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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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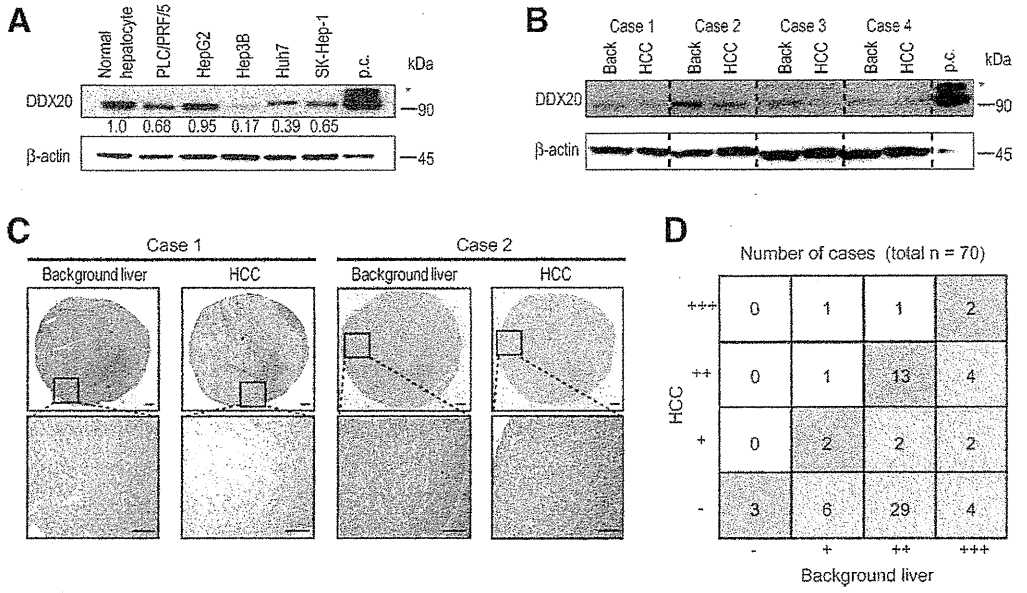

Fig. 1. Reduced DDX20 expression levels in hepatocellular carcinoma. (A) DDX20 protein expression in HCC cell lines. Numbers between the panels indicate DDX20 protein levels normalized to β-actin levels. Lysates of 293T cells transiently transfected with a FLAG-tagged DDX20-expressing plasmid yielded 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

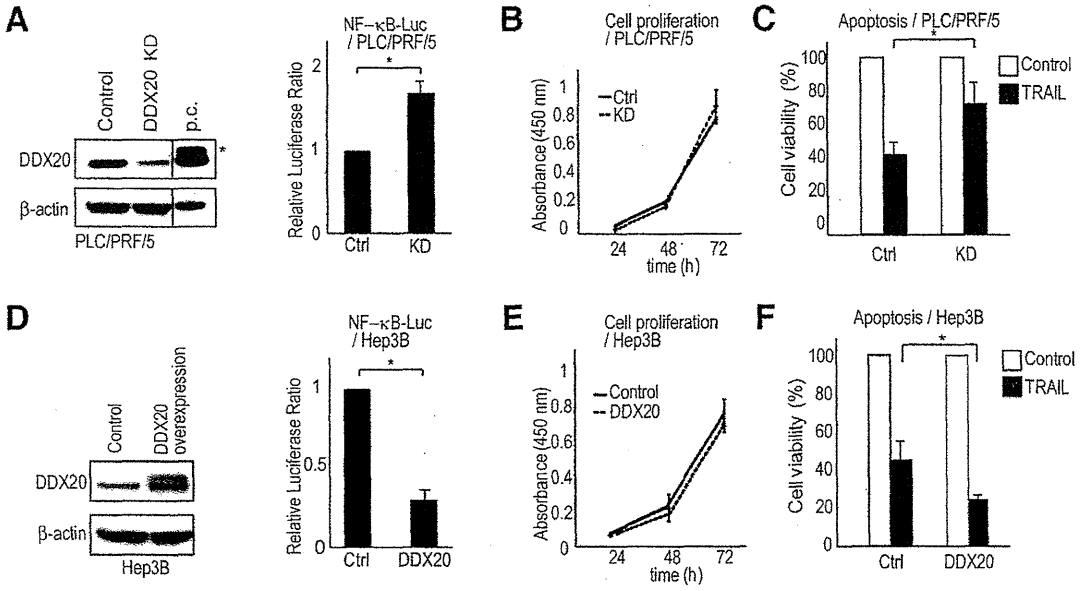

Fig. 2. Modulation of downstream events of the nuclear factor-κB pathway by DDX20. (A) Left: Establishment of stable DDX20-knockdown (DDX20 KD) PLC/PRF/5 cells. *Positive control (p.c.). Right: DDX20 deficiency enhances TNF-α–induced NF-κB activity. NF-κB reporter plasmids were transiently transfected 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

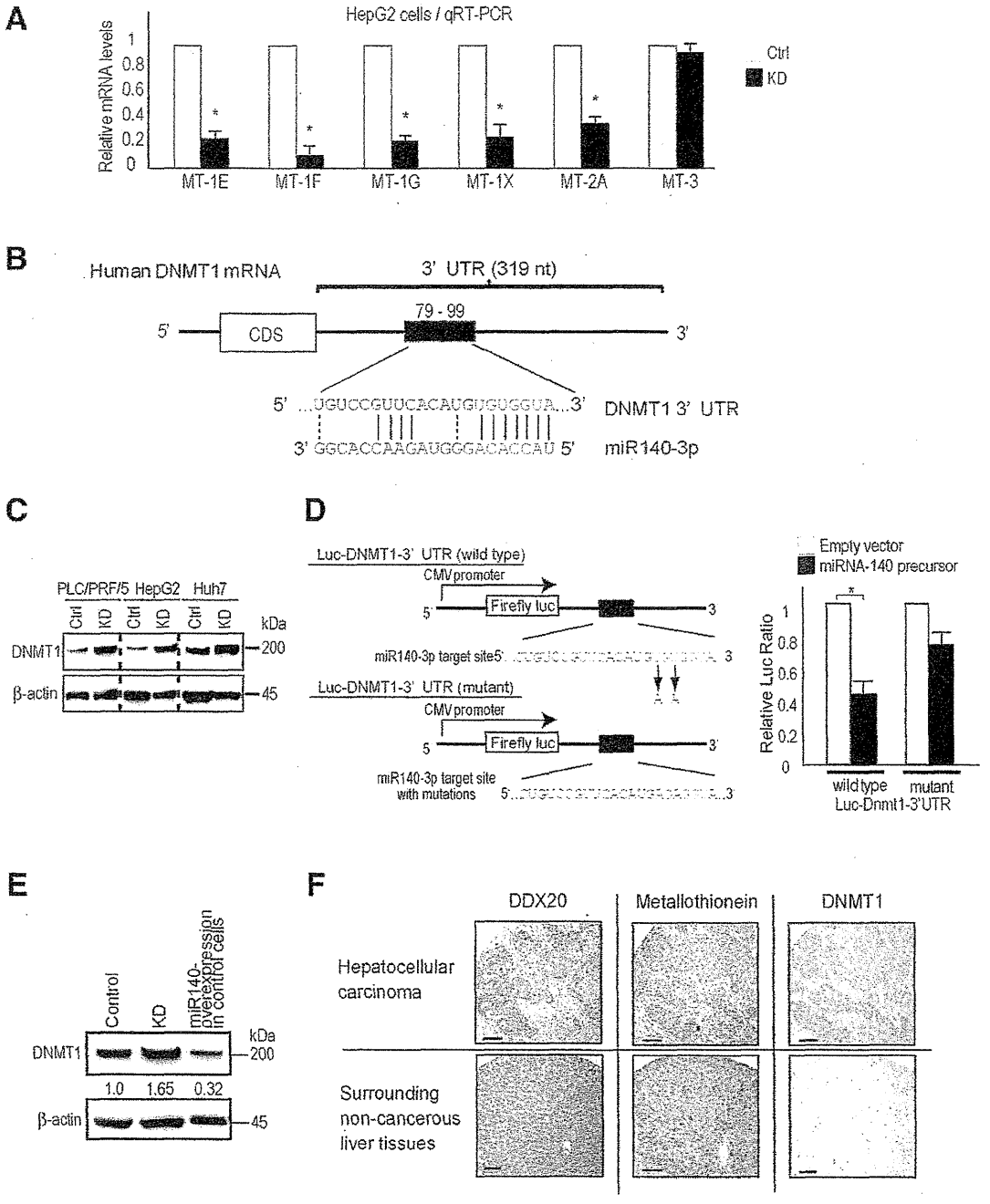

Fig. 3. Targeting of Dnmt1 by miRNA-140-3p and reduced MT expression. (A) The expression levels of MTs were determined using quantitative reverse-transcriptase polymerase chain reaction. The relative expression ratios of the MTs in control (white bars) and DDX20-knockdown (black bars) HepG2 cells were 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

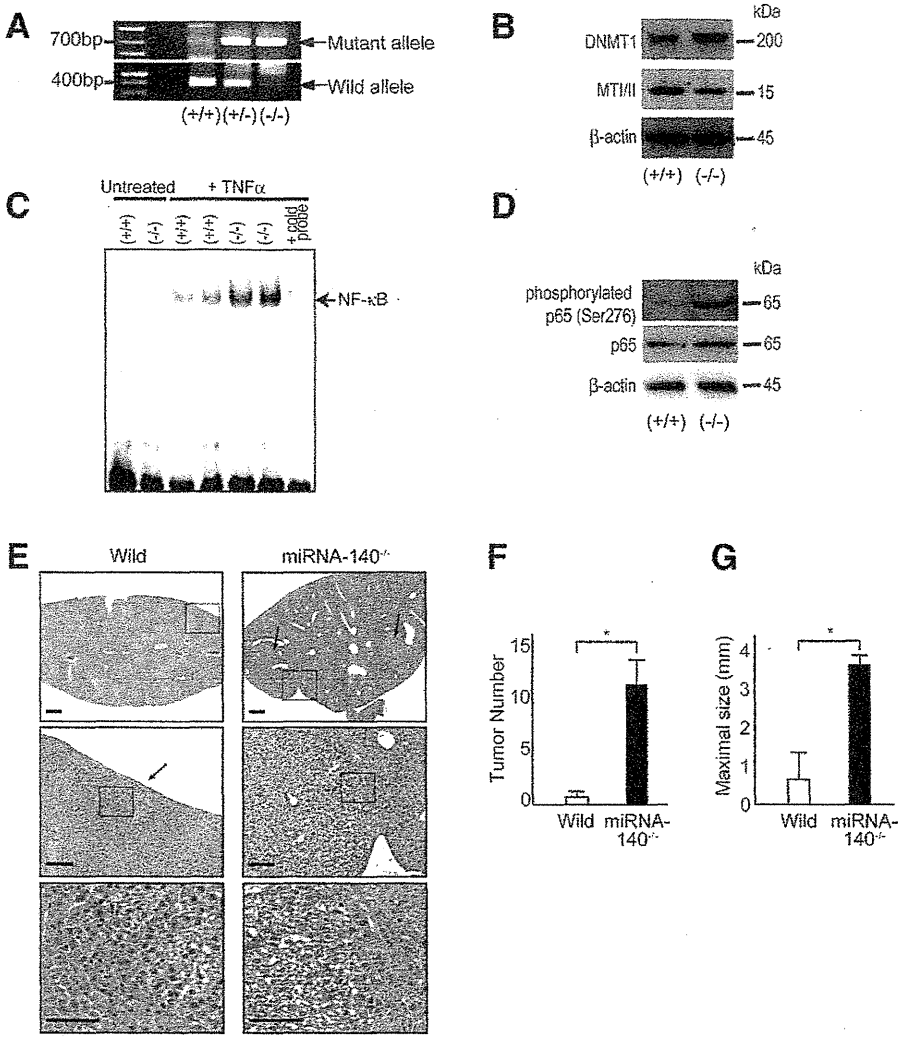

Fig. 4. miRNA-140$^{-/-}$ mice are prone to hepatocarcinogenesis. (A) Representative genotyping of mice with wild-type or mutant alleles. PCR genotyping was performed for miRNA-140 wild-type (419 bp; Wild) and knockout (734 bp; Mutant) alleles. (+/+), wild-type; (+/−), heterozygous; (−/−), knockout. (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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Review

A Worldwide Survey of the Current Daily Practice in Liver Surgery

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Key Words
Liver function · Liver volume · Liver resection · Laparoscopic hepatectomy

Abstract
Background: Liver resection remains the mainstay of curative treatment for liver malignancies. A variety of preoperative assessments and surgical techniques have improved the short- and long-term outcomes of liver resection in patients with liver tumors. Recently, laparoscopic hepatectomies have been increasingly performed. The aim of the present study is to survey the current practice of liver surgery in high-volume centers in the world. ***Methods:*** A questionnaire on the preoperative assessment for liver surgery, open hepatectomy, and laparoscopic hepatectomy was sent to 94 liver centers in the world. ***Results:*** Forty-two centers (45%) responded to this survey (29 Asian, 9 European, and 4 North American centers). All but one of the centers evaluated the future liver remnant (FLR) volume, and 95% of them performed preoperative portal vein embolization to increase the FLR volume. In half of the centers, the required FLR volume was over 30% in patients with normal liver and 50% in patients with cirrhotic liver. To reduce the intraoperative blood loss, half of the centers routinely used Pringle's maneuver, and 85% restricted the intraoperative fluid infusion to reduce the central venous pressure. More than 10 laparoscopic hepatectomies were performed per year in 62% of the centers, and more than 30 were performed in 26%, respectively. Laparoscopic major hepatectomies were performed in 24%. Two-thirds answered that the laparoscopic approach would be feasible in donor hepatectomy. ***Conclusion:*** The evaluation of FLR volume in patients with normal or cirrhotic liver and the usage of preoperative portal vein embolization have become essential practice in more than 90% of the centers. Reduced blood loss has been achieved using Pringle's maneuver, restriction of fluid infusion, and a variety of surgical devises. The laparoscopic approach is increasingly extended to major hepatectomy or donor hepatectomy.

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Introduction

Liver resection represents the mainstay of curative treatment for hepatic malignancies. Recent progress in preoperative assessments and refinements in surgical techniques have dramatically improved the safety and prognostic outcomes of liver surgery over the past two decades. One of the major concerns for liver surgeons is how to evaluate the functional reserve especially in patients with underlying liver damage due to viral hepatitis, steatosis, or chemotherapeutic agents. There has been a variety of liver function tests and grading systems reported in the literature [1–6]. In a previous survey conducted by Breitenstein et al. [7] in 2007, a wide diversity in the application of liver function tests and in the minimal future liver remnant (FLR) volume to be preserved was demonstrated among centers as well as continents. Therefore, it will be meaningful to know the trend of the preoperative assessment to maintain the safety of liver resection toward the year 2012.

A number of surgical devices and techniques for liver resection have been developed in order to reduce the intraoperative blood loss [8–23]. It has been reported that the amount of intraoperative blood loss was associated with the incidence of surgical complications [24, 25], and perioperative blood transfusion have been shown to increase the recurrence rate of liver malignancies after surgical treatment [26]. Several studies have searched an advantage of one surgical device or techniques over others to reduce intraoperative blood loss [9–15]. However, only one study has assessed the trend of the devices and techniques used during liver surgery [27].

On the other hand, the recent wave of the laparoscopic approach has reached to the field of liver resection, which is further diversifying the daily practice of liver surgeons. This survey aimed to address the current trend in liver surgery all over the world, focusing on 3 topics: preoperative assessment, procedures in open hepatectomy, and laparoscopic liver resection.

Methods

Ninety-four leading hepato-pancreato-biliary centers around the world were invited to participate in this survey in July 2012 (72 Asian, 13 European, and 9 North American centers). These centers were selected on the basis of academic achievements and the personal contacts through international conferences. A questionnaire was sent to the centers by e-mail, with a cover letter calling for participation. The survey was closed in October 2012.

The number of open and laparoscopic liver resections performed in each center per year was filled in at the beginning of the questionnaire. The main questionnaire focused on the following 3 topics to evaluate the current practices in liver surgery: preoperative assessment of liver function and FRL volume, procedures and devices used in open liver surgery, and indications and devices applied in laparoscopic liver surgery (tables 1–3).

The derived data are expressed as medians with ranges. The best answer was to be selected in each question, but some centers selected several choices. Hence, the results are demonstrated as the total number of all answers.

Results

Forty-two centers (45%) responded to the survey (29 Asian, 9 European, and 4 North American centers). The total number of liver resections per year was 125 (30–785), and the number of open liver resections per year was 100 (8–700). One-quarter of the centers performed less than 50 liver resections per year, and another one-quarter performed more than 150 liver resections.

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Table 1. Questionnaire on the preoperative assessment of liver function and FLR volume

1	In the preoperative assessment of liver function reserve, which grading system do you use? a. Child-Pugh score b. ICGR15 test c. Presence or absence of portal hypertension d. MELD score e. Others, please describe
2	Do you evaluate the FLR volume by CT volumetry? a. Yes, only before hemihepatectomy b. Yes, before segmentectomy or hemihepatectomy c. No
3	Do you perform preoperative PVE before hepatectomy? a. Yes, based on the balance between the hepatic function and the FRL volume calculated by CT volumetry b. Yes, only before right hemihepatectomy or trisectoriectomy (trisegmentectomy) c. No d. Others, please describe
4	Which percentage of FRL volume do you accept without PVE in patients with normal liver function? a. 20% b. 25% c. 30% d. 35% e. 40%
5	Which percentage of FRL volume do you accept without PVE in patients with impaired liver function? a. 30% or less b. 35% c. 40% d. 45% e. 50% or more

The number of laparoscopic liver resections per year was 14 (0–100). One-quarter of the centers performed more than 30 laparoscopic liver resections. Five centers (12%, 4 Asian centers and 1 European center) did not adopt the laparoscopic approach in liver surgery. The correlation between the number of open and laparoscopic liver resections is shown in figure 1.

Preoperative Assessment of Liver Function and FLR Volume (fig. 2)

Liver Function

To estimate the preoperative liver function, 31 centers (74%, 25 Asian and 6 European centers) adopted the indocyanine green retention test at 15 min (ICGR15). Only 1 out of the 25 Japanese centers did not choose ICGR15 as the preoperative assessment modality. The Child-Pugh score was used in 14 centers (33%, 7 Asian, 5 European, and 2 North American centers). The presence of portal hypertension was taken into account in 7 centers (17%, 3 Asian and 3 European centers as well as 1 North American center). Two centers (1 European and 1 North American) used the model for end-stage liver disease (MELD) score as the sole preoperative assessment.

Volumetric Analysis

All but one of the centers evaluated the FLR volume using computed tomography (CT) volumetry. Twenty-one centers performed CT volumetry prior to segmentectomy or hemihepatectomy or more, and the remaining 20 centers assessed prior to hemihepatectomy or more, respectively.

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Table 2. Questionnaire on the procedures and devices used in open liver surgery

1 Do you perform vascular control by individually dissecting the hepatoduodenal ligaments or in the en bloc fashion by Takasaki et al.'s method [18] prior to division of liver parenchyma?
   a. Individually hepatic artery, portal vein and the bile duct
   b. En bloc ligation of the Glissonian sheath at the hepatic hilum
   c. No

2 Do you apply intermittent Pringle's maneuver during division of the hepatic parenchyma?
   a. Almost always
   b. Sometimes (only when excessive bleeding occurs)
   c. Rarely
   d. No

3 Do you restrict the intraoperative fluid infusion to reduce the pressure of IVC?
   a. Yes
   b. No

4 How do you divide (not seal the portal pedicles) the hepatic parenchyma?
   a. Clump-crushing
   b. CUSA
   c. Harmonic Scalpel
   d. LigaSure
   e. Linear stapler
   f. Tissue Link
   g. Bipolar scissors
   h. Others, please describe

5 How do you seal the thin portal pedicles (less than 3 mm in diameter) or hepatic parenchyma?
   a. Ligation with stitch
   b. Harmonic Scalpel
   c. LigaSure
   d. Linear Stapler
   e. Tissue Link
   f. Bipolar scissors
   g. Hemoclip
   h. Others, please describe

6 Backflow from the hepatic veins is massive. How do you reduce blood loss?
   a. Performing selective hepatic venous control
   b. Performing total or half clamping IV
   c. Performing total vascular exclusion
   d. Blood drawing to reduce CVP
   e. Head up or head down
   f. Nothing special
   g. Others, please describe

7 You need to reconstruct a thick hepatic vein. Which graft do you use?
   a. External iliac vein graft
   b. Prosthetic vein graft
   c. Internal jugular vein
   d. From gonadal vein, making a thick graft
   e. From great saphenous vein, making a thick graft
   f. Cryopreserved vein graft
   g. Others, please describe

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Table 3. Questionnaire on the indications and devices applied in laparoscopic liver surgery

1	What is your 'current' indication for laparoscopic hepatectomy? a. Left lateral segmentectomy and limited resection of the peripheral part of the liver b. Major hepatectomies and/or for tumors in the posterior part of the liver (segment I/VII) c. Others, please describe
2	What will be your 'future' indication for laparoscopic hepatectomy? a. Left lateral segmentectomy and limited resection of the peripheral part of the liver b. Major hepatectomies and/or for tumors in the posterior part of the liver (segment I/VII) c. Others, please describe
3	Do you think the laparoscopic approach is acceptable for donor hepatectomy? a. Yes b. Yes, only for left lateral graft c. No d. Others, please describe
4	Do you apply intermittent Pringle's maneuver during division of the hepatic parenchyma? a. Almost always b. Sometimes (only when excessive bleeding occurs) c. Rarely d. No
5	How do you divide (not seal the portal pedicles) the hepatic parenchyma? a. Clump-crushing b. CUSA c. Harmonic Scalpel d. LigaSure e. Linear stapler f. Tissue Link g. Bipolar scissors h. Others, please describe
6	How do you seal the thin portal pedicles (less than 3 mm in diameter) or hepatic parenchyma? a. Ligation with stitch b. Harmonic Scalpel c. LigaSure d. Linear stapler e. Tissue Link f. Bipolar scissors g. Hemoclip h. Others, please describe

Portal Vein Embolization

Preoperative portal vein embolization (PVE) was performed to induce the compensatory hypertrophy of FLR in 40 centers (95%) based on the results of the liver functional reserve and the extent of the liver resection.

In patients with normal liver function, 17 centers (40%) set 30% of the total liver volume as the critical FLR volume for safe liver resection and performed PVE when the estimated FLR volume is less than 30%. In patients with underlying cirrhosis, 18 centers (43%) set less than 50% of the FLR volume as the critical volume requiring PVE.

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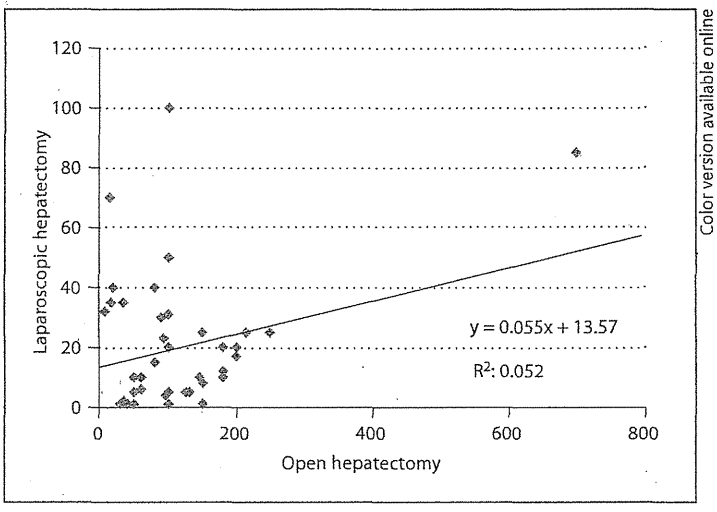

Fig. 1. Correlation between the number of open and laparoscopic liver resections.

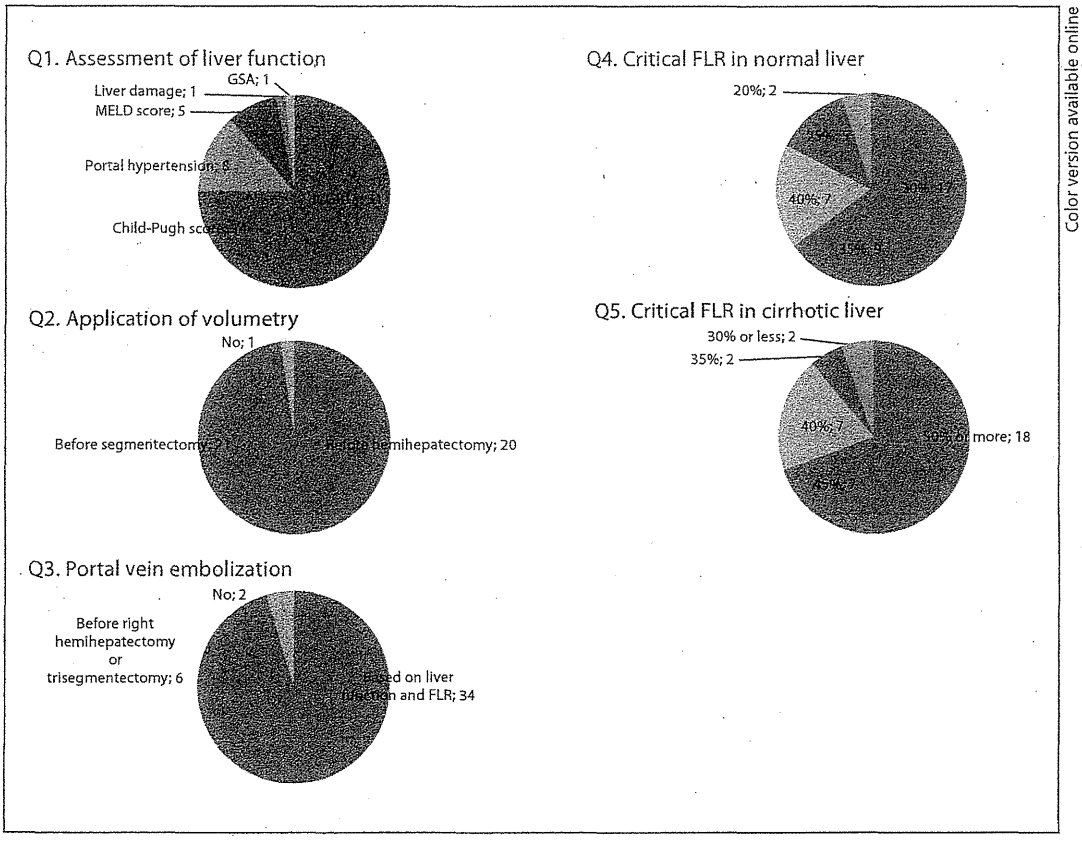

Fig. 2. Preoperative assessment of liver function and FLR volume. GSA = Galactosyl serum albumin scintigraphy.

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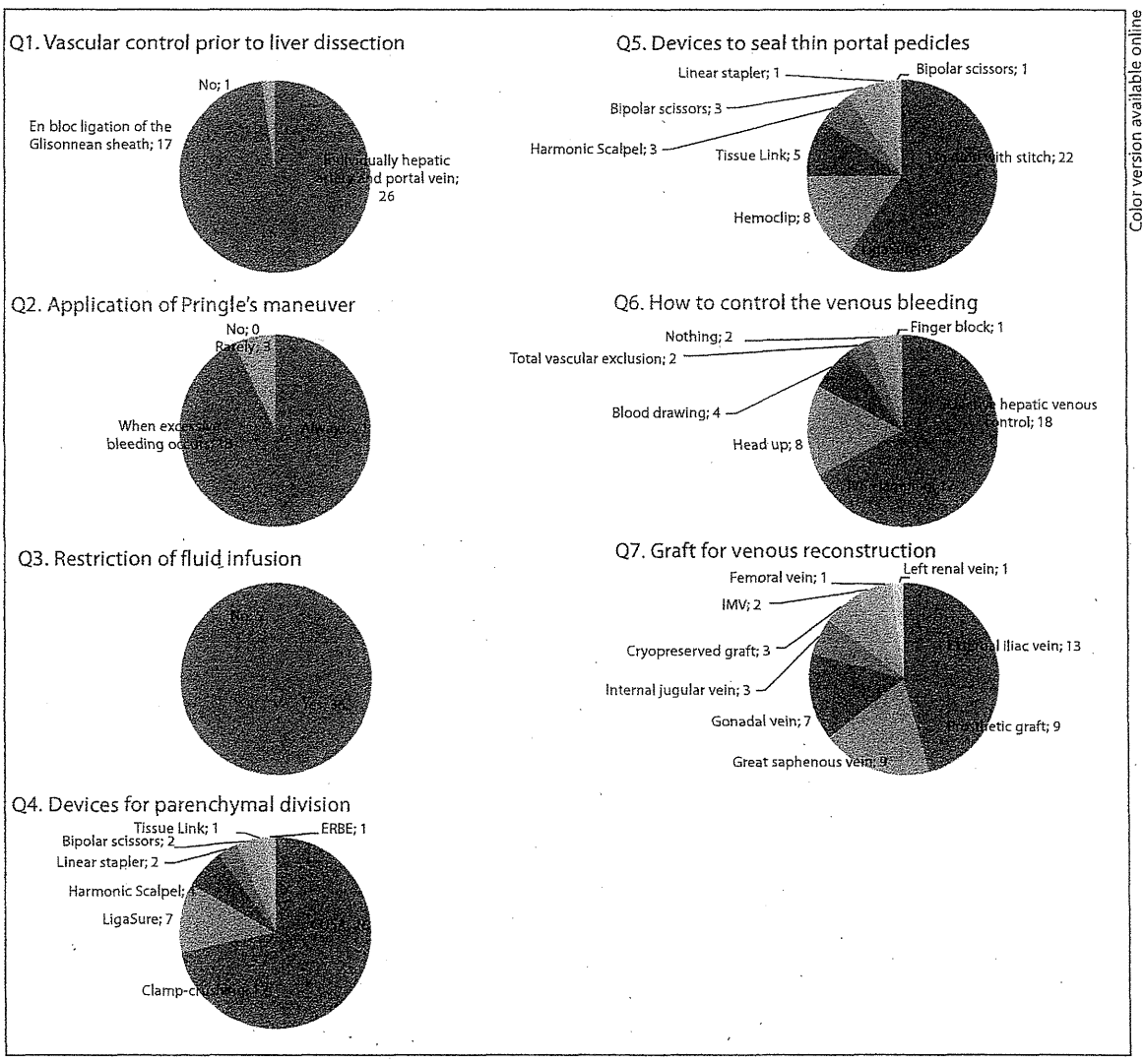

Fig. 3. Open hepatectomy.

Open Hepatectomy (fig. 3)

Surgical Devices Used during Liver Surgery

The most used device to divide the liver parenchyma was CUSA (26 centers, 62%), followed by clamp-crushing methods (17 centers, 40%). To seal the thin portal pedicles, half of the centers (52%) used ligation with stitch. The second most used device was LigaSure (9 centers, 21%).

Inflow Control

The vascular control prior to the division of the liver parenchyma was performed by individually ligating the hepatic artery and the portal vein in 23 centers (48%), while 14 centers (33%) used en bloc ligation of the Glissonian sheath at the hepatic hilum (Takasaki et al.'s method [18]). Pringle's maneuver was used routinely during the division of the liver parenchyma in 21 centers (50%). Three centers (7%) did not adopt the Pringle's maneuver.

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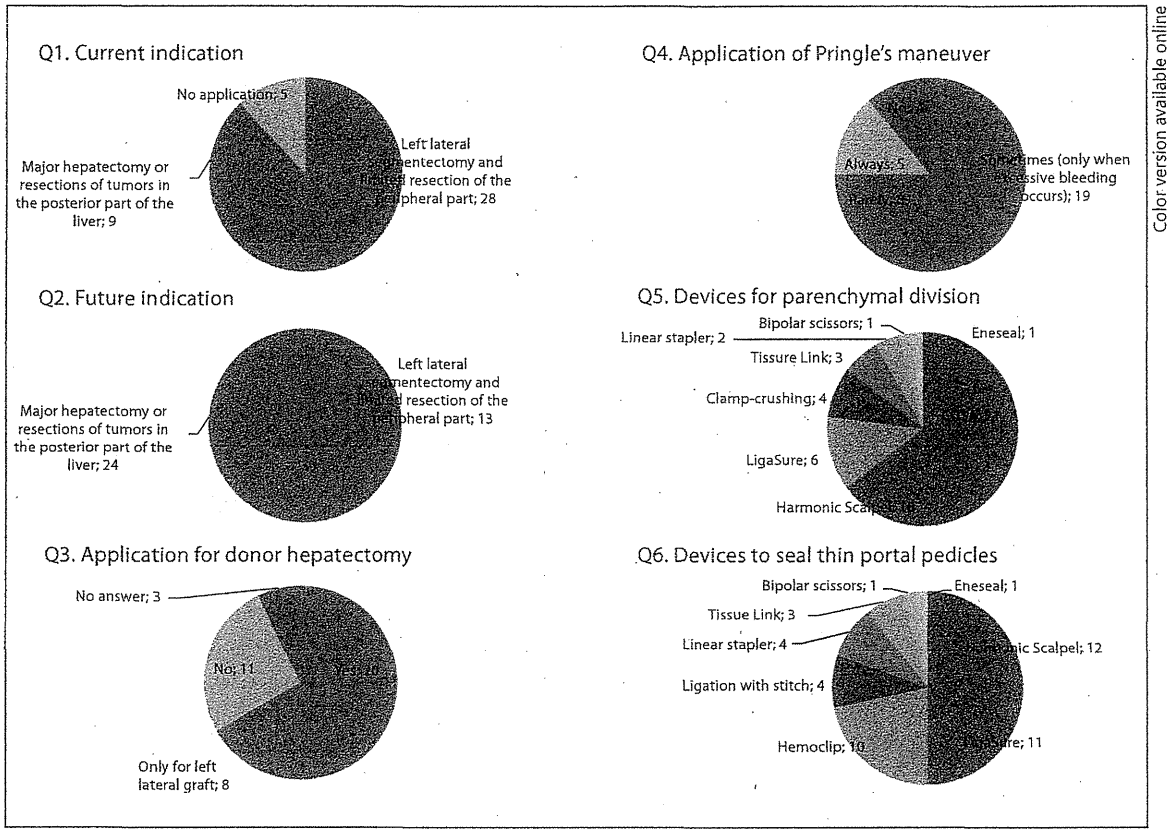

Fig. 4. Laparoscopic approach.

Outflow Control

Thirty-six centers (86%) restricted the intraoperative fluid infusion to reduce the pressure in the inferior vena cava (IVC). In cases when venous bleeding was massive, 18 centers (43%) performed selective hepatic venous control, and 17 centers (40%) performed IVC clamping to reduce the blood loss.

Venous Reconstruction

The most often used graft for venous reconstruction was the external iliac vein (13 centers, 31%). Both prosthetic graft and great saphenous vein were used in 9 centers each (21%).

Laparoscopic Approach (fig. 4)

Indication

Thirty-seven centers (88%) adopted laparoscopic liver resection, but the current indication was limited to left lateral segmentectomy or limited resection of the peripheral part of the liver in 28 of these 37 centers (76%). The remaining 9 centers applied the laparoscopic approach to major hepatectomies or resections of tumors in the posterior part of the liver. In the future, 22 centers are willing to apply the laparoscopic approach to major hepatectomies or resection of tumors in the posterior part of the liver. Twenty-eight centers (67%) answered that the laparoscopic approach is feasible for donor hepatectomy.

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Laparoscopic Procedures

Pringle's maneuver was used routinely only in 5 centers (12%) during the laparoscopic approach. The most frequently used device to divide the liver parenchyma was CUSA [21 centers, 57% (21/37)], followed by Harmonic Scalpel [10 centers, 27% (10/37)]. To seal thin portal pedicles, Harmonic Scalpel, LigaSure, and Hemoclip were used in 12, 11, and 10 centers, respectively.

Discussion

The current survey provides an overview of the current practices of liver surgery, including the laparoscopic approach. The results show that many leading liver centers have applied detailed preoperative assessments and intraoperative procedures to reduce blood loss, and that the laparoscopic approach has been widely used in the world, holding the promise of being applied for the field of transplantation.

The evaluation of the resectional liver volume in patients with normal or cirrhotic liver and the application of PVE have become essential to achieve safe liver resection. The importance of assessing the FLR volume prior to liver resection has been emphasized in many reports because of the significant interpatient variation in liver volumes [28–33]. Lack of liver volume after hepatectomy was reportedly associated with an increased incidence of liver dysfunction not only in patients with cirrhotic liver but also in those with normal liver. In this survey, all but one out of the 45 centers evaluated the FLR volume prior to hemihepatectomy or segmentectomy to prevent postoperative liver failure. Moreover, 95% of the centers performed PVE to increase the FLR volume based on the results of liver function and FLR volume (81%) and/or procedures of liver resection (12%). The rate of the application of PVE (95%) was higher than that reported in the survey conducted by Breitenstein et al. [7] in 2007 (89%). The minimal FLR volume in normal liver was 30–40% in 83% of the centers that answered the question. The critical limit of the FLR volume in this survey was higher than that in the previous survey [median 25% (range 20–40%)] [7]. The safe limit of the FLR volume might have interindividual differences; however, 40% of the FLR volume in normal liver would be a reliable criteria to achieve zero mortality following hepatectomy [34]. In practice, 30% is the standard in the world, although half of the centers answered that 50% of the FLR volume would be necessary in cirrhotic liver, which means that right hemihepatectomy cannot be safely performed in cirrhotic liver without preoperative PVE.

Reduction of intraoperative blood loss is a significant factor affecting the short- as well as long-term outcomes after liver resection [24–26]. Various intraoperative techniques have been widely applied to control the bleeding from both the inflow and outflow system. As for the inflow system, Pringle's maneuver and selective vascular occlusion can reduce the bleeding by limiting the blood flow to the liver [16, 17]. Our survey demonstrated that most of the centers applied Pringle's maneuver routinely (50%) or when excessive bleeding occurs (43%) during open hepatectomy. On the other hand, the frequency of routine use of Pringle's maneuver decreased to 14% in the laparoscopic approach.

Bleeding from the outflow system is a major problem during complex liver resections because the backflow bleeding from the hepatic veins can occasionally be massive [22]. The survey reflects the high interest in the control of bleeding from the outflow system, and 88% of the centers restricted the intraoperative fluid infusion to reduce the central venous pressure. In cases of massive backflow from the hepatic veins, the most frequently used procedures were the control of selective hepatic veins (43%) and IVC clamping (40%). Recently, 2 prospective randomized controlled studies proved the efficacy of IVC clamping

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for reducing blood loss [35, 36]. Total vascular exclusion, in which the infra- and suprahepatic IVCs are clamped, has been reported to be an effective procedure to achieve a bloodless liver resection [20, 21]. However, this procedure is associated with significant hemodynamic changes and requires close monitoring to prevent central hypovolemia. Only 2 centers (5%) applied total vascular exclusion to control backflow venous bleeding [23].

Our survey reflects the worldwide prevalence of laparoscopic liver resection and shows that 88% of the participating centers adapted the laparoscopic approach in liver surgery. Since the first introduction by Gagner et al. [37] in 1992, the application of laparoscopic liver resection has slowly progressed because of the complexity of liver surgery. Two meta-analyses demonstrated the benefits of the laparoscopic approach in terms of reduced operative blood loss and earlier recovery compared with open liver surgery [38, 39]. However, careful interpretation of the results derived from these meta-analyses is required in the light of potential selection bias. Most of the data were extracted from the comparison of the series of left lateral segmentectomy or partial resection of the liver, which accounted for a small part of the wide variety of hepatectomies. The results of this survey well reflect the careful attitude toward the current indications of the laparoscopic approach, that is, 28 out of 37 centers (78%) limited the procedure to left lateral segmentectomy and partial resection of the peripheral lesion. On the other hand, this study also implies that the indications for the laparoscopic approach would expand in the near future. More than half of the centers that limited the laparoscopic procedure to left lateral segmentectomy and partial resection were willing to expand the indication to major hepatectomies. In addition, two-thirds of all centers answered that the application of the laparoscopic approach to donor hepatectomy would be feasible. However, donor mortality is not zero even in open living donor hepatectomies [40]. Hence, one should remain cautious about the application of the laparoscopic approach, whose safety has not been well established regarding major hepatectomy.

Despite the introduction of many devices, a conservative trend was observed regarding the methods to transect the liver parenchyma during open surgery. The clamp-crushing technique and ultrasonic dissectors (CUSA), which were introduced in the 1970s [8] and in the 1990s [9, 10], respectively, were the two major methods favored in many centers. Furthermore, more than half of the centers selected ligation with stitch to seal the thin portal pedicles. Those results might have arisen from the evidence that several randomized trials showed no superiority of other new techniques over classical clamp-crushing [9–15].

A potential selection bias of the centers should be taken into account when interpreting our study results. Asian centers accounted for 69% of the centers that participated in this survey, and the rate is considerably high compared to the previous survey conducted in 2007 (17%) [7]. This regional bias must have led to the high application of ICGR15, which is not widely accepted in Western countries [7]. In addition, the low response rate to the questionnaire (45%) is another limitation of this survey considering the high response rate of the previous survey (75%). A more organized survey through an international liver association could provide a better overview of the current practices in liver surgery, which would help to make liver surgery safer and more standardized in the near future.

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Conflict of Interest

No conflicts of interest to disclose.

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