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Two-Step Forward Genetic Screen in Mice Identifies Ral GTPase-Activating Proteins as Suppressors of Hepatocellular Carcinoma

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BACKGROUND & AIMS: High-throughput sequencing technologies have identified thousands of infrequently mutated genes in hepatocellular carcinomas (HCCs). However, high intratumor and intertumor heterogeneity, combined with large numbers of passenger mutations, have made it difficult to identify driver mutations that contribute to the development of HCC. We combined transposon mutagenesis with a high-throughput screen of a small-hairpin RNA (shRNA) library to identify genes and pathways that contribute to HCC development. **METHODS:** Sleeping beauty transposons were mobilized in livers of transgenic mice predisposed to develop hepatocellular adenoma and HCC owing to expression of the hepatitis B virus surface antigen. This whole-genome mutagenesis technique was used to generate an unbiased catalogue of candidate cancer genes (CCGs). Pooled shRNA libraries targeting 250 selected CCGs then were introduced into immortalized mouse liver cells and the cells were monitored for their tumor-forming ability after injection into nude mice. **RESULTS:** Transposon-mediated mutagenesis identified 1917 high-confident CCGs and highlighted the importance of Ras signaling in the development of HCC. Subsequent pooled shRNA library screening of 250 selected CCGs validated 27 HCC tumor-suppressor genes. Individual shRNA knockdown of 4 of these genes (*Acaa2*, *Hbs1l*, *Ralgapa2*, and *Ubr2*) increased the proliferation of multiple human HCC cell lines in culture and accelerated the formation of xenograft tumors in nude mice. The ability of Ralgapa2 to promote HCC cell proliferation and tumor formation required its inhibition of Rala and Ralb. Dual inhibition of Ras signaling via Ral and Raf, using a combination of small-molecule inhibitor RBC8 and sorafenib, reduced the proliferation of HCC cells in culture and completely inhibited their growth as xenograft tumors in nude mice. **CONCLUSIONS:** In a 2-step forward genetic screen in mice, we identified members of the Ral guanosine triphosphatase–activating protein pathway and other proteins as suppressors of HCC cell proliferation and tumor growth. These proteins might serve as therapeutic targets for liver cancer.

Keywords: Sleeping Beauty; Liver Cancer; RalGAPs; Kinase Inhibitor.

Hepatocellular carcinoma (HCC) is a deadly cancer with a mortality rate ranking third among all cancers worldwide.[1] Although the incidence of HCC in the United States is lower than the world average, it has been increasing for the past 20–30 years.[1] Surgical resection and liver transplantation are the only curable treatments for HCC, but this can be applied to only a limited number of early stage patients. There are very few treatment options for patients with advanced-stage HCC, thus the overall 5-year survival of HCC patients is less than 12%.[2] Sorafenib is the only potentially effective antineoplastic agent against advanced-stage HCC, but this drug prolongs survival by only a few months.[3] To discover effective treatments for HCC and improve the prognosis of HCC patients, it is crucial to comprehensively understand the molecular mechanisms driving HCC.

International cancer genome projects, including the International Cancer Genome Consortium[4] and The Cancer Genome Atlas (TCGA),[5] have used next-generation sequencing to read thousands of human cancer genomes. As a part of this effort, more than 500 cases of HCC have been sequenced. The hope was that this large collection of sequence data would help clarify the molecular pathogenesis of HCC. This extensive collection of sequence data, however, has identified only a few genes, such as *TERT*, *CTNNB1*, *TP53*, and *ARID2*, that are mutated in more than 5% of HCC patients. In contrast, infrequent mutations (<5% of patients) were identified in more than 10,000 genes,[6] suggesting the existence of high intratumor and intertumor heterogeneity in addition to a number of passenger mutations. Therefore, although it is not arduous to sequence

Abbreviations used in this paper: **CCG, candidate cancer genes; CIS, common transposon insertion site; gCIS, gene-centric common transposon insertion site–calling method; GTPase, guanosine triphosphatase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFLC, immortalized fetal liver cell; mRNA, messenger RNA; PCR, polymerase chain reaction; RalGAP, Ral guanosine triphosphatase-activating protein; shRNA, short hairpin RNA; siRNA, small interfering RNA; SB, sleeping beauty; TCGA, The Cancer Genome Atlas; TSG, tumor-suppressor gene.**

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large numbers of individual cancer genomes in the $1000 genome era, it is still a challenge to determine the true drivers of HCC. Indeed, an analysis of 4742 human cancer genome sequences have indicated that thousands more tumor samples will need to be sequenced to generate a statistically reliable catalogue of cancer genes mutated in more than 2% of patients.[7]

To help discriminate between drivers and passengers of HCC, we previously performed a sleeping b*eauty* (SB) transposon mutagenesis screen in a hepatitis B virus (HBV)-induced mouse model of HCC.[8] Cells that acquire a survival advantage through transposon-mediated random gene activation or inactivation become enriched over time in these mice, which eventually give rise to tumors. Sequencing of these tumors enabled us to identify 21 candidate early stage drivers and a much larger number of candidate late-stage drivers.[8] Importantly, this collection of candidate cancer genes (CCGs) was enriched for genes that are mutated, deregulated, or function in signaling pathways important for human HCC, with a striking 1199 genes being linked to cellular metabolic processes. This may explain in part the large intertumor and intratumor heterogeneity observed in HCC patients. Many different combinations of mutant cancer genes may exist that are sufficient to drive HCC. Taken together, these data, and current limitations of human cancer genome sequencing, led us to perform additional transposon mutagenesis screens to generate a comprehensive catalogue of HCC CCGs. We then used a high-throughput short hairpin RNA (shRNA) library screening method to show the value of this resource for identifying new drivers of HCC. Collectively, our studies show that 2-step forward genetic screens in mice provide a rich resource for identifying new drivers of HCC, as well as new potential therapeutic drug targets.

Materials and Methods

Mice

Rosa26-loxP-STOP-loxP-SB11 transposase knock-in mice (Gt[ROSA]26Sortm2[sb11]Njen),[9] *T2/Onc3* (12740) transposon transgenic mice (TgTn[sb-T2/Onc3]12740Njen),[9] B6.Cg-Tg(Alb-cre)21Mgn/J (strain 003574; The Jackson Laboratory, Bar Harbor, ME) and C57BL/6J-Tg(Alb1HBV)44Bri/J (strain 002226; The Jackson Laboratory) were used in this study. Mice homozygous or heterozygous for the Alb-Cre transgene and heterozygous for the hepatitis B surface antigen (HBsAg) transgene were crossed to mice homozygous for Rosa26-lsl-SB11 and T2/Onc3 (12740) to generate Alb-Cre/+; T2Onc3/+; Rosa26-lsl-SB11/+; HBsAg/+ mice and Alb-Cre/+; T2Onc3/+; Rosa26-lsl-SB11/+ mice, which were used for the SB screen. Mice of both sexes were used for these experiments but only tumors from male mice were used to identify transposon insertion sites. Mice were monitored twice weekly for general health and tumor burden. Palpation was used to estimate tumor size. Mice were euthanized and necropsied when the tumor size was estimated at 2 cm diameter or more, or when mice showed symptoms of morbidity/moribundity, including ruffled coat, lack of activity, distended abdomen, or breathing difficulty, whichever occurred first. Liver tumors seen grossly were saved for histopathologic examination and molecular analysis. Adenoma and HCC were diagnosed histopathologically by a veterinarian pathologist based on published histologic criteria.[10] Adenomas and carcinomas were combined for molecular analysis. Mice were housed in a specific pathogen–free facility with a 12-hour light/dark cycle. All mouse procedures were approved by our Institutional Animal Care and Use Committee (Institute of Molecular and Cell Biology and Houston Methodist Research Institute).

Identification of Transposon Insertion Sites

Identification of transposon insertion sites was performed using splinkerette polymerase chain reaction (PCR) to produce barcoded PCR products that were pooled and sequenced using the 454 GS-Titanium (Roche, Branford, CT) platform.[8] All informative sequence reads were mapped to the B6 mouse genome (mm9) and merged together to identify nonredundant SB insertion sites. Cloning and mapping of the transposon insertion sites was performed as previously described.[8] The likelihood of local hopping of the SB transposon is increased on the chromosome where the transposon concatamer is located.[8] Thus, all insertions on chromosome 9, donor site of T2/Onc3, were removed from the data set for subsequent analysis. A total of 359,425 and 145,280 mapped reads corresponding to 62,482 and 23,746 nonredundant insertion sites were identified from 106 tumors in 16 Liver-Onc3/HBsAg mice and 48 tumors from 7 Liver-Onc3 mice, respectively (Supplementary Data Sets 1 and 2). Common transposon insertion site (CISs) were identified using the gene-centric CIS-calling method (gCIS), which looks for a higher density of transposon insertions within the coding regions of all RefSeq genes than predicted by chance.[8] Multiple insertions were located in noncoding areas of targeted genes. T2/Onc transposons have bidirectional splice acceptors and a splice donor following a strong promoter. Therefore, a transposon inserted into the noncoding region of a target gene stops endogenous transcription of the target gene at the upstream exon, and the transposon also drives its transcription from the downstream exon if inserted into sense orientation of the target gene.

Comparative Oncogenomics to Select CCGs for Pooled shRNA Library Screen

For the pooled shRNA library screen, genes were prioritized further from the 1917 high-confidence CCGs using the following criteria. For the somatic mutation cohort, nonsynonymous somatic mutation data of human HCC was obtained from 4 original articles[11–14] and the International Cancer Genome Consortium data portal (https://dcc.icgc.org/). A total of 6949 nonsynonymous mutations in 5025 genes from 121 HCC patients were used for the comparison and CCGs with 3 or more nonsynonymous mutations were selected. For the gene expression cohort, normalized microarray gene expression data of human HCCs and nontumor liver tissues in GSE14520, GSE25097, and GSE36376 was downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) (PMID: 22202459, 21955977, and 23800896, respectively). CCGs with 1.5-fold or more down-regulation of messenger RNA (mRNA) abundance in HCCs compared with nontumor liver tissue in 2 or 3 data sets were selected. For the trunk driver