

A model of liver carcinogenesis originating from hepatic progenitor cells with accumulation of genetic alterations

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Activation-induced cytidine deaminase (AID) contributes to inflammation-associated carcinogenesis through its mutagenic activity. In our study, by taking advantage of the ability of AID to induce genetic aberrations, we investigated whether liver cancer originates from hepatic stem/progenitor cells that accumulate stepwise genetic alterations. For this purpose, hepatic progenitor cells enriched from the fetal liver of AID transgenic (Tg) mice were transplanted into recipient “toxin-receptor mediated conditional cell knockout” (TRECK) mice, which have enhanced liver regeneration activity under the condition of diphtheria toxin treatment. Whole exome sequencing was used to determine the landscape of the accumulated genetic alterations in the transplanted progenitor cells during tumorigenesis. Liver tumors developed in 7 of 11 (63.6%) recipient TRECK mice receiving enriched hepatic progenitor cells from AID Tg mice, while no tumorigenesis was observed in TRECK mice receiving hepatic progenitor cells of wild-type mice. Histologic examination revealed that the tumors showed characteristics of hepatocellular carcinoma and partial features of cholangiocarcinoma with expression of the AID transgene. Whole exome sequencing revealed that several dozen genes acquired single nucleotide variants in tumor tissues originating from the transplanted hepatic progenitor cells of AID Tg mice. Microarray analyses revealed that the majority of the mutations (>80%) were present in actively transcribed genes in the liver-lineage cells. These findings provided the evidence suggesting that accumulation of genetic alterations in fetal hepatic progenitor cells progressed to liver cancers, and the selection of mutagenesis depends on active transcription in the liver-lineage cells.

Tumorigenesis comprises multiple processes with a stepwise accumulation of genetic alterations that drive the progressive transformation of normal cells into highly malignant derivatives.¹ Recent studies of a large number of genomes in human cancer tissues clarified that cancer cells generally possess hundreds of somatic mutations and dysregulated gene expression profiles.^{2–4} Although the origin of cancer cells remains mostly unsolved at present, it might be difficult for fully differentiated cells to acquire these large numbers of

nucleotide alterations during their limited life-span to achieve malignant transformation. In contrast, stem/progenitor cells have a long lifetime to supply the differentiated progenies in each organ. Thus, it appears reasonable to assume that long-lived tissue stem/progenitor cells can accumulate genetic alterations and hence could be the origin of tumor cells. Consistent with this hypothesis, a number of studies have provided evidence that the mutations would most likely result in expansion of the altered stem cells, perpetuating and

Key words: liver cancer, hepatic progenitor cells, activation-induced cytidine deaminase (AID), mutation, liver carcinogenesis

Abbreviations: AFP: alpha-fetoprotein; AID: activation-induced cytidine deaminase; hHB-EGF: human heparin binding epidermal growth factor-like growth factor; CK: cytokeratin; DT: diphtheria toxin; GFP: green fluorescent protein; HCC: hepatocellular carcinoma; ICC: intrahepatic cholangiocarcinoma; MAPK: mitogen-activated protein kinase; PPAR: peroxisome proliferator-activated receptor; PCR: polymerase chain reaction; SNV: single nucleotide variant; Tg: transgenic; TRECK: toxin-receptor mediated conditional cell knockout. Additional Supporting Information may be found in the online version of this article.

S.K.K. and A.N. contributed equally to this work.

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What's new?

The accumulation of stepwise genetic aberrations is a defining feature of cancer. To better understand this process in liver cancer, the present study leveraged the mutagenic ability of activation-induced cytidine deaminase (AID) by using fetal hepatic progenitor cells from AID transgenic mice. The progenitor cells were transplanted into “toxin-receptor mediated conditional cell knockout” mice, where they accumulated genetic alterations sufficient to induce liver tumor formation, for both HCC and cholangiocarcinoma. The landscape of accumulated alterations was revealed by whole exome sequencing. The findings lend support to the idea that cancer arises from tissue stem/progenitor cells.

increasing the chances of additional mutations, leading to malignant transformation.^{5–8}

Several studies have provided evidence that hepatocellular carcinoma (HCC) might originate from hepatic stem/progenitor cells.^{9–12} A histologic study of clinical specimens also revealed that a substantial number of human HCC tissues have bipotential characteristics with coexpression of biliary and hepatocytic markers such as cytokeratin 7 (CK7), CK19, alpha-fetoprotein (AFP) and albumin.^{13,14} Conversely, all cholangiocarcinoma tissues examined showed hepatocellular differentiation in part of the tumor and expression of hepatic progenitor cell markers.¹⁵ Findings from a recent study also suggested that human HCC could arise as a consequence of the dysregulated proliferation of hepatic progenitor cells when the TGF- β and IL-6 signaling pathway was disrupted.¹⁶

Activation-induced cytidine deaminase (AID) can induce genetic alterations in human genome DNA sequences.^{17,18} Under physiological condition, AID is expressed almost exclusively in B lymphocytes, and plays a critical role not only in class switch recombination but also in somatic hypermutation of immunoglobulin genes. We recently demonstrated that inflammatory stimulation triggers aberrant AID expression in epithelial cells and initiates and/or promotes oncogenic pathways by inducing genetic alterations in various tumor-related genes.^{19,20} Indeed, AID expression is induced by proinflammatory cytokine stimulation and/or hepatitis C virus infection through NF- κ B activation in hepatocytes,²¹ and the resultant AID upregulation leads to the accumulation of somatic mutations in *TP53* and *c-MYC* genes, both of which are frequently mutated in human cancer tissues.^{21,22} These findings suggest that aberrant AID production induced by chronic inflammation in the liver contributes to hepatocarcinogenesis via the accumulation of genetic aberrations in tumor-related genes.²³

The fact that it usually takes over a year for AID transgenic (Tg) mice to accumulate the genetic aberrations required for carcinogenesis^{24,25} prompted us to speculate that constitutive expression of AID in the cells with long life-span might possess the higher risk for malignant transformation compared to that in the cells with the limited life-span. Therefore, in our study, we took advantage of the AID-mediated stepwise genotoxicity that recapitulates human hepatitis-associated carcinogenesis to investigate whether liver cancer originates from fetal hepatic progenitor cells with constitutive AID expression. Accordingly, we separated hepatic

progenitor cells enriched from the fetal liver of AID Tg mice followed by transplantation into recipient mice and examined whether recipient mice receiving AID-expressing hepatic progenitor cells develop liver tumors. Furthermore, to unveil the overall landscape of genetic alterations that accumulate in hepatic progenitor cells during the process of malignant transformation, we applied whole exome sequencing and determined the whole picture of genetic aberrations that accumulated in liver cancer cells originating from hepatic stem/progenitor cells.

Material and Methods**Animals**

The “toxin-receptor mediated conditional cell knockout” mice, which are homozygous for the albumin enhancer/promoter driven-human heparin binding epidermal growth factor-like growth factor (hHB-EGF) alleles, achieve the specific and conditional ablation of hepatocytes under the treatment of diphtheria toxin (DT).²⁶ AID Tg mice were previously described.²⁴ All animals were maintained in a specific pathogen-free facility at the Kyoto University Faculty of Medicine. All animal experiments were approved by the ethics committee for animal experiments and performed under the Guidelines for Animal Experiments of Kyoto University.

Isolation of enriched hepatic progenitor cells, cell transplantation and administration of diphtheria toxin

Hepatic progenitor cells were obtained from the fetal liver of pregnant wild-type, AID Tg and green fluorescent protein (GFP) Tg mice on gestational day 13.5 and were enriched through sphere formation as previously described.²⁷ Briefly, after the digestion of fetal liver tissues using a 0.5% collagenase solution (Invitrogen, Carlsbad, CA), fetal liver cells were subjected to floating culture to form spheres in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum. After 16 h incubation, the formed spheres were selected by gravity sedimentation and inoculated on Type-I collagen-coated culture plates (Asahi Glass, Chiba, Japan). After 24 h of incubation, floating hematopoietic cells were removed by washing and adhered cells were collected using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, MO) for 3 min. The dissociated cells were counted and suspended in a Ca²⁺-free Hank's balanced salt solution (Invitrogen) with fetal calf serum at a density of 5.0

$\times 10^6$ cells/mL as the enriched hepatic progenitor cells. To characterize the enriched hepatic progenitor cells, expression levels of fetal liver stem/progenitor markers, including albumin, AFP, DLK1, CK19 and CD133 were examined using both immunohistochemistry and RT-PCR. In addition, the lack of expression of the hematopoietic cell marker CD45 in sphere-derived hepatic progenitor cells was also confirmed by both immunostaining and RT-PCR.

To achieve efficient engraftment of transplanted hepatic progenitor cells to livers of the recipient mice, we used TRECK mice as a liver-specific regeneration model.²⁶ TRECK mice express DT receptor under control of the albumin promoter, and treatment with DT selectively and efficiently ablates the hepatocytes, resulting in enhanced liver regeneration and efficient colonization of transplanted hepatic progenitor cells.²⁷ The enriched hepatic progenitor cells were transplanted into 7- to 9-week old TRECK mice using an intrasplenic approach.^{27,28} We injected 0.2 mL of a cell suspension containing 1.0×10^6 hepatic progenitor cells. The DT was purified as described previously²⁶ and a total of 75 ng/kg DT was administered by intraperitoneal injection into recipient mice twice a week for 25 weeks from the day of cell transplantation.

Whole exome capture and massively-parallel sequencing

Massively-parallel sequencing was performed using the Illumina Genome Analyzer Iix (Illumina, San Diego, CA) as described.²⁹ End-repair of DNA fragments, addition of adenine to the 3' ends of DNA fragments, adaptor ligation and PCR amplification were performed according to the instructions. Exome capture was performed according to the NimbleGen Arrays Users Guide (Roche, Basel, Switzerland). The DNA library was hybridized to the custom designed NimbleGen Seq Cap arrays targeting a total of 17,089 genes, including 157,728 exons. These libraries were enriched independently using a minimal PCR amplification step of 18 cycles with Phusion High-Fidelity DNA polymerase. The concentration of enriched DNAs was measured by Quant-iT PicoGreen Reagent and Kits (Invitrogen) to make a working concentration of 10 nM. Cluster generation and sequencing was performed for 76 cycles on the Illumina Genome Analyzer Iix as described using the pair-end protocol and collecting 76 bases from each read.²⁹ The obtained images were analyzed and base-called using GA pipeline software version 1.4 with the default settings provided by Illumina. All sequence reads were deposited in the DNA Data Bank of Japan Sequence Read Archive; accession number DRA000601.

RNA preparation and hybridization to the microarray

Total RNA was extracted from adult mice (12-week old) liver tissues, bone marrow and the fetal liver at Day 13.5 of gestation using RNeasy Mini Kit (Qiagen, Valencia, CA). The details of the procedures for hybridization to the microarray were described previously.³⁰ RNA amplification and labeling

were performed according to the manufacturer's instructions (Agilent Technologies, Palo Alto, CA). Array image acquisition and feature extraction were performed using an Agilent G2505C scanner with feature extraction software (Agilent Technologies). Microarray data were deposited in the GEO database; accession number GSE39213.

Genome Analyzer sequence data analysis and variant filtering.

Semiquantitative reverse transcription PCR and quantitative real-time genomic and reverse transcription-PCR

Histology and immunohistochemistry

Southern blot analysis

Statistical analysis

These procedures are described in Supporting Information Materials and Methods.³¹⁻³³

Results

Enrichment of hepatic progenitor cells derived from fetal mouse liver

Enriched hepatic progenitor cells were obtained from the fetal liver of wild-type, AID Tg and GFP Tg mice through the formation of cell spheres, and the dissociated cells were cultured, counted and then transplanted into recipient mice (Fig. 1a). To characterize the sphere-derived hepatic cells used for the transplantation procedure, we first examined the expression of various marker genes in the fetal liver of wild-type mice. Immunohistochemistry revealed that expression of both the liver cell marker albumin and the hematopoietic cell marker CD45 were detectable in the fetal liver tissues (Fig. 1b). Cells expressing DLK1, a cell surface marker for hepatic stem/progenitor cells, comprised ~10% of the total cells of the fetal liver parenchyma (Supporting Information Fig. 2a). The enriched cell population specifically contained cells expressing the hepatocyte-lineage cell markers such as albumin and AFP, but no expression of CD45 was detectable in these sphere-forming cells (Fig. 1c). In addition, we confirmed that almost the entire enriched sphere-derived cell population expressed E-cadherin and DLK1, and a subset of those enriched cells expressed CK19 and CD133 (Supporting Information Fig. 2b). On the other hand, the floating cells that did not form spheres strongly expressed CD45 (Fig. 1d). RT-PCR also revealed that the sphere-forming cells prepared for the transplantation procedure expressed albumin, AFP, DLK1, CK19 and CD133 transcripts, but not CD45 (Fig. 1e). Similar results were obtained in the fetal liver of AID Tg mice (data not shown). These expression profiles of the collected sphere-derived cells were consistent with those found in previous studies³⁴ and indicated that the enriched cells derived from the fetal liver fully contained hepatic lineage progenitor cells.

Efficient engraftment of transplanted hepatic progenitor cells in the recipient liver

To enhance engraftment of the transplanted cells in the liver, we used TRECK mice as a liver-specific regeneration model.

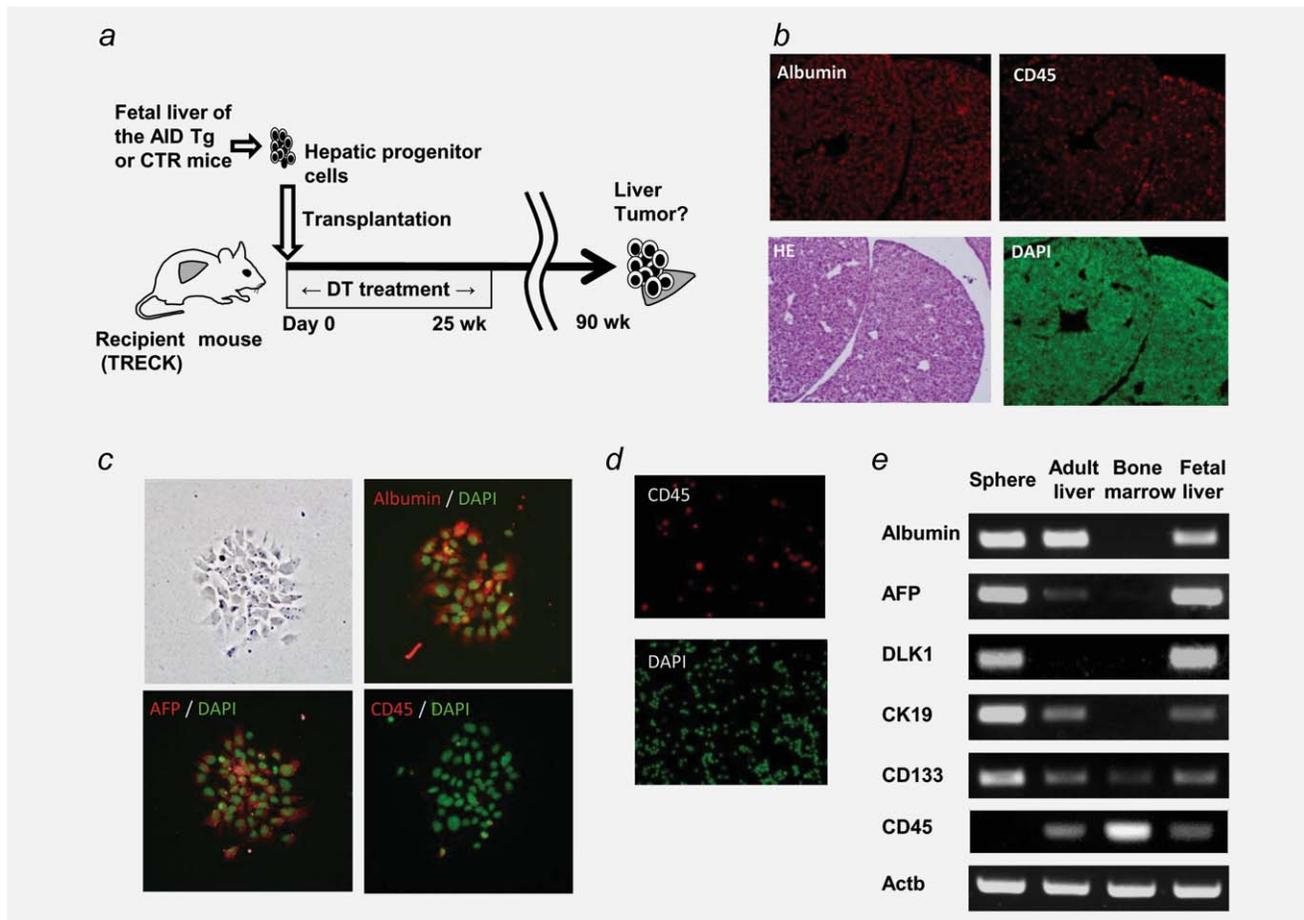


Figure 1. Enrichment of hepatic stem/progenitor cells from the fetal liver. (a) Schematic diagram showing the transplantation of the enriched hepatic stem/progenitor cells of AID Tg mice or control (CTR) mice into the recipient TRECK mice. DT was administered intraperitoneally twice a week to recipient (TRECK) mice for 25 weeks from the day of cell transplantation. The phenotypes were examined 90 weeks after transplantation. (b) Microscopic image (H&E staining) of the fetal liver tissues. Immunohistochemical staining for both the liver cell marker albumin and the hematopoietic cell marker CD45 are shown. (c) Immunohistochemical staining of the enriched cell population from the fetal liver via sphere formation for albumin, AFP and CD45. (d) Immunohistochemical staining of floating cells that did not form spheres for CD45. (e) Representative RT-PCR for the various phenotypic expression: albumin, AFP, DLK1, CK19, CD133, CD45 and control Actb (β -actin). Total RNA was extracted from the spheres of the enriched cell population from the fetal liver, adult liver tissue, bone marrow and fetal liver tissue.

These mice express hHB-EGF precursor, which functions as a DT receptor, under the control of an albumin promoter, and thus the hepatocytes of these mice are selectively ablated by the administration of DT.²⁶ We confirmed that the transcripts of hHB-EGF were specifically detectable in the liver of the TRECK mice (Fig. 2a), and immunohistochemistry also revealed that hHB-EGF protein expression was present in the TRECK mouse liver tissues (Fig. 2b). Serum alanine aminotransferase levels of a TRECK mouse were increased at 24 h after 75 ng/kg of DT administration, peaked at 48–72 h and subsequently returned to basal levels after 120 h (Fig. 2c). After repeated trials, we found that twice-weekly DT administration maintained the sublethal liver injury, resulting in the constitutive hepatic regeneration process. Under these experimental conditions, DT-mediated ablation of hepatocytes in TRECK mice resulted in the expansion of cells expressing E-

cadherin, EpCAM and HNF4 α accompanied by an increased number of the Ki67-positive cells, suggesting enhanced proliferation activity of hepatocyte-lineage cells including hepatic progenitor cells and mature hepatocytes in the TRECK liver tissues (Fig. 2d, and data not shown).

To examine the repopulation of the transplanted cells in the recipient liver, the hepatic progenitor cells of the GFP Tg fetal livers obtained in a similar way were introduced into the TRECK mice, followed by the repeated DT administration. At Day 7, the GFP-positive cells were observed as clusters, and at Day 30 the cluster of the GFP-positive cells was large enough to view macroscopically (Fig. 2e). Moreover, the cluster of hepatocytes derived from the transplanted GFP-positive enriched hepatic progenitor cells was detectable in the recipient liver even 90 days after the transplantation while no such cells were observed in the liver of mice

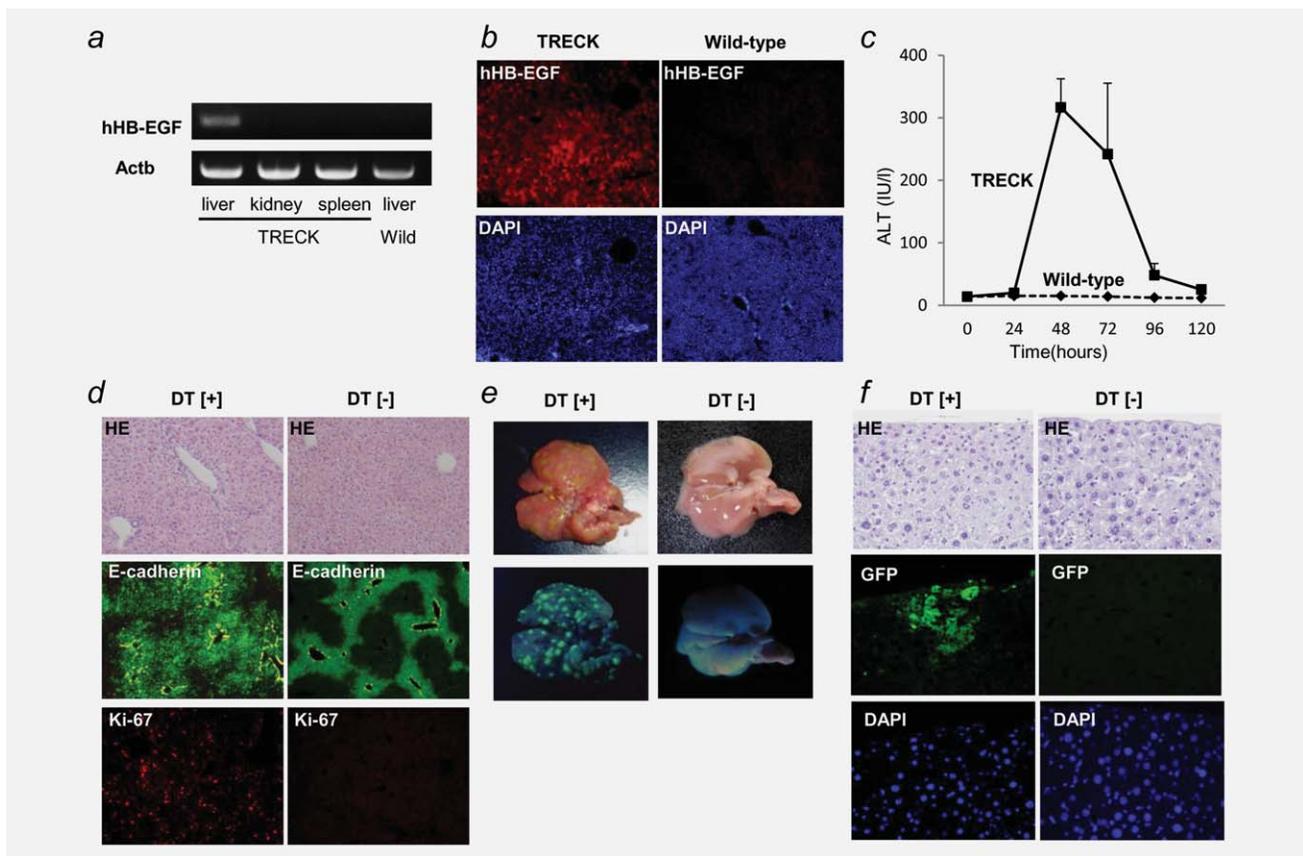


Figure 2. Efficient engraftment of the transplanted hepatic progenitor cells in the recipient liver. (a) The hHB-EGF expression in the liver, kidney and spleen of a TRECK mouse and the liver of a wild-type mouse determined by semiquantitative RT-PCR analysis. Upper, hHB-EGF expression; Lower, control 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. 2f). 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. 3a). 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. 3b, 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. 3b, lower panel).

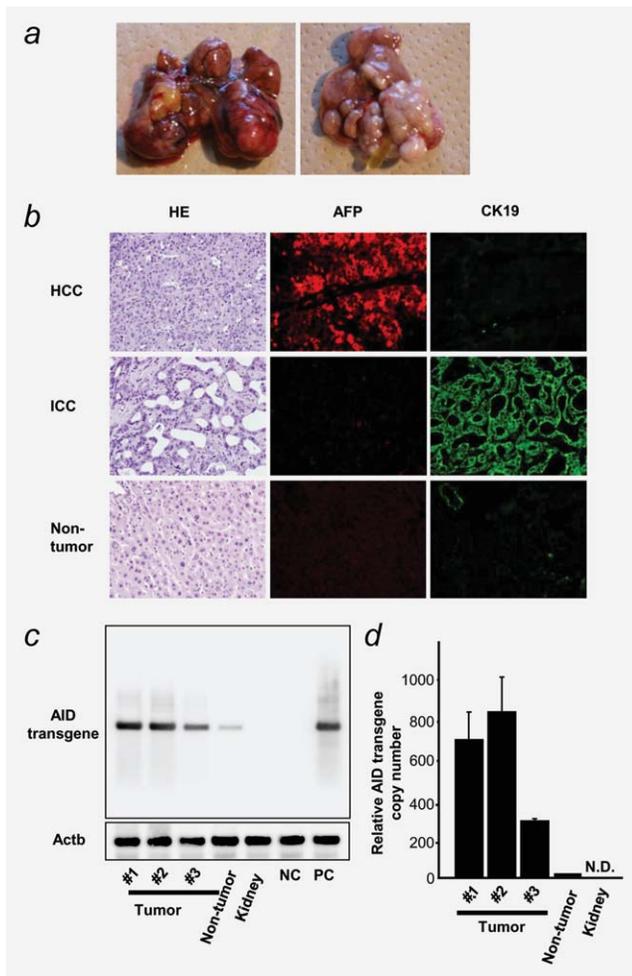


Figure 3. Development of tumors in livers receiving hepatic progenitor cells from AID Tg mice. (a) Macroscopic images of tumors that developed in recipient mice receiving progenitor cells from AID Tg mice. (b) Microscopic images of a liver tumor that developed in a recipient mouse receiving hepatic progenitor 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. 3c). 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. 3d). 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. 2c). 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 2d, 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%)

¹Genes 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 step-wise 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.

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