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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.[1,2] 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.[3–6] 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.[7,8] 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.[9] 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.[10–12] 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.[13] 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.[14,15] 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.[16,17] 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.[18–20] 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.[21,22] Fragmented DNA (more than 5 μg) 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 2×76 base pairs was performed on the Illumina Genome Analyzer IIx (using their SBS Kits v5). Data collection and base calling were performed using SCS v2.9/RTA 1.9, and the resultant data files were converted to the FASTQ format.

Selected Exome Capture and Massively Parallel Sequencing

Fragmented DNA (1 μg) 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.[21] 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