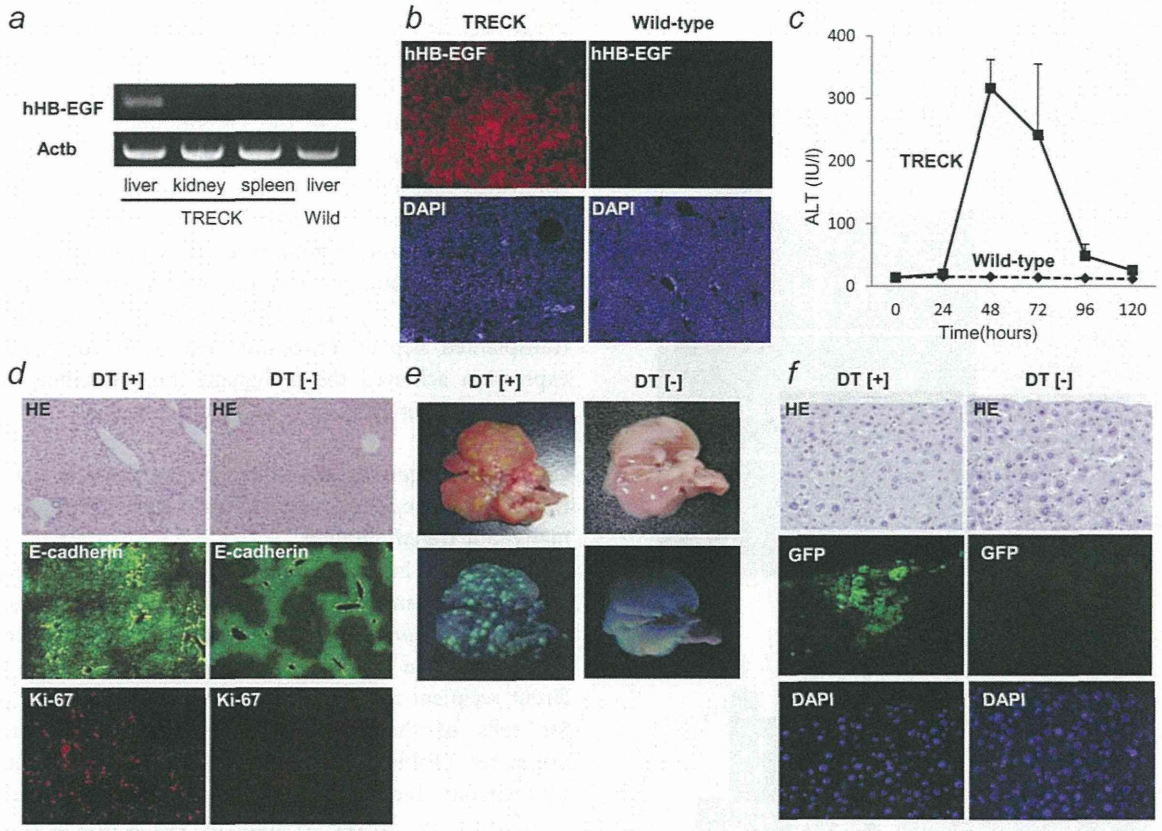

Figure 2. Efficient engraftment of the transplanted hepatic progenitor cells in the recipient liver. (*a*) The hHB-EGF expression in the liver, kidney and spleen of a TRECK mouse and the liver of a wild-type mouse determined by semiquantitative RT-PCR analysis. Upper, hHB-EGF expression; Lower, control Actb expression. (*b*) Immunohistochemical staining for human hHB-EGF in the liver of the TRECK and wild-type mice. Upper, hHB-EGF immunofluorescence; Lower, DAPI staining. (*c*) Time-course changes in ALT values of the TRECK and wild-type mice after the first DT administration. Vertical bars show SD. (*d*) Immunostaining analysis of liver tissue specimens of a TRECK mouse with (DT [+]) or without (DT [−]) DT administration. Upper, H&E staining; Middle, E-cadherin immunofluorescence; Lower, Ki-67 immunofluorescence. (*e*) Macroscopic image of a representative liver receiving GFP-positive hepatic progenitor cells at 30 days after transplantation. (F) Histologic analysis of liver tissue specimens receiving GFP-positive hepatic progenitor cells at 90 days after transplantation. Upper, H&E staining; Middle, GFP immunofluorescence; Lower, DAPI staining.

without DT administration (Fig. 2*f*). These findings indicated that the transplanted cells efficiently engrafted and continued to proliferate in the recipient livers treated with DT as time progressed.

Transplanted hepatic progenitor cells with constitutive AID expression progressed to liver cancers

Next, the enriched hepatic progenitor cells from AID Tg mice were transplanted into 13 recipient (TRECK) mice, and the DT was administered to the recipient mice for 25 weeks. Two mice died in a week after transplantation, while the remaining 11 mice were viable and thus subjected to phenotypic analyses. We found that liver tumors developed in 7 of 11 (63.6%) recipient mice that received the enriched hepatic progenitor cells of the AID Tg mice 90 week after cell transplantation (Fig. 3*a*). Among them, four mice developed multiple tumors and three developed a single large nodule. On the other hand, none of the 13 recipient mice receiving hepatic progenitor cells from wild-type or GFP Tg mice showed tumorigenesis during the same observation period, while only one mouse developed a tumor with the characteristics of lipoma. Moreover, all the five recipient mice examined that received the mature hepatocytes of adult AID Tg mice at 6 months of age showed no phenotypic changes in the liver tissues. Histologic examination revealed that all the tumors examined showed the characteristics of well-to-moderately differentiated HCC. Interestingly, one tumor showed not only the enhanced AFP expression but also the ductal formation of tumor cells accompanied by the expression of CK19, indicating the features of intrahepatic cholangiocarcinoma (ICC) (Fig. 3*b*, upper and middle panel). In addition, partial positivity for MUC1 immunostaining in the tumor indicated that the tumor contained the mucin-producing area (data not shown). On the other hand, no histologic changes were observed in the non-tumorous region of liver tissues receiving the AID-expressing hepatic progenitor cells (Fig. 3*b*, lower panel).

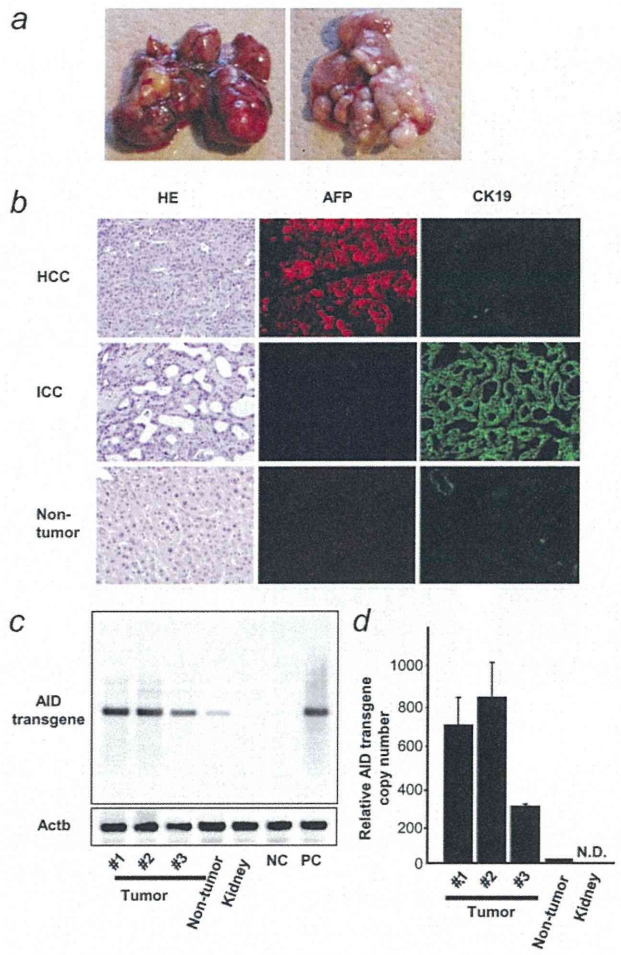

Figure 3. Development of tumors in livers receiving hepatic progenitor cells from AID Tg mice. (*a*) Macroscopic images of tumors that developed in recipient mice receiving progenitor cells from AID Tg mice. (*b*) Microscopic images of a liver tumor that developed in a recipient mouse receiving hepatic progenitor cells from an AID Tg mouse. Upper, AFP-positive part; Middle, CK19-positive part; Lower, non-tumorous liver tissue. Immunohistochemical staining for H&E, AFP and CK19 are shown. (*c*) Southern blot analysis for the AID transgene. DNA was extracted from three liver tumor tissues (Tumor #1, 2, 3), a non-tumor liver tissue (Non-tumor), the kidney of the corresponding animal, a liver of a TRECK mouse (Negative control; NC) and a liver of an AID Tg mouse (Positive control; PC), followed by the amplification and hybridization to the probe specific for the AID transgene. (*d*) Results of quantitative genomic PCR for AID transgene in three liver tumor tissues, a non-tumor liver tissue and the kidney of the corresponding animal. N.D. means not detected.

To examine whether the cancers that developed in recipient mice liver were derived from the transplanted hepatic progenitor cells, we examined the expression of the AID Tg mice-specific transgene in three randomly selected tumors that developed in the recipient livers. Southern blotting analyses revealed strong signals of the AID transgene in the tumor tissues (Fig. 3*c*). Weak signal of the AID transgene was also detected in the non-tumorous region, suggesting continuous engraftment of the transplanted hepatic progenitor-derived cells in the recipient mouse liver. In contrast, there were no detectable signals of the AID transgene in organs other than the liver of recipient mice, such as kidney, or in liver tissues of the TRECK mice without receiving the transplantation. Quantitative genomic PCR analyses also confirmed that all tumor tissues examined strongly expressed the AID transgene (Fig. 3*d*). Moreover, the expression level of hHB-EGF in the tumor tissue was significantly lower than that in the surrounding non-tumorous liver tissue (Supporting Information Fig. 2*c*). These findings suggested that the transplanted hepatic progenitor cells with constitutive AID expression achieved the malignant transformation and progressed to either HCC or cholangiocarcinoma.

Landscape of genetic alterations accumulated in the transplanted hepatic progenitor cells during the process of malignant transformation

To unveil the landscape of genetic alterations that accumulated in the transplanted hepatic progenitor cells during the process of tumorigenesis, we determined the sequences of the whole exome in two independent liver cancers from two different recipient mice and the corresponding hepatic progenitor cells of the same AID Tg mice from which they originated (Table 1). As a control, we also determined the whole exome sequences of the livers of their littermates with a wild-type phenotype. A total of 94.2% of the reads were properly aligned to the reference mouse genome and accordingly we obtained about 4.4 Gb of the aligned sequence data per sample on average after exome enrichment. 77.6% of the captured target exons were covered by 20× or more coverage depth read with a high quality genotype call. The variant filtering process is summarized in Supporting Information Figure 1. We identified 24 [23 single nucleotide variants (SNVs) and one indel] and 162 (160 SNVs and two indels) somatic mutations in HCC#1 and HCC#2, of which the number of mutated genes with SNVs were 23 (HCC#1) and 105 (HCC#2), respectively (Table 2, and Supporting Information Table 4). As shown in Supporting Information Figure 2*d*, C/G to T/A substitution pattern was dominant, consistent with the previous finding that AID induces C/G to T/A transition into the genome.[23,24] The candidate variants were then validated by conventional direct population Sanger sequencing (Supporting Information Fig. 3), and we finally confirmed that 20 (HCC#1) and 87 (HCC#2) SNVs were non-synonymous variants. Among them, there were no genes commonly mutated in both tumors. Interestingly, 19 of 23 (82.6% in HCC#1) and 80 of 105 (76.2% in HCC #2) genes with SNVs were those reported in human liver cancer tissues (International Cancer Genome Consortium; http://www.icgc.org/). Although tumor-suppressor Trp 53 gene also acquired mutations in both tumors, the nucleotide alteration rate was less than 20%. Pathway analyses using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (http://www.genome.jp/kegg/) revealed that 11 (HCC#1) and

Table 1. Overview of exome sequencing data

	Wild-type liver	HPCs	HCC #1	HCC #2
Total reads	40,531,478	66,249,904	74,974,839	71,744,206
Aligned reads	39,836,233	64,288,711	73,520,622	70,334,812
Aligned sequence (bp)	2,895,739,984	4,640,585,570	5,299,669,630	4,893,614,870
Median read depth	47	69	83	69
1× coverage	26,389,043	26,497,041	26,651,266	26,676,550
8× coverage	25,737,539	25,954,163	26,050,816	25,993,659
20× coverage	24,034,052	25,453,341	25,554,806	25,105,923
30× coverage	21,708,378	24,777,302	24,956,955	23,893,453

Whole exome sequencing were performed for the liver of wild-type mouse, the hepatic progenitor cells (HPCs) of the AID Tg mouse, and liver cancer tissues (HCC#1 and HCC#2) developed in the two different TRECK mice transplanted with HPCs. Total reads, aligned reads, aligned sequence(bp), median read depth and the number of the captured target exons which were 1×, 8×, 20× and 30× or more coverage depth read were shown for each sample.
Abbreviations: HCC: hepatocellular carcinoma; HPC: hepatic progenitor cell.

Table 2. Characteristics of single nucleotide variants (SNVs) identified in the liver cancers derived from the hepatic progenitor cells of the AID Tg mice

	HCC#1	HCC#2
Number of SNVs (single nucleotide variants)	23	160
Number of mutated genes with SNVs	23	105
Number of SNVs with nonsynonymous variants	20 of 23 (86.7%)	87 of 160 (54.3%)
Number of mutated genes with SNVs reported in human liver cancer tissues	19 of 23 (82.6%)	80 of 105 (76.2%)
Number of mutated genes with SNVs categorized into well-known pathways in KEGG	11 of 23 (47.8%)	66 of 105 (62.8%)
Number of mutated genes with SNVs highly expressed in fetal or adult liver relative to bone marrow	21 of 22[1](95.4%)	85 of 99[1](85.8%)

[1]Genes that were not present in the microarray panel were excluded from the analysis.
Abbreviations: KEGG: Kyoto Encyclopedia of Genes and Genomes; SNV: single nucleotide variant.

66 (HCC#2) genes were categorized into the well-known signaling pathways, including peroxisome proliferator-activated receptor (PPAR) and mitogen-activated protein kinase (MAPK) signaling, and cell adhesion function (Table 3).

Although it is widely recognized that the mutational profiles of the tumor-related genes differ between different tissues, the mechanisms of those organ-specific differences in the mutated genes during the process of tumorigenesis remain unclear. We speculated that the genes that acquired mutations in HCC tissues might be preferentially and actively transcribed in hepatic lineage cells, because it has been shown that AID-induced mutagenic activity is directly proportional to the transcription levels of the target gene.[35–37] Therefore, we analyzed the gene expression profiles in the fetal and adult liver using microarray, and examined whether the mutated genes in HCC tissues were transcribed at relatively higher levels in liver-lineage cells compared with hematopoietic lineage cells. Among the mutated genes identified, transcription levels of 95.4% and 85.8% of the genes in HCC#1 and HCC#2, respectively, were higher in fetal and/or adult liver tissues than in bone marrow-derived hematopoietic cells (Table 2, and Supporting Information Table 4), indicating that the genes actively transcribed in fetal and/or adult liver cells might have preferentially acquired the mutations through the genotoxic activity of AID. Consistently, quantitative RT-PCR analyses revealed that all the mutated genes analyzed were actively transcribed in adult liver tissues (Supporting Information Fig. 4). In contrast, representative genes that are actively transcribed in hematopoietic tissues,[38] such as *Cd4*, *Cd5* and *Tgfbr2*, showed no mutations in liver tumors and less or no transcription in the liver compared with other organs (Supporting Information Fig. 4). We also confirmed that 19 (82.6% in HCC#1) and 93 (88.6% in HCC#2) of the mutated genes were actively transcribed in the liver tissues based on the mouse whole transcriptome analysis.[39] Together, these findings suggest that the acquisition of mutations during hepatocarcinogenesis strongly depends on the transcription of target genes in the liver-lineage cells.

Discussion

Recently, recognition of the role of tissue stem/progenitor cells in the carcinogenesis process led to a new hypothesis that cancer arises from tissue stem/progenitor cells.[40] Indeed,

Table 3. Categorization of the mutated genes in HCCs using the KEGG (Kyoto encyclopedia of genes and Genomes) database

HCC#1				
Insulin signaling pathway (2)	Sorbs1	Prkar1b		
Pathways in cancer (2)	Stk36	Erbb2		
Adherens junction (2)	Sorbs1	Erbb2		
HCC#2				
Metabolic pathways (12)	Akr1c6	Dgat2	Sephs2	Chka
	Cyp2e1	Maob	Nt5e	Pck1
	Rdh8	St3gal6	Tk1	Tkt
PPAR signaling pathway (7)	Fads2	Slc27a2	Apoa1	Apoa2
	Fabp5	Pck1	Scd1	
MAPK signaling pathway (6)	Jun	Jund	Fos	
	Dusp4	Rac3	Gadd45g	
Pathways in cancer (6)	Jun	Nfkbia	Ceppa	
	Fzd7	Rac3	Fos	
Toll-like receptor signaling pathway (3)	Jun	Nfkbia	Fos	
Hepatitis C (3)	Ldlr	Nfkbia	Socs3	
Adipocytokine signaling pathway (3)	Nfkbia	Pck1	Socs3	
Wnt signaling pathway (3)	Fzd7	Jun	Rac3	

Values in parenthesis show the number of the genes categorized into each pathway.
Abbreviations: MAPK: mitogen-activated protein kinase; PPAR: peroxisome proliferator-activated receptor.

genetically-engineered fetal progenitor cells lacking the tumor-suppressor gene function have been shown to play a role as the origin of liver cancer.[9,11,41] Whether the stepwise accumulation of genetic alterations on hepatic stem/progenitor cells contributes to the development of tumor cells, however, remains unknown. In our study, we demonstrated that engrafted hepatic progenitor cells originated from the AID Tg mice progressed to liver tumors, including both HCC and cholangiocarcinoma, through the accumulation of somatic mutations in a variety of target genes.

Several previous studies demonstrated that the transplanted putative fetal liver stem/progenitor cells are capable of repopulating the liver that encounter extensive liver injury favoring the proliferation and survival of transplanted hepatocytes.[42–44] The DT receptor has been identified as a membrane-anchored form of the HB-EGF precursor.[26] Recently, it was shown that transplanted hepatic progenitor cells derived from the fetal liver were efficiently engrafted and repopulated in the liver of recipient HB-EGF-expressing mice with DT stimulation.[27,28] Using this model, efficient engraftment of the transplanted cells in recipient mice with HB-EGF expression in the liver enabled us to examine the fate of transplanted hepatic progenitor cells with constitutive AID expression. Notably, liver tumors with histologic features of human HCC developed in the recipient mice that received the hepatic progenitor cells derived from the AID Tg mice, while no tumorigenesis was observed in the recipient mice transplanted with hepatic progenitor cells of control mice. The findings that the tumors contained the AID transgene indicated that these tumors were derived from the transplanted hepatic progenitor cells accompanied with the AID-induced genetic aberrations. Interestingly, one of those tumors showed both the characteristics of HCC and cholangiocarcinoma in a single nodule, suggesting that the hepatic progenitor cells with the accumulation of genetic aberration could possess the potential to progress both HCC- and cholangiocarcinoma-lineage tumor cells. Alternatively, it might be possible that AID-mediated genetic alterations contribute to modifying the differentiation status of tumor cells, leading to either HCC or bile duct cancers from common progenitor cells.

Sequencing of whole genomes, whole exomes and whole transcriptomes of cancer samples has recently become feasible using deep sequencing technologies. In this study, to obtain the overall picture of genetic alterations accumulated in the hepatic progenitor cells of the AID Tg mice that achieved malignant transformation, we performed whole exome sequencing of the transplanted progenitor cells and the resultant tumor tissues, and unveiled the landscape of genetic alterations that accumulated during tumorigenesis. We found that various genetic aberrations, mainly SNVs, were highly accumulated in the tumors, further supporting the putative involvement of aberrant AID activity in the development of HCC. One thing to be noted is that approximately 80% of mutated genes detected in the liver cancer tissues developed in the recipient mice have been reported to

be mutated in human HCC tissues (International Cancer Genome Consortium; http://www.icgc.org/), although it is not possible to draw a definitive conclusion from analyses of the limited number of HCCs that developed in the recipient mice. Functional annotation analyses revealed that many of the genes that acquired genetic aberrations are categorized into several important signaling pathways, including those involved in the regulation of cell proliferation, cell metabolism and cell adhesion. Thus, it could be suggested the stepwise dysregulation of cell function caused by the accumulation of genetic aberrations in hepatic progenitor cells appears to play a pivotal role in the development of tumor cells.

We previously revealed that genetic changes induced by the genotoxic activity of AID show organ-specific profiles and suggested the possibility that the target preference of AID-induced mutagenesis contributes to the diversity of tissue-specific oncogenic pathways.[23] One possible explanation for the target selection for mutagenesis is that AID preferentially induces mutations in the actively transcribed genes in each cell, because AID likely induces somatic mutations on the single-strand DNA exposed during the transcription process.[35–37] Consistent with this hypothesis, we confirmed in this study that the majority of genes with SNVs were the actively transcribed genes in liver-lineage cells. However, we also observed that the transcription level of the gene is not solely responsible for the acquisition of AID-mediated genotoxicity, because one of the most actively transcribed hepatotrophic genes, albumin, did not accumulate SNVs in liver tumor cells (data not shown). Consistently, extensive sequencing of various genes in B lymphocytes revealed that only 25% of the transcribed genes accumulated SNVs in an AID-dependent manner.[45] Mutational hotspots preferentially attacked by AID genotoxicity frequently possess unique sequence characteristics, so-called RGYW/WRCY motifs (where W = A or T, R = A or G and Y = C or T) in transcribed targets.[46] Moreover, a recent study reported clusters of various types of repeat sequences in the vicinity of cleaved sites in AID target genes.[47] Thus, target selection of AID-mediated mutagenesis might require both active transcription and sequence characteristics of the genes.

In conclusion, the findings in our study suggested that mutagenic activity of AID might contribute to the malignant transformation of hepatic progenitor cells to liver cancer cells via the induction of genetic alterations. Some of the actively transcribed genes in the liver-lineage cells preferentially accumulated SNVs and might contribute to the development of tumor cells. However, based on the model used in our study, we could not fully determine whether the developed tumors derived directly from the fetal hepatic progenitor cells or via mature hepatocytes, because the transplanted fetal progenitor cells differentiated into mature hepatocytes in the recipient liver.[27] Moreover, the truly significant driver mutations responsible for hepatocarcinogenesis remain unclear. Thus, further elucidation of the precise step of the AID-induced accumulation of genetic aberrations will be required to identify the genetic alterations that possess the key to the carcinogenesis process. In addition, the fractionation by fluorescence-activated cell sorting would be essential to identify the subset of hepatic stem/progenitor cells that play a role in the origin of tumor cells.

Acknowledgements

The authors are grateful for the support of Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists. They thank Dr. I. Ikai, Dr. T. Machimoto and Dr. R. Kamimura for helpful suggestion and Dr. K. Terasawa, Dr. Y. Fujii, Ms. K. Matsubara and Ms. C. Kakimoto for help with the analyses.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
2. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* 2002;31:339–46.
3. Hussain SP, Schwank J, Staib F, et al. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene* 2007;26:2166–76.
4. Guichard C, Amaddeo G, Imbeaud S, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat Genet* 2012;44:694–8.
5. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414:105–11.
6. Pardal R, Clarke MF, Morrison SJ. Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer* 2003;3:895–902.
7. Burkert J, Wright NA, Alison MR. Stem cells and cancer: an intimate relationship. *J Pathol* 2006;209:287–97.
8. Mishra L, Banker T, Murray J, et al. Liver stem cells and hepatocellular carcinoma. *Hepatology* 2009;49:318–29.
9. Dumble ML, Croager EJ, Yeoh GCT, et al. Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma. *Carcinogenesis* 2002;23:435–45.
10. Zender L, Spector MS, Xue W, et al. Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. *Cell* 2006;125:1253–67.
11. Xue W, Zender L, Miething C, et al. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 2007;445:656–60.
12. Yamashita T, Ji J, Budhu A, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* 2009;136:1012–24.
13. Durnez A, Verslype C, Nevens F, et al. The clinicopathological and prognostic relevance of cytokeratin 7 and 19 expression in hepatocellular carcinoma. A possible progenitor cell origin. *Histopathology* 2006;49:138–51.
14. Roskams T. Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. *Oncogene* 2006;25:3818–22.
15. Komuta M, Spee B, Vander Borght S, et al. Clinicopathological study on cholangiolocellular carcinoma suggesting hepatic progenitor cell origin. *Hepatology* 2008;47:1544–56.
16. Tang Y, Kitisin K, Jogunoori W, et al. Progenitor/stem cells give rise to liver cancer due to aberrant TGF-beta and IL-6 signaling. *Proc Natl Acad Sci U S A* 2008;105:2445–50.
17. Muramatsu M, Kinoshita K, Fagarasan S, et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* 2000;102:553–63.
18. Honjo T, Kinoshita K, Muramatsu M. Molecular mechanism of class switch recombination: linkage with somatic hypermutation. *Annu Rev Immunol* 2002;20:165–96.
19. Matsumoto Y, Marusawa H, Kinoshita K, et al. Helicobacter pylori infection triggers aberrant

expression of activation-induced cytidine deaminase in gastric epithelium. *Nat Med* 2007;13:470–6.
20. Endo Y, Marusawa H, Kou T, et al. Activation-induced cytidine deaminase links between inflammation and the development of colitis-associated colorectal cancers. *Gastroenterology* 2008;135:889–98.
21. Endo Y, Marusawa H, Kinoshita K, et al. Expression of activation-induced cytidine deaminase in human hepatocytes via NF-kappaB signaling. *Oncogene* 2007;26:5587–95.
22. Kou T, Marusawa H, Kinoshita K, et al. Expression of activation-induced cytidine deaminase in human hepatocytes during hepatocarcinogenesis. *Int J Cancer* 2007;120:469–76.
23. Chiba T, Marusawa H, Ushijima T. Inflammation-associated cancer development in digestive organs: mechanisms and roles for genetic and epigenetic modulation. *Gastroenterology* 2012;143:550–63.
24. Okazaki IM, Hiai H, Kakazu N, et al. Constitutive expression of AID leads to tumorigenesis. *J Exp Med* 2003;197:1173–81.
25. Morisawa T, Marusawa H, Ueda Y, et al. Organ-specific profiles of genetic changes in cancers caused by activation-induced cytidine deaminase expression. *Int J cancer* 2008;123:2735–40.
26. Saito M, Iwawaki T, Taya C, et al. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol* 2001;19:746–50.
27. Machimoto T, Yasuchika K, Komori J, et al. Improvement of the survival rate by fetal liver cell transplantation in a mice lethal liver failure model. *Transplantation* 2007;84:1233–9.
28. Ishii T, Yasuchika K, Machimoto T, et al. Transplantation of embryonic stem cell-derived endodermal cells into mice with induced lethal liver damage. *Stem cells* 2007;25:3252–60.
29. Nasu A, Marusawa H, Ueda Y, et al. Genetic heterogeneity of hepatitis C virus in association with antiviral therapy determined by ultra-deep sequencing. *PLoS One* 2011;6:e24907.
30. Fujiwara M, Marusawa H, Wang HQ, et al. Parkin as a tumor suppressor gene for hepatocellular carcinoma. *Oncogene* 2008;27:6002–11.
31. Ishii T, Yasuchika K, Fujii H, et al. In vitro differentiation and maturation of mouse embryonic stem cells into hepatocytes. *Exp Cell Res* 2005;309:68–77.
32. Nakatani T, Mizuhara E, Minaki Y, et al. Helt, a novel basic-helix-loop-helix transcriptional repressor expressed in the developing central nervous system. *J Biol Chem* 2004;279:16356–67.
33. Toda Y, Kono K, Abiru H, et al. Application of tyramide signal amplification system to immunohistochemistry: a potent method to localize antigens that are not detectable by ordinary method. *Pathol Int* 1999;49:479–83.
34. Yasuchika K, Hirose T, Fujii H, et al. Establishment of a highly efficient gene transfer system for mouse fetal hepatic progenitor cells. *Hepatology* 2002;36:1488–97.
35. Yoshikawa K, Okazaki IM, Eto T, et al. AID enzyme-induced hypermutation in an actively transcribed gene in fibroblasts. *Science* 2002;296:2033–6.
36. Chaudhuri J, Tian M, Khuong C, et al. Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature* 2003;422:726–30.
37. Ramiro AR, Stavropoulos P, Jankovic M, et al. Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. *Nat Immunol* 2003;4:452–6.
38. Kotani A, Okazaki IM, Muramatsu M, et al. A target selection of somatic hypermutations is regulated similarly between T and B cells upon activation-induced cytidine deaminase expression. *Proc Natl Acad Sci U S A* 2005;102:4506–11.
39. Mortazavi A, Williams BA, McCue K, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 2008;5:621–8.
40. Sell S, Leffert HL. Liver cancer stem cells. *J Clin Oncol* 2008;26:2800–5.
41. Katz SF, Lechel A, Obenauf AC, et al. Disruption of Trp53 in livers of mice induces formation of carcinomas with bilineal differentiation. *Gastroenterology* 2012;142:1229–39.
42. Nierhoff D, Ogawa A, Oertel M, et al. Purification and characterization of mouse fetal liver epithelial cells with high in vivo repopulation capacity. *Hepatology* 2005;42:130–9.
43. Oertel M, Menthena A, Chen YQ, et al. Purification of fetal liver stem/progenitor cells containing all the repopulation potential for normal adult rat liver. *Gastroenterology* 2008;134:823–32.
44. Duncan AW, Dorrell C, Grompe M. Stem cells and liver regeneration. *Gastroenterology* 2009;137:466–81.
45. Liu M, Duke JL, Richter DJ, et al. Two levels of protection for the B cell genome during somatic hypermutation. *Nature* 2008;451:841–5.
46. Rogozin IB, Pavlov YI, Bebenek K, et al. Somatic mutation hotspots correlate with DNA polymerase eta error spectrum. *Nat Immunol* 2001;2:530–36.
47. Kato L, Begum NA, Burroughs AM, et al. Non-immunoglobulin target loci of activation-induced cytidine deaminase (AID) share unique features with immunoglobulin genes. *Proc Natl Acad Sci U S A* 2012;109:2479–84.

Hepatology Research

Hepatology Research 2013; 43: 67–71 doi: 10.1111/j.1872-034X.2012.01020.x

Original Article

Efficacy and safety of prophylaxis with entecavir and hepatitis B immunoglobulin in preventing hepatitis B recurrence after living-donor liver transplantation

Yoshihide Ueda,[1] Hiroyuki Marusawa,[1] Toshimi Kaido,[2] Yasuhiro Ogura,[2] Kohei Ogawa,[2] Atsushi Yoshizawa,[2] Koichiro Hata,[2] Yasuhiro Fujimoto,[2] Norihiro Nishijima,[1] Tsutomu Chiba[1] and Shinji Uemoto[2]

Departments of [1]Gastroenterology and Hepatology and [2]Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Aim: Hepatitis B recurrence after liver transplantation can be reduced to less than 10% by combination therapy with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). The aim of this study was to evaluate the efficacy and safety of prophylaxis with entecavir (ETV), which has higher efficacy and lower resistance rates than LAM, combined with HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

Methods: Twenty-six patients who received ETV plus HBIG (ETV group) after LDLT for hepatitis B virus (HBV)-related end-stage liver disease were analyzed by comparing with 63 control patients who had received LAM plus HBIG (LAM group).

Results: The survival rates of the patients treated with ETV plus HBIG was 73% after both 1 and 3 years, and there was no statistical difference between the patients in the ETV group and LAM group. No HBV recurrence was detected during the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 4% at 3 years and 6% at 5 years in the LAM group. No patients had adverse effects related to ETV administration.

Conclusion: ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Key words: entecavir, hepatitis B, liver transplantation, living donor

INTRODUCTION

THE RECURRENCE OF hepatitis B virus (HBV) infection after liver transplantation for HBV-related diseases resulted in poor outcomes before the development of effective prophylaxis with lamivudine (LAM) and hepatitis B immunoglobulin (HBIG). Without the prophylaxis, the majority of patients developed recurrent infections due to HBV in the early phases after liver transplantation, and the recurrence resulted in rapidly progressive liver injury, early graft loss and reduced survival.[1–3] The development of prophylaxis dramatically reduced the post-transplant recurrence of hepatitis B and markedly improved prognosis. The most widely used prophylaxis so far has been a combination therapy of LAM and i.v. HBIG.

In the non-transplant setting, the long-term use of LAM resulted in high rates of emergence of resistance to the drug, with rates ranging 14–32% after 1 year and 60–70% after 5 years of treatment. In most cases, the resistance was the result of selection of LAM-resistant mutations in the YMDD motif of the DNA polymerase domain of HBV.[4] Moreover, the emergence of HBV strains with mutations that allow escape from hepatitis B surface antibody (anti-HBs) recognition has been reported in patients vaccinated for HBV,[5,6] in patients with chronic hepatitis B[7,8] and in liver transplant recipients after HBIG administration.[9–11] Therefore, the emergence of LAM resistance and HBIG resistance might

Correspondence: Dr Yoshihide Ueda, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Email: yueda@kuhp.kyoto-u.ac.jp

Received 13 February 2012; revision 6 March 2012; accepted 28 March 2012.

increase the risk of recurrence during long-term administration of LAM and HBIG, although the rate of HBV recurrence in liver transplant recipients who received prophylaxis with LAM and HBIG for more than 10 years has not been reported to date. At present, several nucleoside analogs are available for the treatment of chronic hepatitis B[4]. Among them, there is entecavir (ETV), a carbocyclic analogue of 2′-deoxyguanosine, which has been shown to have higher efficacy than LAM in patients with chronic hepatitis B. In addition, ETV has a higher genetic barrier to resistance than LAM. The resistance to ETV requires at least three mutations including rtM204V/I, which causes LAM-resistance, rtL180M, and a mutation at one of the following codons: rtT184, rtS202 or rtM250.[4] Therefore, ETV is now used as a first-line therapy in the treatment of chronic hepatitis B worldwide. Data available in the published work suggest that, in transplant recipients, ETV plus HBIG represents a better prophylaxis protocol than LAM plus HBIG for long-term prevention of HBV recurrence after liver transplantation. However, the efficacy and safety of this treatment is largely unknown.

The aim of this study was to evaluate the efficacy and safety of prophylaxis with ETV and HBIG in preventing hepatitis B recurrence after living-donor liver transplantation (LDLT).

METHODS

Patients

WE RETROSPECTIVELY ANALYZED the medical records of 97 patients who underwent LDLT for HBV-related end-stage liver diseases from September 2002 to December 2010. Of these, eight patients were excluded from our study because they had breakthrough hepatitis due to HBV with LAM-resistant mutations and were prescribed LAM plus adefovir before liver transplantation. Accordingly, 89 patients were enrolled in this study.

Prophylaxis with ETV or LAM combined with HBIG

Lamivudine plus HBIG therapy was given to all recipients with HBV-related end-stage liver diseases from September 2002 to November 2006, as reported previously.[12] From December 2006, we changed the protocol for prophylaxis to ETV plus HBIG. ETV at a dose of 0.5 mg/day or LAM at a dose of 100 mg/day was given before transplantation, usually when the patient was referred to the hospital and scheduled for transplantation. Preoperative ETV or LAM prophylaxis was followed by combination with HBIG after transplantation. The first application of HBIG at a dose of 200 IU/kg body mass was administrated i.v. during the anhepatic phase of LDLT, and repeated every day for the first 5 days post-surgery. HBV serological markers were examined at weekly intervals for the first 2 months after the transplant, then at monthly intervals, and 1000 IU of HBIG was periodically administrated to maintain the serum anti-HBs titers at more than 500 IU/L during the first 6 months and 200 IU/L thereafter throughout the follow-up period.[12]

Immunosuppression

Tacrolimus and low-dose steroid therapy were administrated to induce immunosuppression in most patients.[13] Mycophenolate mofetil was administrated to patients who experienced refractory rejection or required reduction of tacrolimus dose due to adverse events. Patients who received ABO blood-type-incompatible transplants were treated with rituximab, plasma exchange, and hepatic artery or portal vein infusion with prostaglandin E1 and methylprednisolone.[14]

Diagnosis of HBV activation

Activation of HBV was diagnosed when hepatitis B surface antigens (HBsAg) and/or HBV DNA became positive in the serum of the patients. After LDLT, HBsAg, anti-HBs and serum HBV DNA were measured at least at 3 monthly intervals. Serological HBV markers, including HBsAg, anti-HBs, hepatitis B core antibody, hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe), were measured by chemiluminescent enzyme immunoassay (Fuji Rebio, Tokyo, Japan). Serum HBV DNA titer was analyzed using a commercial polymerase chain reaction (PCR) assay (Amplicor HBV Monitor; Roche, Branchburg, NJ, USA). LAM-resistant YMDD mutant virus was detected by the PCR enzyme-linked mini-sequence assay.[15]

Statistical analysis

Baseline characteristics are shown in Table 1. For continuous variables, medians and ranges are given, and the significance of the data was analyzed with the Wilcoxon rank sum test. For categorical variables, counts are given, and the data were analyzed with the χ^2-test. Survival rates and the rates of patients who showed HBV activation after LDLT were estimated using the Kaplan–Meier method and compared using log–rank tests. $P < 0.05$ was considered significant.

Table 1 Baseline characteristics of 90 patients

	Entecavir + HBIG (n = 26)	Lamivudine + HBIG (n = 63)	P-value
Age (years)	55 (33–68)	53 (26–64)	0.062†
Men/women	19/7	46/17	0.995‡
Primary disease			0.595‡
Acute liver failure	6 (23%)	9 (14%)	
Liver cirrhosis, HCC$^-$	6 (23%)	20 (32%)	
Liver cirrhosis, HCC$^+$	14 (54%)	34 (54%)	
HBV markers before LDLT			
HBsAg$^+$	24 (92%)	61 (97%)	0.350‡
HBeAg$^+$	6 (23%)	18 (29%)	0.595‡
HBV DNA before LDLT	<2.6 (<2.6–7.6<)	3.7 (<2.6–7.6<)	0.010†
<2.6 log IU/mL	14 (54%)	19 (30%)	0.024‡
Follow-up period (months)	25.1 (0.2–58.6)	70.6 (0.5–109.2)	<0.001†

Qualitative variables are shown in number; and quantitative variables expressed as median (range).
†Wilcoxon rank sum test.
‡χ^2-Test.
HBeAg, hepatitis B e antigen; HBIG, hepatitis B immunoglobulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LDLT, living-donor liver transplantation.

RESULTS

Patient characteristics

TWENTY-SIX PATIENTS who received ETV plus HBIG (ETV group) after LDLT for HBV-related end-stage liver disease were included in this study. Baseline characteristics of these patients are listed in Table 1 and compared with those of 63 control recipients who received LAM plus HBIG (LAM group) at our institute already present in our database. The two groups of patients did not differ significantly by age, sex, primary diseases or serological markers for HBV before LDLT. Serum HBV DNA levels before LDLT were significantly lower in the ETV group than in the LAM group. Fourteen of 26 patients (54%) showed less than 2.6 log IU/mL of serum HBV DNA in the ETV group. Median follow-up period was 25.1 months (range, 0.2–58.6) in the ETV group, whereas it was 70.6 months (range, 0.5–109.2) in the LAM group.

Efficacy and safety of prophylaxis with ETV plus HBIG

Survival rates of the patients treated with ETV plus HBIG estimated by Kaplan–Meier analysis was 73% at both 1 and 3 years (Fig. 1a). There was no difference between the ETV group and the LAM group, in which survival rates were 81% at 1 year, 78% at 3 years and 73% at

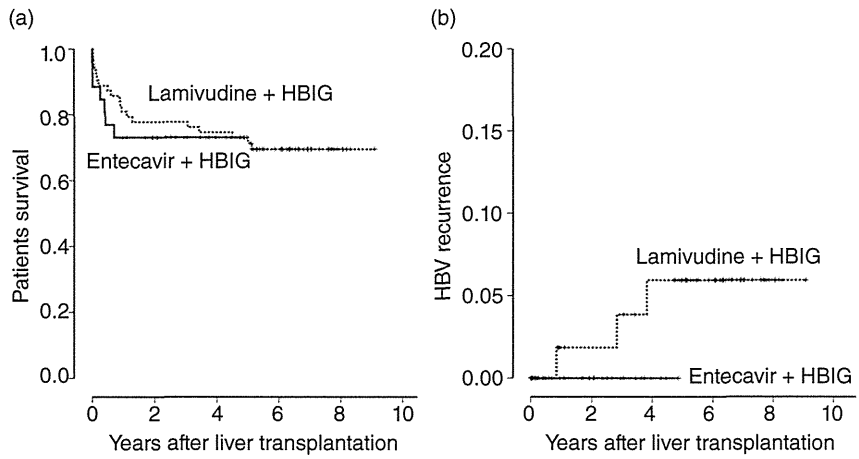

Figure 1 (a) Post-transplantation survival rates and (b) hepatitis B virus (HBV) recurrence after living-donor liver transplantation in HBV positive recipients who received entecavir and hepatitis B immunoglobulin (HBIG) (solid line), or lamivudine and HBIG (dotted line), estimated by Kaplan–Meier method.

5 years. Causes of death in patients in the ETV group were pneumonia ($n = 2$), sepsis ($n = 1$), pulmonary hemorrhage ($n = 1$), cerebral hemorrhage ($n = 1$), graft liver failure ($n = 1$) and multiple organ failure ($n = 1$), none of which were related to ETV. No HBV recurrence was detected in the median follow-up period of 25.1 months in the ETV group, whereas the HBV recurrence rate was 2% at 1 year, 4% at 3 years and 6% at 5 years in the LAM group (Fig. 1b). Three patients in the LAM group had HBV recurrence at 10, 34 and 46 months after LDLT. The emergence of HBV with LAM-resistant mutations in the YMDD motif was confirmed in two of the three patients. HBV mutations of another patient could not be determined because of the low level of serum HBV DNA. As the follow-up period of the ETV group was shorter than that of the LAM group and the HBV recurrence in the LAM group occurred in long-term follow-up after LDLT, the rate of HBV recurrence was not significantly different between the ETV and LAM groups. No patients had adverse events due to ETV administration.

DISCUSSION

IN THIS STUDY, we demonstrated that ETV combined with HBIG provides effective and safe prophylaxis in preventing hepatitis B recurrence after LDLT.

Two studies of patients receiving a combination of ETV and HBIG after liver transplantation have been previously reported.[16,17] One study demonstrated that 30 recipients who received ETV plus HBIG prophylaxis had no recurrence of HBV and no adverse effect relating to ETV.[17] The other study showed that no HBV recurrence was observed in two recipients with HBV-associated cirrhosis receiving ETV, tenofovir and HBIG.[16] Both studies showed the efficacy and safety of prophylaxis with ETV and HBIG in preventing short-term recurrence of HBV after liver transplantation. The current study confirmed their results for longer follow-up periods. Our results showed that prophylaxis with ETV and HBIG has similar efficacy and safety to that with LAM and HBIG, but did not show any further advantage of ETV compared to LAM treatment. Longer follow up might be needed to reveal the difference of HBV recurrence rate. One characteristic of our present report is that all patients in this study underwent LDLT. Our results suggest that prophylaxis with ETV and HBIG in patients after LDLT has similar efficacy and safety to patients after deceased-donor liver transplantation demonstrated in the previous reports.[16,17] More recently, efficacy of ETV monotherapy in preventing recurrence of HBV for liver transplant recipients with chronic hepatitis B was reported.[18] The study demonstrated that most patients showed disappearance of HBsAg and undetectable serum HBV DNA after liver transplantation without HBIG. Although long-term efficacy of ETV monotherapy needs be confirmed, both our data and previous reports suggest that ETV is an effective and safe antiviral agent in the post-transplant setting.

ACKNOWLEDGMENTS

THIS WORK WAS supported by Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (no. 21229009 and 23590972), Health and Labor Sciences Research Grants for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan, and a grant from Bristol-Myers-Squibb.

REFERENCES

1 Davies SE, Portmann BC, O'Grady JG *et al.* Hepatic histological findings after transplantation for chronic hepatitis B virus infection, including a unique pattern of fibrosing cholestatic hepatitis. *Hepatology* 1991; **13**: 150–7.
2 O'Grady JG, Smith HM, Davies SE *et al.* Hepatitis B virus reinfection after orthotopic liver transplantation. Serological and clinical implications. *J Hepatol* 1992; **14**: 104–11.
3 Todo S, Demetris AJ, Van Thiel D, Teperman L, Fung JJ, Starzl TE. Orthotopic liver transplantation for patients with hepatitis B virus-related liver disease. *Hepatology* 1991; **13**: 619–26.
4 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
5 Carman WF, Zanetti AR, Karayiannis P *et al.* Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325–9.
6 Hsu HY, Chang MH, Liaw SH, Ni YH, Chen HL. Changes of hepatitis B surface antigen variants in carrier children before and after universal vaccination in Taiwan. *Hepatology* 1999; **30**: 1312–7.
7 Kohno H, Inoue T, Tsuda F, Okamoto H, Akahane Y. Mutations in the envelope gene of hepatitis B virus variants co-occurring with antibody to surface antigen in sera from patients with chronic hepatitis B. *J Gen Virol* 1996; **77** (Pt 8): 1825–31.
8 Yamamoto K, Horikita M, Tsuda F *et al.* Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J Virol* 1994; **68**: 2671–6.