable tumour but because of refusal of surgery. Those who preferred surgery would have gone directly to the surgical department, which has extensive experience in hepatic resection (27).

It is not easy to compare outcomes between ethanol injection and surgical resection. Indications are different between the two treatments. Furthermore, indications for each treatment are different from institution to institution. Thus, a case adjudged to be treatable by ethanol injection or surgical resection at an institution may not be given the same treatment at another. The best-known indication criteria may be those proposed in the BCLC protocol (23), which states that surgical resection should be restricted to patients with performance status 0, Child–Pugh class A, single HCC, normal portal pressure and normal serum bilirubin level. In patients satisfying

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those criteria, the 5-year survival rate is expected to be >70% (20). In this study, 5-year survival rate of the patients satisfied the criteria was 72.8%, which appears satisfactory when compared with outcomes following surgical resection. Furthermore, in patients with solitary HCC, ≤ 3 cm in diameter, and Child–Pugh A, 5- and 10-year survival rates were 69.2% and 36.7% respectively. In patients treated by surgical resection, 5- and 10-year survival rates were 34.4–70.0% and 10.5–52.0% respectively (22, 33–39). Although this is an observational study with no control, survivals following ethanol injection appear comparable to those reported following surgical resection.

A randomized controlled trial showed no significant difference in survival between ethanol injection and surgical resection (40). Several non-randomized controlled trials also reported similar overall survival between the two treatments (5–7, 40–43), whereas others reported higher survival with resection (44). Further studies are necessary to resolve this issue of comparing ablation with resection.

We made strenuous efforts to standardize the procedure of ethanol injection because many physicians performed ethanol injection at our institution. In addition to proficient practice of ethanol injection, detailed preoperative planning, cautious postoperative evaluation of therapeutic effect and careful follow-up are vital to achieve satisfactory outcomes.

Source population in this study may represent selection bias, as we performed ethanol injection on most patients who were hospitalized at our department; however, many patients with unfavourable tumour conditions for ethanol injection might not have been referred to us. Therefore, caution is required when extrapolating our findings to the general population of HCC patients.

A second limitation is that study population cannot be clearly defined. This study was based on daily clinical practice over a 20-year period. Indication criteria of ethanol injection changed over time, mainly because of the introduction of the other ablations: microwave ablation and radiofrequency ablation. Furthermore, various treatments besides percutaneous ablations were available for HCC, such as surgical resection and chemoembolization, with frequently overlapping indications.

One further limitation is the fact that this was a single-centre study. To extrapolate the findings in this study to patients at other institutions, consideration should be given to differences in the indications, methods, expertise, performance of available ultrasound and CT equipment and others. Treatment outcome may be influenced by the physicians' expertise and the institution's volume of care. We performed over 2000 ethanol injection treatments, which may represent a much greater number of treatments than those in most other institutions.

In conclusion, our 20-year experience shows that ethanol injection was potentially curative, resulting in long-term survival over 20 years. Findings in this study may suggest that other ablation therapies, such as radiofrequency ablation, will achieve similar or even better long-term results in HCC.

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Antagonism of Sphingosine 1-Phosphate Receptor 2 Causes a Selective Reduction of Portal Vein Pressure in Bile Duct-Ligated Rodents

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Sinusoidal vasoconstriction, in which hepatic stellate cells operate as contractile machinery, has been suggested to play a pivotal role in the pathophysiology of portal hypertension. We investigated whether sphingosine 1-phosphate (S1P) stimulates contractility of those cells and enhances portal vein pressure in isolated perfused rat livers with Rho activation by way of S1P receptor 2 ($S1P_2$). Rho and its effector, Rho kinase, reportedly contribute to the pathophysiology of portal hypertension. Thus, a potential effect of $S1P_2$ antagonism on portal hypertension was examined. Intravenous infusion of the $S1P_2$ antagonist, JTE-013, at 1 mg/kg body weight reduced portal vein pressure by 24% without affecting mean arterial pressure in cirrhotic rats induced by bile duct ligation at 4 weeks after the operation, whereas the same amount of $S1P_2$ antagonist did not alter portal vein pressure and mean arterial pressure in control sham-operated rats. Rho kinase activity in the livers was enhanced in bile duct-ligated rats compared to sham-operated rats, and this enhanced Rho kinase activity in bile duct-ligated livers was reduced after infusion of the $S1P_2$ antagonist. $S1P_2$ messenger RNA (mRNA) expression, but not $S1P_1$ or $S1P_3$, was increased in bile duct-ligated livers of rats and mice and also in culture-activated rat hepatic stellate cells. $S1P_2$ expression, determined in $S1P_2^{LacZ/+}$ mice, was highly increased in hepatic stellate cells of bile duct-ligated livers. Furthermore, the increase of Rho kinase activity in bile duct-ligated livers was observed as early as 7 days after the operation in wildtype mice, but was less in $S1P_2^{-/-}$ mice. *Conclusion*: S1P may play an important role in the pathophysiology of portal hypertension with Rho kinase activation by way of $S1P_2$. The $S1P_2$ antagonist merits consideration as a novel therapeutic agent for portal hypertension. (HEPATOLOGY 2012;56:1427-1438)

Portal hypertension is a major complication of liver cirrhosis, being a leading cause of death or cause for liver transplantation.[1,2] The management of patients with portal hypertension is still a clinical problem; nonselective beta-adrenergic blockers, the most commonly used pharmacological treatment for portal hypertension, have significant limitations due to adverse events and unpredictable response.[3] Furthermore, the mean decrease in portal vein pressure in response to beta-adrenergic blockers is only ≈15%.[4] Therefore, it is clear that new treatment strategies are needed to improve the prognosis of patients with advanced portal hypertension.

It is well known that the enhanced pressure of the portal vein is caused by the increased intrahepatic vascular resistance. Fibrosis and regenerative nodule formation are classical mechanisms that account for

Abbreviations: BDL, bile duct ligation; S1P, sphingosine 1-phosphate; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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