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Kuroki M, Ariumi Y, Hijikata M, Ikeda M, Dansako H, Wakita T, <u>Shimotohno K</u> , Kato N.	PML tumor suppressor protein is required for HCV production.	Biochem Biophys Res Commun	430	592-597	2013



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## 肝炎等克服緊急対策研究事業

### 肝移植後 C 型肝炎に対する治療法の標準化を目指した 臨床的ならびに基礎的研究

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## BASIC AND TRANSLATIONAL—LIVER

### Leptin Receptor Somatic Mutations Are Frequent in HCV-Infected Cirrhotic Liver and Associated With Hepatocellular Carcinoma

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**BACKGROUND & AIMS:** Hepatocellular carcinoma develops in patients with chronic hepatitis or cirrhosis via a stepwise accumulation of various genetic alterations. To explore the genetic basis of development of hepatocellular carcinoma in hepatitis C virus (HCV)-associated chronic liver disease, we evaluated genetic variants that accumulate in nontumor cirrhotic liver. **METHODS:** We determined the whole exome sequences of 7 tumors and background cirrhotic liver tissues from 4 patients with HCV infection. We then performed additional sequencing of selected exomes of mutated genes, identified by whole exome sequencing, and of representative tumor-related genes on samples from 22 cirrhotic livers with HCV infection. We performed *in vitro* and *in vivo* functional studies for one of the mutated genes. **RESULTS:** Whole exome sequencing showed that somatic mutations accumulated in various genes in HCV-infected cirrhotic liver tissues. Among the identified genes, the leptin receptor gene (*LEPR*) was one of the most frequently mutated in tumor and nontumor cirrhotic liver tissue. Selected exome sequencing analyses detected *LEPR* mutations in 12 of 22 (54.5%) nontumorous cirrhotic livers. *In vitro*, 4 of 7 (57.1%) *LEPR* mutations found in cirrhotic livers reduced phosphorylation of STAT3 to inactivate *LEPR*-mediated signaling. Moreover, 40% of *Lepr*-deficient (C57BL/KsJ-*db/db*) mice developed liver tumors after administration of thioacetamide compared with none of the control mice. **CONCLUSIONS:** Based on analysis of liver tissue samples from patients, somatic mutations accumulate in *LEPR* in cirrhotic liver with chronic HCV infection. These mutations could disrupt *LEPR* signaling and increase susceptibility to hepatocarcinogenesis.

**Keywords:** Liver Cancer; Whole Exome Sequencing; Genetics; STAT3.

Chronic inflammation plays an important role in the development of various human cancers. Indeed, many human cancers are closely associated with chronic inflammation, such as *Helicobacter pylori*-associated gastric cancer and inflammatory bowel disease-associated colorectal cancer.<sup>1,2</sup> On the other hand, tumor cells are

believed to be generated by a stepwise accumulation of genetic alterations in various tumor-related genes during the process of inflammation-associated carcinogenesis.<sup>3–6</sup> Thus, it is reasonable to assume that somatic mutations latently accumulate in inflamed tissues, where the risk of tumorigenesis is high. Consistent with this hypothesis, several studies have shown frequent somatic mutations in nontumorous inflammatory tissues.<sup>7,8</sup> To clarify the mechanisms of inflammation-associated carcinogenesis, it is important to unveil the genetic alterations that occur in the inflamed tissues before tumor development. The diversity of mutated genes and the low frequency of genetic alterations compared with tumor tissues, however, are obstacles to revealing the landscape of accumulated genetic aberrations in chronically inflamed nontumorous tissues.

Several possible molecular mechanisms have been proposed for the genetic alterations occurring in the inflammatory condition.<sup>9</sup> We recently showed that the expression of activation-induced cytidine deaminase (AID), a DNA/RNA mutator enzyme family member, links inflammation to an enhanced susceptibility to genetic aberration during the development of various gastrointestinal and hepatobiliary cancers.<sup>10–12</sup> One clear example of inflammation-associated cancer is human hepatocellular carcinoma (HCC). HCC arises in the background of chronic inflammation caused by hepatitis C virus (HCV) infection.<sup>13</sup> We showed that aberrant AID expression triggered by HCV infection and the resultant inflammatory response leads to the generation of somatic mutations in various tumor-related genes in the inflamed liver tissues.<sup>14,15</sup> The target genes of AID-mediated mutagenesis in the inflamed hepatocytes, however, remain unclear.

Recent advances in sequencing technology have enabled us to reveal the whole picture of human genome sequences in association with the risk of development of a variety of human diseases, including cancers.<sup>16,17</sup> Whole exome capture

**Abbreviations used in this paper:** AID, activation-induced cytidine deaminase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; Ig, immunoglobulin; TAA, thioacetamide.

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has identified several candidate driver genes in various human cancers.<sup>18–20</sup> Although deep sequencing on tumor tissues provides the most comprehensive analysis of the cancer genome, the genetic alterations accumulated in chronically inflamed tissues might provide an additional opportunity to clarify the early genetic changes required for carcinogenesis. In the present study, we applied whole exome sequencing to not only the tumor but also nontumorous liver tissues infected with HCV and found that somatic mutations of the leptin receptor gene (*LEPR*) latently underlie a subset of the cirrhotic liver tissues, providing the putative genetic basis for HCV-associated hepatocarcinogenesis.

## Materials and Methods

### Whole Exome Capture and Massively Parallel Sequencing

Massively parallel sequencing was performed as described previously.<sup>21,22</sup> Fragmented DNA (more than 5  $\mu$ g) was used to prepare each DNA sequencing library. The DNA libraries were prepared according to the instructions provided with the Illumina Preparation Kit (Illumina, San Diego, CA). Whole exome sequence capture was then performed using SeqCap EZ Human Exome Library v2.0 (Roche, Madison, WI) according to the manufacturer's instructions. Cluster generation was performed on the Illumina cluster station (using their TruSeq PE Cluster Kit v5). Paired-end sequence for  $2 \times 76$  base pairs was performed on the Illumina Genome Analyzer IIx (using their SBS Kits v5). Data collection and base calling were performed using SCSv2.9/RTA 1.9, and the resultant data files were converted to the FASTQ format.

### Selected Exome Capture and Massively Parallel Sequencing

Fragmented DNA (1  $\mu$ g) was used to prepare each DNA sequencing library. The DNA libraries were prepared using TruSeq DNA Sample Prep Kits (Illumina) according to the manufacturer's protocol. Selected gene capture (*TP53*, *CTNNB1*, *LEPR*) was performed using the SeqCap EZ Choice library (Roche) according to the manufacturer's recommendations. Cluster generation and multiplexed paired-end sequencing for  $2 \times 71 + 7$  base pairs was performed as described previously. Data collection and base calling were performed as described previously and demultiplexed using CASAVA version 1.8.2 software (Illumina) with the default settings.

Sequence data analysis and variant filtering, patients, cell culture and transfection, immunoblotting analysis, and animal experiments are described in [Supplementary Methods](#) and [Supplementary Figures 1 and 2](#).

## Results

### Whole Exome Sequencing Identified the Mutation Signature of Synchronous HCCs in Patients With Chronic HCV Infection

To explore the genetic basis of HCV-associated hepatocarcinogenesis, we first determined the whole exome sequences in matched pairs of HCC and background liver tissues obtained from 4 patients with chronic HCV infection

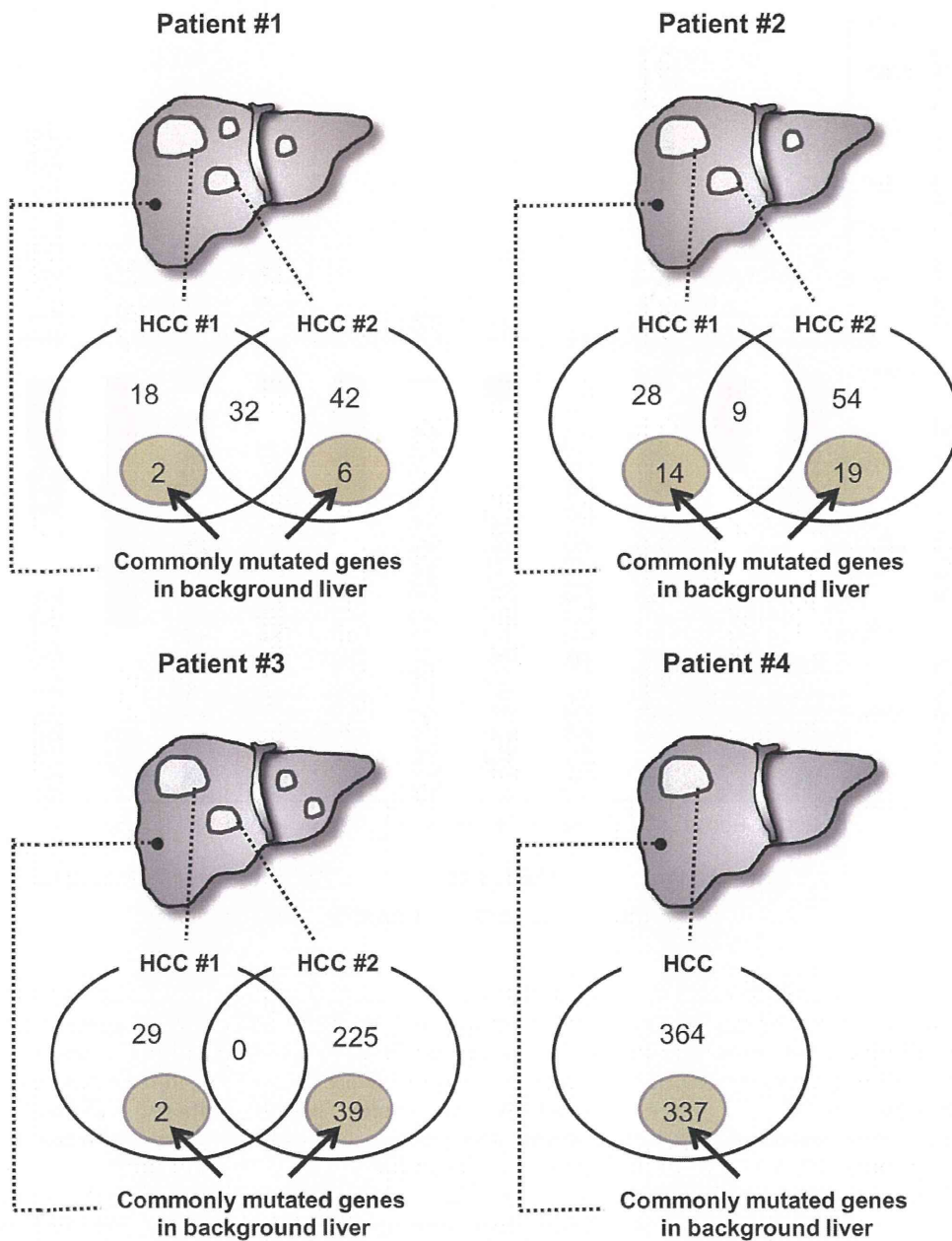
([Supplementary Table 1](#), patients 1–4). Three of these patients had multiple HCCs, and one had a solitary HCC in the liver. To compare the mutation signature in synchronous HCCs that developed in the same background liver, we determined the whole exome sequences of 2 representative HCCs in 3 cases and a solitary HCC in the remaining case ([Figure 1](#)). These 7 HCCs from 4 patients comprised 2 well-differentiated and 5 moderately differentiated HCCs, and the background liver tissue showed the histological characteristics of cirrhosis. To subtract the normal variants of each individual from the somatic mutations, we also determined the whole exome sequences of matched peripheral lymphocytes in each patient.

On average, we generated approximately 3.1 gigabases of sequence per sample, 80.1% of which were aligned with the human reference genome (Human Genome Build 37.3), and the mean coverage in the targeted regions was 33.8-fold ([Supplementary Table 2](#)). The variant filtering process is summarized in [Supplementary Figure 1](#), and the overall error rate in our current platform was confirmed to be less than 0.2%, as described previously.<sup>21</sup> Overall, a total of 970 nucleotide positions in 768 different genes were mutated at a frequency of more than 20% of reads in the 7 HCC tissues ([Supplementary Table 3](#)). Among them, 79 genes were recurrently mutated in 2 or more tumor tissues (data not shown). These genes included representative tumor-related genes associated with HCC such as *TP53* (mutated in 2 of 7 tumors). Pathway analyses using Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>) revealed that metabolic pathway-related genes were most frequently damaged in HCC tissues (5 of 7 tumors) ([Supplementary Table 4](#)).

Interestingly, the mutation signature was remarkably different between the synchronously developed HCCs in each patient ([Figure 1](#)). In patient 3, none of the genes were commonly mutated in the 2 tumors examined, while 29 and 225 genes acquired independent somatic mutations in each tumor, respectively. In contrast, 32 genes (64.0% of mutated genes of HCC 1 in patient 1) and 9 (24.3% of mutated genes of HCC 1 in patient 2) were commonly mutated in the synchronously developed HCCs of those patients, indicating that the synchronous HCCs that developed in patient 1 or 2 shared a common pattern of genetic aberrations. These findings may suggest that the synchronous tumors in patients 1 and 2 were derived from common tumor-precursor cells or developed through intrahepatic metastasis, whereas the tumors in patient 3 developed independently in a multicentric manner.

### Somatic Mutations Accumulated in the Cirrhotic Liver With HCV Infection

Whole exome sequencing also revealed a large number of nucleotide alterations in nontumorous cirrhotic liver tissues. In some cases, the total number of mutated genes in nontumorous liver was higher than that in tumor tissues, while the mutation frequency in nontumorous tissues tended to be lower than that in the matched tumor tissues ([Figure 2](#)). Sorting Intolerant From Tolerant (SIFT) functional impact predictions (<http://provean.jcvi.org/index.php>) revealed that the mean percentage of somatic



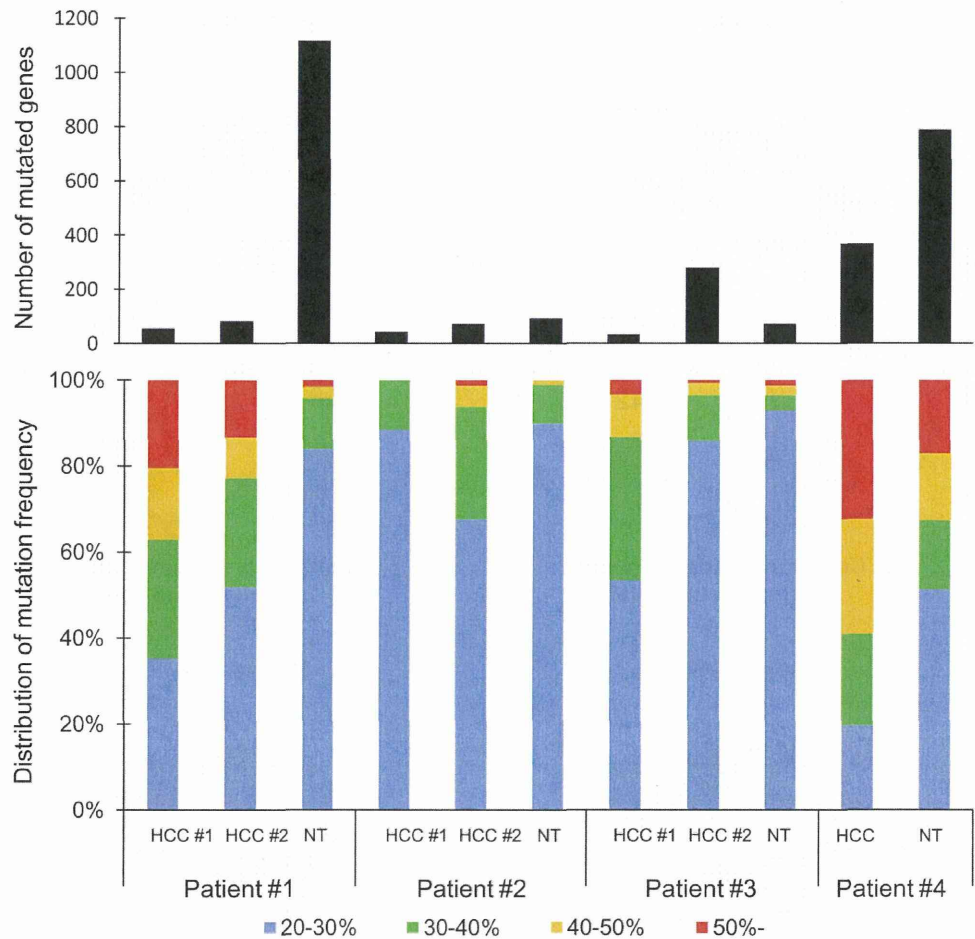
**Figure 1.** Schematic diagram showing the number of mutated genes in tumors and the number of genes commonly mutated in both tumor and the matched nontumorous liver tissues. Patients 1, 2, and 3 had synchronously developed HCCs, and patient 4 had a solitary HCC. Venn diagrams represent the number of mutated genes in each HCC tissue determined by whole exome sequencing. The numbers of genes commonly mutated in the synchronously developed multiple HCCs were 32, 9, and 0 in patients 1, 2, and 3, respectively. Among the mutated genes in HCC (at a frequency of more than 20% of reads), the number of genes commonly mutated in both HCC and matched nontumorous background liver (at a frequency of more than 5% of reads) is shown in shaded circles.

mutations predicted to be “damaging” in tumorous and nontumorous tissues was 20.4% and 13.1%, respectively, suggesting that somatic mutations that accumulated in nontumorous tissues included “passenger” mutations with less functional significance more frequently than those that accumulated in tumor tissues (Supplementary Table 3). We also identified a total of 448 indels in 7 HCC tissues (Supplementary Table 5), while fewer indels were detected in all of the nontumorous cirrhotic liver tissues examined (Supplementary Table 6). Consistent with previous studies,<sup>19</sup> we found that one-third of the mutations that accumulated in the exome sequences of HCC tissues were enriched as C>T; G>A transition, followed by A>G; T>C.

Similar to tumor tissues, C>T; G>A transition mutations were most frequently detected in nontumorous cirrhotic tissues (Supplementary Figure 3).

The aim of this study was to identify the somatic mutations in the nontumorous HCV-positive cirrhotic liver that may contribute to tumorigenesis. Therefore, we focused on the genes commonly mutated in both tumor and nontumorous liver tissues from the same patient. Because few genes commonly acquired somatic mutations with a frequency of more than 20% both in the tumor and the matched nontumorous liver tissues, we selected potential somatic mutations in nontumorous tissues that represented more than 5% of the total reads for further evaluation





**Figure 2.** The number of mutated genes and the distribution of mutation frequency in tumor and nontumorous cirrhotic liver tissues. The number of mutated genes (upper panel) and the distribution of mutation frequency (lower panel) detected by whole exome sequencing in each sample are shown (at a frequency of more than 20% of reads). Patients 1, 2, and 4 had more mutated genes in nontumorous liver tissue than those in HCC, while the mutation frequency at each nucleotide position in the majority of nontumorous cirrhotic liver tissues was <30%. NT, nontumorous cirrhotic liver.

(Supplementary Figure 1). The 5% threshold in nontumorous liver was chosen because common polymorphisms in each patient were excluded by determining the nucleotide changes with a frequency of more than 5% in the matched normal samples, such as peripheral lymphocytes.<sup>18,23</sup>

Based on these criteria, nucleotide positions that were commonly mutated in both the tumor (at a frequency of more than 20% of reads) and the matched background liver (at a frequency of more than 5% of reads) of each patient were detected (Figure 1). Among them, we focused on 40 mutations that result in amino acid changes (Supplementary Table 7) and found that only 2 genes, *LEPR* and *ZNF408*, were recurrently mutated with a frequency greater than 5% of reads in nontumorous cirrhotic livers from 2 of the 4 patients (listed as the top 2 genes in Supplementary Table 7). Of these 2 genes, we focused on *LEPR*, which has mutations that have been correlated with various human diseases, such as obesity and metabolic disorders.<sup>24</sup>

### Identification of *LEPR* as the Recurrently Mutated Gene in Cirrhotic Livers With HCV Infection

We designed a selected sequence capture system that enabled us to enrich the whole exonic sequences of the

*LEPR* followed by deep sequencing. In addition, selected exonic capture of *TP53* and *CTNNB1*, the representative driver genes for hepatocarcinogenesis,<sup>19,20,25</sup> was performed on the same cohort. Accordingly, the selected exonic sequencing was applied to 22 additional HCV-positive cirrhotic liver tissues, 10 HCC tissues, and matched peripheral lymphocytes from 22 patients (Supplementary Table 1, patients 5–26). Selected exome sequencing generated a mean coverage of 996-, 1656-, and 2348-fold on *LEPR*, *TP53*, and *CTNNB1*, respectively (Supplementary Table 8). The variant filtering process is summarized in Supplementary Figure 2, and we detected both high-frequency (at a frequency of more than 20% of reads) and low-frequency (at a frequency of 1%–20% of reads) mutations separately.

High-frequency mutations in *TP53* and *CTNNB1* were detectable in 1 of 10 (10%) and 1 of 10 (10%) of the HCCs, respectively (Table 1), and these rates in the HCCs were consistent with recent deep-sequencing studies.<sup>19,20</sup> None of the nontumorous liver tissues possessed high-frequency mutations in *TP53* or *CTNNB1*; however, low-frequency mutations of *TP53* and *CTNNB1* were detected in 17 of 22 (77.3%) and 12 of 22 (54.5%) of the nontumorous livers, respectively. These findings indicated that somatic mutations in the representative cancer driver genes latently

**Table 1.** Number of Tumor Tissues and Nontumorous Cirrhotic Liver Tissues With Somatic Mutations at High and Low Frequencies in *TP53*, *CTNNB1*, and *LEPR*

	<i>LEPR</i>	<i>TP53</i>	<i>CTNNB1</i>
High-frequency mutations (>20%)			
Tumor (n = 10)	0	1	1
Nontumor (n = 22)	1 <sup>a</sup>	0	0
Low-frequency mutations (1%–20%)			
Tumor (n = 10)	9	8	9
Nontumor (n = 22)	12 <sup>a</sup>	17	12

<sup>a</sup>One patient had both high-frequency and low-frequency mutations in *LEPR*.

accumulated with a relatively low frequency in the cirrhotic livers with HCV infection.

Interestingly, we also found high- and/or low-frequency mutations in *LEPR* in both tumor and nontumorous liver tissues. Indeed, 9 of 10 (90%) tumors and 12 of 22 (54.5%) nontumorous cirrhotic livers possessed high- and/or low-frequency mutations in *LEPR* (Table 1). Notably, some somatic mutations were commonly detected in different positions of the same patient's liver. For example, C1084T (reference position: 65557165) mutations of *LEPR* were detected in the right, left, and caudate lobes of one patient (Supplementary Table 1, patient 11), suggesting that some of the hot spots of the acquired somatic mutations in the *LEPR* gene are commonly present in hepatocytes of the same liver underlying HCV infection. On the other hand, no mutations in *LEPR* were identified by deep sequencing analysis of noncirrhotic liver tissues from patients with chronic HCV infection or liver tissue from patients without HCV infection. (Supplementary Table 9). To confirm the somatic mutations present in *LEPR* in the nontumorous liver, we validated the candidate mutations by Sanger sequencing. For this purpose, we determined the sequences of exons 9 and 10 of *LEPR* of at least 50 randomly picked clones that were amplified from the nontumorous liver tissues of each patient. Although it was difficult to detect all the low-frequency mutations using the conventional cloning-sequencing method, we confirmed that somatic mutations were recurrently accumulated in *LEPR* of nontumorous cirrhotic liver tissues (Supplementary Figure 4).

### *LEPR* Mutations Found in HCV-Positive Cirrhotic Liver Resulted in the Disruption of Downstream Signaling

Selected exome sequencing detected low-frequency mutations at a total of 650 nucleotide positions of *LEPR* in 12 of 22 (54.5%) HCV-positive cirrhotic liver tissues. Although the nucleotide changes were unevenly distributed throughout the whole *LEPR* exonic sequences, we detected 67 nucleotide alterations at the immunoglobulin (Ig) domain of *LEPR*, 38 of which (56.7%) were recurrently

mutated in 2 or more patients (Figure 3A). Among them, nonsynonymous mutations that caused the amino acid changes were detected at 62 of the 67 (92.5%) nucleotide positions, and 10 of the 62 were also mutated in at least one HCC tissue examined in this study. Histological examination revealed no significant association between the presence of *LEPR* mutations and the level of fatty changes in the liver tissue (data not shown).

To explore the functional relevance of *LEPR* mutations detected in HCV-positive cirrhotic liver tissues, we randomly selected 7 *LEPR*s with a mutated Ig domain from 62 nonsynonymously mutated *LEPR*s and examined the downstream signaling properties of the mutated *LEPR* in vitro.

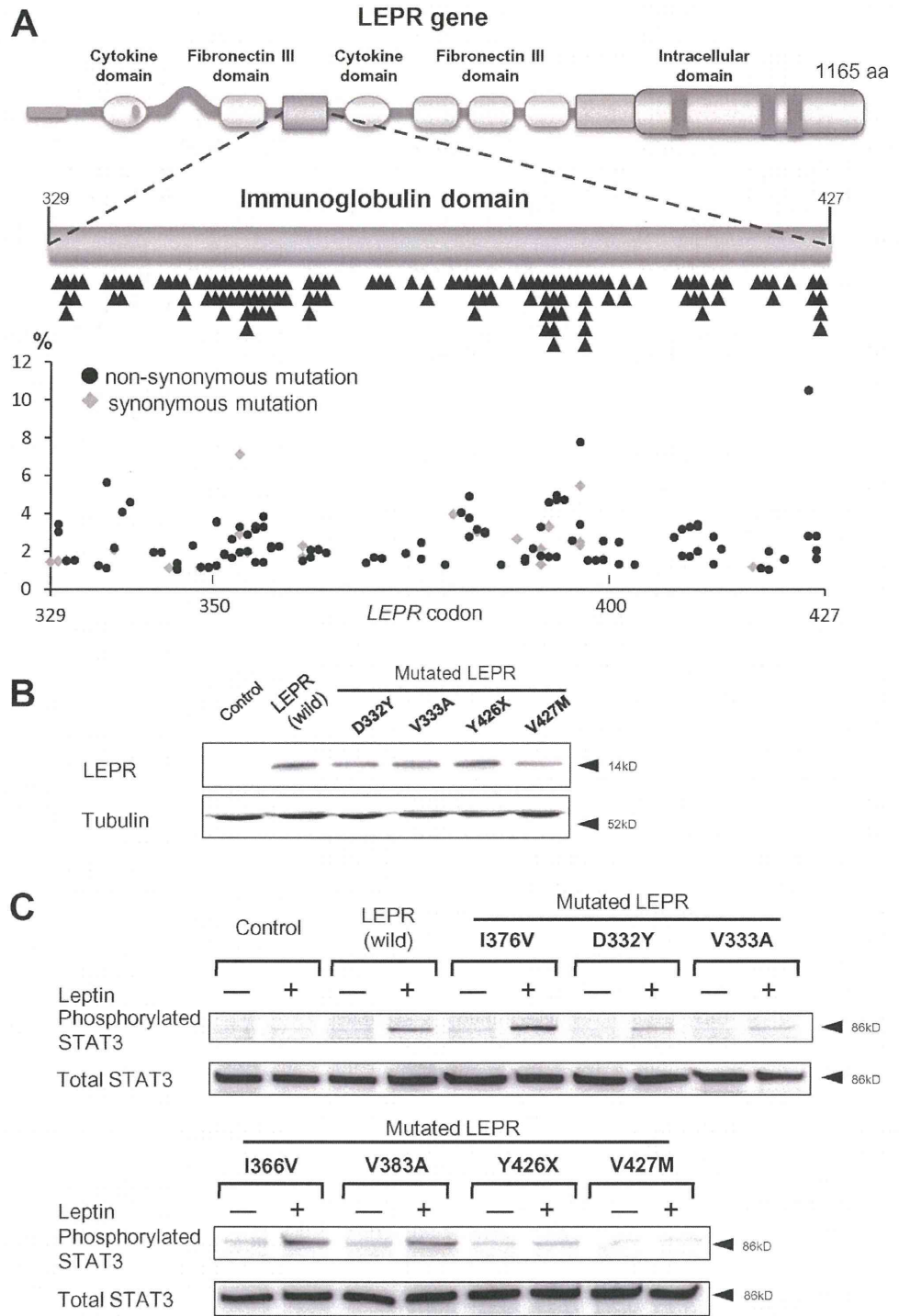
Accordingly, we subcloned the mutated *LEPR*s and constructed expression plasmids encoding those mutant *LEPR*s (Figure 3B). We first confirmed that only a small amount of endogenous *LEPR* expression was observed in both HEK293 and HepG2 cells (Supplementary Figure 5) and the induction of the phosphorylation of STAT3 by wild-type *LEPR* in the presence of recombinant human leptin (Figure 3C). In contrast, 4 of 7 (57.1%) mutations in the Ig domain of *LEPR* resulted in the reduction or loss of STAT3 phosphorylation in vitro (Figure 3C). To clarify the functional significance of *LEPR* mutations, the cell proliferation rate was determined in HepG2 cells expressing either wild-type or mutated *LEPR*s that were identified in HCV-positive cirrhotic liver tissues using the lentivirus system.<sup>26</sup> Up-regulation of cyclin D1 and/or E transcripts as well as enhanced cell proliferation were observed in the cells with expression of the mutated *LEPR* gene compared with wild-type cells, while there was no difference in the expression levels and subcellular localization between wild-type and mutated *LEPR* protein (Supplementary Figure 6). These findings indicate that some of the somatic mutations that latently accumulated in the Ig domain of *LEPR* of the cirrhotic liver tissue might cause dysfunction of *LEPR*-mediated signaling in the cells with those somatic mutations.

### *LEPR* Dysfunction Enhanced Susceptibility to Tumorigenesis

To determine the functional relevance of *LEPR* dysfunction on development of liver cancer, we examined whether disruption of the *LEPR* gene contributes to liver tumorigenesis using a genetically altered mouse model, the *Lepr*-deficient C57BL/KsJ-*db/db* mouse (hereafter referred to as *db/db* mouse).<sup>27</sup> Thioacetamide (TAA), a putative carcinogen, is well established to induce liver fibrosis and tumorigenesis in a murine model.<sup>28</sup> Thus, we conducted an assay to evaluate whether *LEPR* insufficiency alters the effects of TAA-mediated tumorigenesis. Accordingly, TAA was prepared at a concentration of 0.02%, a relatively low dose compared with the carcinogenic dose,<sup>29</sup> and administered in drinking water to mice for 24 weeks. The body weight of the *db/db* mice was about twice that of their lean littermates, and *db/db* mice had hepatomegaly even after normalizing the liver weight to the body weight (Figure 4A). Histological



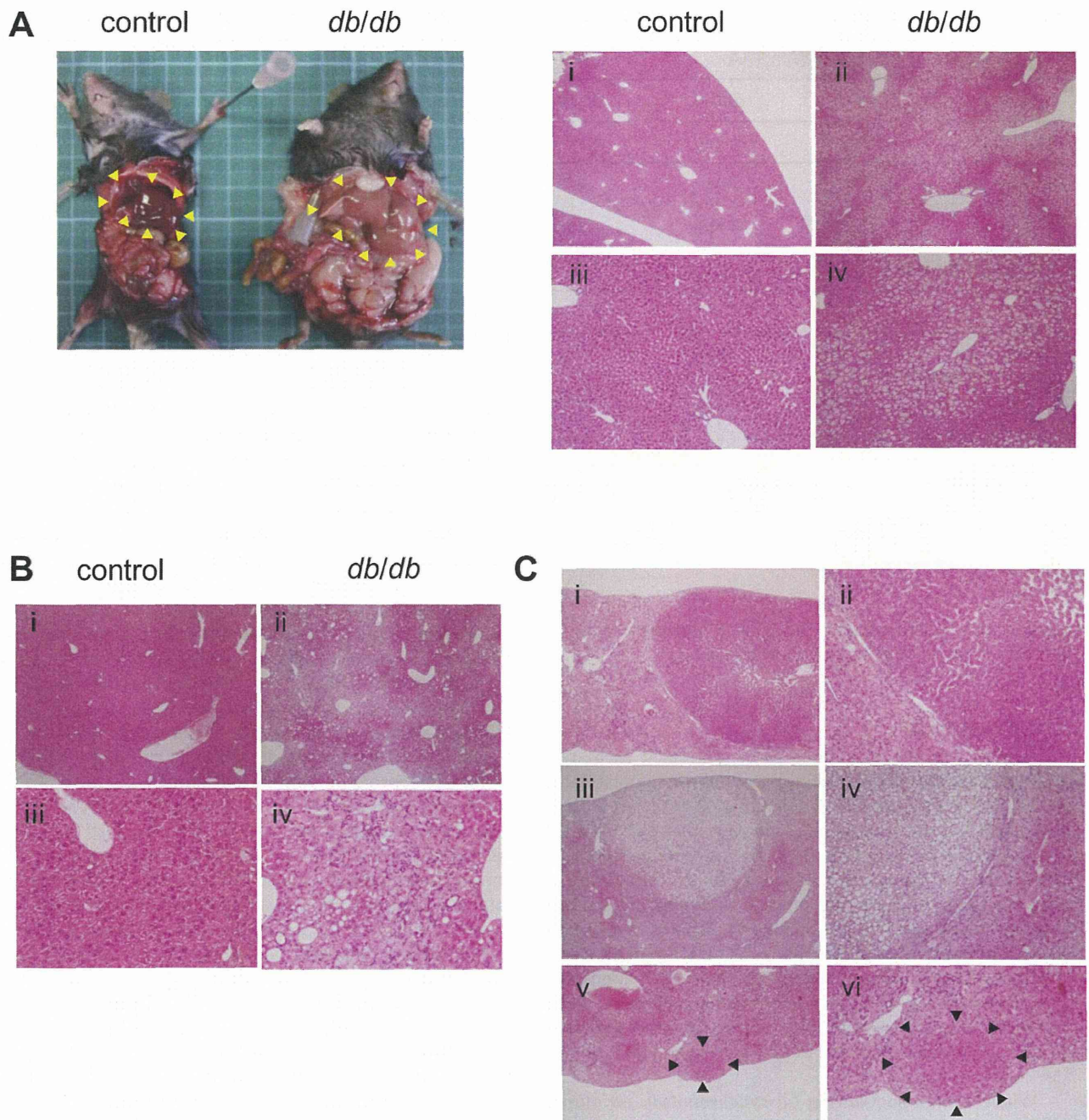
**Figure 3.** Distribution of mutations in the *LEPR* sequence in HCV-positive cirrhotic liver tissues. (A) Schematic diagram of the *LEPR* gene (top panel) and the Ig domain (middle panel). Mutated positions in the Ig domain are indicated by black triangles. A total of 38 of 67 (56.7%) mutated nucleotide positions of the Ig domain were recurrently mutated in 2 or more HCV-positive cirrhotic liver tissues. Frequencies of non-synonymous (black circles) and synonymous (gray diamonds) mutations at each nucleotide position of the Ig domain of each sample are shown (lower panel). Nonsynonymous mutations were detected at 62 of the 67 nucleotide positions. (B and C) HEK293 cells were transfected with constructs encoding wild-type or representative various mutated *LEPRs* that were identified in HCV-positive cirrhotic liver tissues. Control: empty vector. (B) Immunoblotting was performed on the lysate of the cells expressing either wild-type or a mutated Ig domain (D332Y, V333A, Y426X, and V427M) of the *LEPR* gene using anti-Myc antibodies. (C) After transfection, the cells were treated with or without recombinant leptin protein. Total protein was isolated and immunoblot analysis was performed using anti-phospho-STAT3 (upper panel) and anti-total STAT3 (lower panel).



examination revealed the accumulation of lipids within individual hepatocytes in the *db/db* mouse liver, a typical feature of steatosis (Figure 4A).

After administering TAA, blood levels of alanine aminotransferase were substantially elevated in *db/db* mice compared with control mice (Supplementary Table 10).

Consistently, histological examination revealed that inflammatory activity was more severe in the liver of *db/db* mice than in the liver of control mice (Figure 4B). None of the control mice treated with TAA showed tumorigenesis 24 weeks after administration of TAA. In contrast, macroscopic liver nodules developed in 4 of 10 (40%) *db/db* mice



**Figure 4.** Tumors developed in *db/db* mice treated with TAA. (A) Representative macroscopic (left panel) and microscopic (right panel) images (H&E stain) of the liver from a *db/db* mouse and a littermate control mouse without administration of TAA. The liver of the *db/db* mouse is enlarged and yellowish compared with the control (yellow arrowheads). Histological analysis of the liver tissue of (ii and iv) *db/db* mouse and (i and iii) control mouse. Original magnification: 4× (upper panels) and 10× (lower panels). (B) Microscopic images (H&E stain) of (i and iii) control mice and (ii and iv) *db/db* mice treated with TAA for 24 weeks. In the *db/db* mice, inflammatory cell infiltration was extensively observed in the liver tissues underlying prominent steatosis (ii and iv). Original magnification: 4× (upper panels) and 20× (lower panels). (C) Microscopic images (H&E stain) of nodules that developed in *db/db* mice treated with TAA for 24 weeks (i–vi). Liver cancers developed in 2 *db/db* mice (i–iv). Arrowheads indicate hepatocyte hyperplasia (v and vi). Original magnification: 4× (left panels) and 10× (right panels).

that received the same dose of TAA during the same observation period (Table 2). Histological examination revealed that 2 *db/db* mice with liver nodules developed

well-differentiated HCC (Figure 4C). In addition, the remaining nodules that developed in *db/db* mouse liver showed features of hepatocyte hyperplasia. These findings