Research Article

Cancer Prevention Research

Prevalence of Aflatoxin-Associated *TP53R249S* Mutation in Hepatocellular Carcinoma in Hispanics in South Texas

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Abstract

We aimed to determine whether aflatoxin dietary exposure plays a role in the high incidence of hepatocellular carcinoma (HCC) observed among Hispanics in South Texas. We measured *TP53R249S* somatic mutation, hallmark of aflatoxin etiology in HCC, using droplet digital PCR and RFLP. *TP53R249S* mutation was detected in 3 of 41 HCC tumors from Hispanics in South Texas (7.3%). We also measured *TP53R249S* mutation in plasma cell-free DNA (cfDNA) from 218 HCC patients and 96 Hispanic subjects with advanced fibrosis or cirrhosis, from South Texas. The mutation was detected only in Hispanic and Asian HCC patients, and patients harboring *TP53R249S* mutation were significantly younger and had a shorter overall survival. The mutation was not detected in any Hispanic subject with advanced fibrosis or cirrhosis. Genes involved in cell-cycle control of chromosomal replication and in BRCA1-dependent DNA damage response were enriched in HCCs with *TP53R249S* mutation. The E2F1 family members, E2F1 and E2F4, were identified as upstream regulators. *TP53R249S* mutation was detected in 5.7% to 7.3% of Hispanics with HCC in South Texas. This mutation was associated with a younger age and worse prognosis. *TP53R249S* was however not detected in Hispanics in South Texas with cirrhosis or advanced fibrosis. Aflatoxin exposure may contribute to a small number of HCCs in Hispanics in South Texas, but the detection of *TP53R249S* mutation in plasma cfDNA is not a promising biomarker of risk assessment for HCC in subjects with cirrhosis or advanced fibrosis in this population. *Cancer Prev Res; 11(2); 103–12. ©2017 AACR.*

Introduction

Liver cancer is the second leading cause of cancer-related mortality worldwide with an estimated 745,000 deaths in 2012 (1, 2). In the United States, in contrast to the 25% decline in overall cancer mortality from 1991 to 2014, deaths from liver cancer have increased at the highest rate of all cancer sites and liver cancer incidence has increased sharply, second only to thyroid cancer (3). There are significant geographic and ethnic variations in the incidence of hepatocellular carcinoma (HCC), the major form of liver cancers, with the highest rates observed in Hispanics in South Texas (4, 5). The main risk factor of HCC is liver cirrhosis. Other risk factors contributing to HCC include chronic hepatitis B or C virus (HBV, HCV) infection, alcohol abuse, nonalcoholic steatohepatitis (NASH) and aflatoxin exposure (6). We previously reported that the prevalence of cirrhosis in Hispanics in South Texas is 0.94%, which is 4-fold higher than the national prevalence (7). Risk factors independently associated with cirrhosis in this population are central obesity, diabetes, HCV, and alcohol with a remarkable population attributable fraction of 65.3% from central obesity. In 20% of the cases, no known risk factor was identified (7).

In this article, we explored the possibility that aflatoxin exposure could be a contributing risk factor for HCC in Hispanics in South Texas. Aflatoxin is a mycotoxin

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produced by the common fungi *Aspergillus flavus* and *Aspergillus parasiticus* and is classified as group 1 carcinogen by the World Health Organization (8). Aflatoxin B1 (AFB1) is the most carcinogenic type of aflatoxin. AFB1 consumption and HCC are epidemiologically linked in much of the developing world, including Southeast Asia, China, and sub-Saharan Africa (9). The highly mutagenic AFB1-DNA adduct induces primarily G:C→T:A mutations and the molecular hallmark of AFB1 exposure and HCC risk is a specific mutation at codon 249 of the TP53 gene (10). This mutation is a single-base substitution at the third base of codon 249 (AGG to AGT), which replaces an arginine by a serine (*R249S*) (11). In regions with high aflatoxin exposure such as Gambia and Qidong in China, *TP53R249S* mutation has been detected in 35% to 61% of HCC tumors (12–14). In moderate aflatoxin exposure regions such as Thailand, *TP53R249S* mutation is present in 8% to 27% of HCC (15, 16). This mutation is detected in 19% of HCCs in Mexico and 16% of HCCs in Brazil, suggesting a medium exposure of aflatoxin in these countries (17, 18). In low aflatoxin exposure regions such as in Europe and the United States, *TP53R249S* mutation was only rarely found if at all, and patients with that mutation were often immigrants from Asia or Africa (19). *TP53R249S* mutation can also be detected in circulating cell-free DNA (cfDNA), with good correlation with aflatoxin exposure. *TP53R249S* mutation has been detected in cfDNA from 40% of HCC patients in Qidong, from 36% to 53% of HCC patients in Gambia and from 26% to 34% of HCC patients in Thailand (20–24). The *TP53R249S* mutation was not detected in cfDNA from European patients with HCC and was never measured in cfDNA from patients with HCC in the United States (24). Aflatoxin exposure is not believed to play any role in hepatocarcinogenesis in the United States. However, aflatoxin contamination has been reported in foods from Texas, particularly after periods of drought (25), and in an early study, AFB1 levels have been detected in HCC patients treated at MD Anderson Cancer Center in Houston (26). Although 1% of the general U.S. population had detectable levels of AFB1–albumin adduct in years following the severe drought of 1998, a study performed among Hispanics in Bexar County, Texas, showed that AFB1–albumin adduct was detected in 21% of the participants with a median level almost 5-fold higher than that detected in the U.S. population (27). A recent study in South Texas reported that HCC patients had higher serum and urine aflatoxin levels than matched controls (28). Therefore, because pockets of exposed persons have been reported in South Texas, in particular among Hispanics, and because of the high incidence of HCC in this population, we aimed to determine the prevalence of the *TP53R249S* mutation in HCCs from Hispanics in South Texas, particularly in counties with high liver cancer rates, and addressed the role aflatoxin exposure may play in the etiology of HCC in this population.

Materials and Methods

Patients and biospecimens

The study was approved by the Institutional Review Boards of all collaborating institutions. Formalin-fixed paraffin-embedded (FFPE) HCCs of 41 Hispanic patients were collected at the University of Texas Medical Branch, Galveston, and at Doctors Hospital at Renaissance, Edinburg (Texas). The demographic and clinical parameters of the 41 Hispanic patients with HCC are described in Supplementary Table S1. DNA and RNA extraction was performed from the tumor areas and from distant nontumoral liver, using QIAamp DNA FFPE Tissue Kit (Qiagen) and High Pure FFPET RNA Isolation Kit (Roche). Plasma samples from 218 histologically confirmed HCC patients were collected between 2002 and 2010, prior to treatment at MD Anderson Cancer Center (Houston, TX). The demographic and clinical parameters of the 218 HCC patients are described in Supplementary Table S2. Plasma samples were also collected from 96 participants of the Cameron County Hispanic Cohort (CCHC) with aspartate transaminase (AST) to platelet ratio index (APRI) scores ≥ 1, indicative of the presence of cirrhosis/advanced fibrosis. The demographic and clinical parameters of the 96 CCHC subjects were described previously (7). All these subjects were enrolled in 4 Texas counties with high rates of liver cancer. The 2014 liver cancer mortality rates in all 4 counties were significantly higher than the statewide rate of $8.74_{/100,000}$, ranging from $9.51_{/100,000}$ to $14.30_{/100,000}$. Dietary information was obtained from a survey administered to CCHC participants ($n = 2,606$) and showed that 56% of Mexican Americans in Cameron/Webb are consuming corn tortillas at least once a day, including 19.1% consuming 3 or more corn tortillas daily. cfDNA was extracted from all plasma samples (500 μL) using QIAamp Circulating Nucleic Acid Kit (Qiagen). FFPE tumor DNA and cfDNA samples were quantified using Qubit Fluorometer and dsDNA High-Sensitivity Assay Kit (Thermo Fisher Scientific). Their quality was assessed using a fragment analyzer and high sensitivity Genomic DNA Analysis Kit (Advanced Analytical Technologies). RNA samples were assessed for quantity using Qubit Fluorometer with RNA High-Sensitivity Assay Kit (Thermo Fisher Scientific) and for quality using Tapestation with High-Sensitivity RNA Kit (Agilent Technologies).

Droplet digital PCR

TP53R249S mutation was detected using the QX200 droplet digital PCR (ddPCR) system (Bio-Rad Laboratories) and the following assays: dHsaCP2000088 for wild-type (wt) *TP53* and dHsaCP2000087 for *TP53R249S* allele.

Mutant and wt *TP53* alleles were differentiated by the fluorophores attached to the probes, with HEX fluorescence for wt *TP53* alleles and FAM fluorescence for mutant *TP53R249S* alleles. Four microliters of DNA was used in each reaction. Thermocycling conditions were: 95°C for 10 minutes, followed by 40 cycles of 94°C for 30 seconds and 55°C for 1 minute, followed by 98°C for 10 minutes. The sealed plates were then placed in the droplet reader for detection of complete ddPCR reactions in individual droplets. The data were analyzed using QuantaSoft software (Bio-Rad Laboratories, Inc.). Samples with a R249S allele fraction $\geq$0.1% and with at least 2 visible mutant signals were considered positive for the mutation as described previously (29, 30).

RFLP

Exon 7 of TP53 was amplified with two rounds of PCR as described previously (24). Both PCR reactions involved a 15-minute hotstart DNA polymerase (KAPA Biosystems) activation at 95°C, 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final 5-minute extension at 72°C. The pairs of primers were: F1-CTTGCCACAGGTCTCCCCAA and R1-AGGGGTCAGCGGCAAGCAGA; F2-AGGCGCACTGGCCTCATCTT and R2-TGTGCAGGGTGGCAAGTGGC. The PCR products were then digested by HaeIII restriction endonuclease (Promega Corporation) and separated on 3% agarose gel stained with ethidium bromide. PLC/PRF/5 and HepG2 hepatoma cell lines were used as positive and negative controls for *TP53R249S* mutation, respectively (31).

Gene expression profiling by RNA-Seq

Barcoded, Illumina-compatible stranded total RNA libraries were prepared using the TruSeq Stranded Total RNA Sample Preparation Kit (Illumina). DNase I–treated RNA (250 ng) was depleted of cytoplasmic and mitochondrial ribosomal RNA using Ribo-Zero Gold (Illumina). After purification, the RNA was fragmented using divalent cations, and double-stranded cDNA was synthesized using random primers. The ends of the resulting double-stranded cDNA fragments were repaired, 5′-phosphorylated, 3′-A tailed and Illumina-specific indexed adapters were ligated. The products were purified and enriched with 12 cycles of PCR to create the final cDNA library. The libraries were quantified using the Qubit dsDNA HS Assay Kit and assessed for size distribution using Agilent Tapestation (Agilent Technologies), then multiplexed using 6–7 libraries per pool. Library pools were quantified by qPCR and sequenced on a HiSeq4000 sequencer using 75-bp paired-end format. Sequence files were generated in FASTQ format, and reads were mapped to human genome 19 and then aligned by TopHat2 (32). Htseq-count was used to generate gene read counts for each sample, and R package "DESeq2" was used to normalize the data. Genes with zero counts across all samples were removed. Feature-by-feature linear models were used to adjust for batch effects. Gene expression profiles were compared with feature-by-feature *t* tests, using a beta-uniform mixture (BUM) models to fit the resulting distribution of *P* values and allow for estimation of the FDR.

The Cancer Genomic Atlas HCC database analysis

Liver HCC [The Cancer Genomic Atlas (TCGA)] was chosen on the cBioPortal online platform (http://www.cbioportal.org/; refs. 33, 34). The 373 sequenced HCC tumors were selected as the patient set. We queried for samples with *TP53R249S* mutation and identified 11 patients with *TP53R249S* mutation. Clinical and demographic information of all 373 HCC patients were downloaded from the cBioPortal website. Overall survival and disease-free survival were examined to compare the prognosis of the patients with *TP53R249S* mutation with those without that mutation. The results are displayed as Kaplan–Meier plots with P values from a log-rank test. Using the cBioPortal Enrichment module, we retrieved mRNA expression data from all HCC tumors and compared genes from tumors with *TP53R249S* mutant to nonmutant tumors using Student *t* tests.

Statistical analysis of clinical variables

Statistical difference between each group was assessed by Student *t* tests for continuous variables and by Fisher exact tests for categorical variables using GraphPad 6.0 software and R Version 3.3. A value of $P < 0.05$ was considered statistically significant.

Results

Prevalence of *TP53R249S* mutation in HCCs from Hispanics in South Texas

We obtained FFPE tumor samples from 41 Hispanic patients with HCC treated at two institutions in South Texas. Demographic and clinical parameters of these 41 patients are summarized in Supplementary Table S1. These patients were predominantly male (82.9%; 34/41), 37.1% were obese, and 58.3% had diabetes. These HCCs were associated with HCV (75%) or NAFLD/NASH (15%), and 61.3% of these patients had underlying cirrhosis. The distribution of well, moderately, and poorly differentiated tumors was 38.9%, 41.7%, and 19.4%, respectively. Following DNA extraction, we measured *TP53R249S* mutation using ddPCR in these 41 HCCs. Droplets positive for mutant alleles, positive for wt alleles, and double-positive for both mutant and wt alleles were clearly separated as shown in Fig. 1. Black, blue, green, and orange dots represent empty droplets, *TP53R249S*-positive droplets, wt DNA-positive droplets, and double-positive wt and *TP53R249S* droplets, respectively. The assay was first validated using HepG2 hepatoma cell line, not harboring the *TP53R249S* mutation and PLC/PRF/5 hepatoma cell line, harboring the *TP53R249S* mutation (Fig. 1A–C). *TP53R249S* mutation was detected in 3 of the 41 HCCs

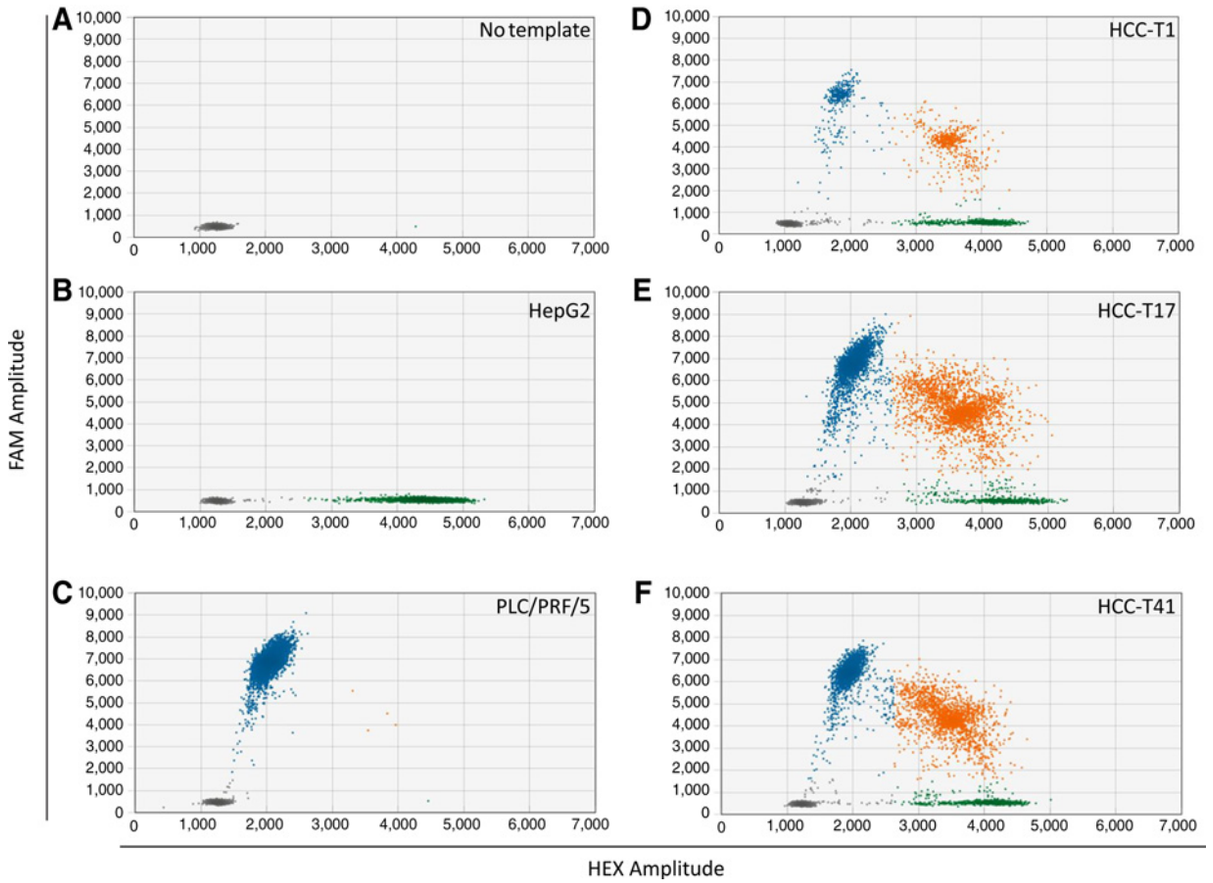

Figure 1.

TP53R249S mutation detection in HCC tumors by ddPCR. Droplet population observed for *TP53R249S* assay in no template control (**A**), HepG2 cell line (**B**), not harboring the *TP53R249S* mutation, PLC/PRF/5 cell line (**C**), harboring the *TP53R249S* mutation, and HCC samples positive for *TP53R249S* mutation (**D–F**). HEX amplitude is up to 7,000 on *x*-axis and FAM amplitude up to 10,000 on the *y*-axis of each panel. Key for dots: black, empty droplets; blue, mutant DNA FAM-positive droplet; green, wild-type DNA HEX-positive droplets; orange, wild-type and mutant DNA double-positive droplets.

Table 1. Demographic and clinical characteristics of Hispanic HCC patients with *TP53R249S* mutation

	Without *TP53R249S* mutation	With *TP53R249S* mutation	*P*
Age	64.1 (40–92)	55.7 (42–78)	0.306
Male	81.60%	100%	1.000
Obese (BMI ≥ 30)	33.30%	100%	0.131
Diabetes	60.60%	33.30%	0.559
HCV			1.000
Yes	72.7%	100%	
No	27.30%	0%	
NAFLD/NASH			1.000
Yes	16.70%	0%	
No	83.30%	100%	
AFP (ng/mL)	886.1 (1.4–12,700)	50.4 (22–98.6)	0.704
Cirrhosis	62.10%	66.7%	1.000
Multiple tumors	19.20%	66.7%	0.136
Differentiation			0.397
Well	42.4%	0%	
Moderate	39.4%	66.70%	
Poor	18.2%	33.30%	

NOTE: Data are presented as mean (range) or frequency %.

(7.3%; HCC-T1, HCC-T17, and HCC-T41), with 33.5%, 66.5%, and 62.5% mutant allele fractions, respectively (Fig. 1D–F). *TP53R249S* mutation was not detected in nontumor-adjacent liver. The presence of *TP53R249S* mutation in these 3 tumors was further confirmed by RFLP (Supplementary Fig. S1). The assay was again validated using HepG2 and PLC/PRF/5 cell lines (Supplementary Fig. S1). After digestion by restriction enzyme HaeIII, two bands (92 and 66 bp) bands are generated in wt samples, such as HepG2, whereas samples harboring the *TP53R249S* mutation, in which the restriction site is destroyed, yield only one band of 158 bp as observed for PLC/PRF/5 (Supplementary Fig. S1). For all 3 HCC tumors identified positive for *TP53R249S* mutation by ddPCR, the PCR products resulted following restriction enzyme digestion, in a mixture of uncleaved 158-bp fragment and cleaved 92 and 66 bp fragments, confirming the presence of *TP53R249S* mutation in the HCC-T1, HCC-T17, and

HCC-T41 DNA samples (Supplementary Fig. S1). There was a very good agreement observed between the ratio of uncleaved to cleaved fragments detected by RFLP and the mutant allele fractions measured by ddPCR for all 3 tumors. No difference in gender, the presence of diabetes, HCV, NAFLD/NASH, cirrhosis or obesity, tumor differentiation, and site of recruitment was identified between the HCCs with *TP53R249S* mutation and those wt for that mutation (Table 1). All subjects with mutated *TP53R249S* were obese (100%) compared with 33% for those subjects without *TP53R249S* mutation, and 66.7% of them had multiple tumors compared with 19.20% for those subjects without *TP53R249S* mutation. The age average of the patients with *TP53R249S* mutation was younger (mean age, 55.7) than the age average of the patients without *TP53R249S* mutation (mean age, 64.1).

Prevalence of *TP53R249S* mutation in plasma cfDNA in HCC patients, seeking care at MD Anderson Cancer Center

We then measured *TP53R249S* mutation in cfDNA isolated from plasma collected from 218 patients with HCC, seeking care at MD Anderson Cancer Center. The ethnicity/race distribution among these 218 HCC patients was 54.6% (119) Non-Hispanic White, 16.1% (35) Hispanic, 4.6% (10) Asian, 7.8% (17) Black, and 17% (37) unknown. The demographic and clinical parameters of these 218 HCC patients are shown in Supplementary Table S2. The majority of the patients were male (72%). Among them, 37.9% were obese, 40.8% had diabetes, 32.5% were positive for HCV, 20.9% were positive for HBV, and 10.9% had NAFLD/NASH. Underlying cirrhosis was present in 54.5% of the patients, with 19.1% alcoholic cirrhosis and 35.4% nonalcoholic cirrhosis. No known risk factor (HCV, HBV, NAFLD/NASH, alcohol) could be identified in 7% of the patients. The distribution of well, moderately, and poorly differentiated tumors is 39.6%, 34.3%, and 26.1%, respectively. Among the 218 cfDNAs analyzed by ddPCR, 4 were positive for *TP53R249S* mutation (HCC-P177, HCC-P182, HCC-P199, and HCC-P207), with mutant fractions of 36.7%, 17.6%, 1.2%, and 44.4%, respectively (Fig. 2). The presence of the *TP53R249S* mutation in these 4 cfDNA samples was further confirmed by RFLP (Supplementary Fig. S2). Here again, there was a very good agreement observed between the ratio of uncleaved to cleaved fragments detected by RFLP and the mutant allele fractions measured by ddPCR for all 4 cfDNA samples. There was no difference in gender, etiology, alpha-fetoprotein levels, degree of differentiation, presence of cirrhosis, child score, or tumor stage among patients with *TP53R249S* mutation compared with those negative for this mutation (Table 2). There was a trend of younger age for those with *TP53R249S* mutation compared with those without mutation (55.8 vs. 65.1) and of shorter overall survival in patients with *TP53R249S* mutation (Supplementary Fig. S3). There was a highly significant difference in ethnicity/race distribution between patients with *TP53R249S* mutation and those negative for this mutation ($P = 0.002$). The *TP53R249S* mutation was indeed only detected in Hispanics and Asians, while Blacks, non-Hispanic Whites, and patients with unknown ethnicity were all negative for *TP53R249S*. The frequency of *TP53R249S* mutation in cfDNA from Hispanic HCC patients was 5.7%. Among the Hispanic HCC patients with *TP53R249S*

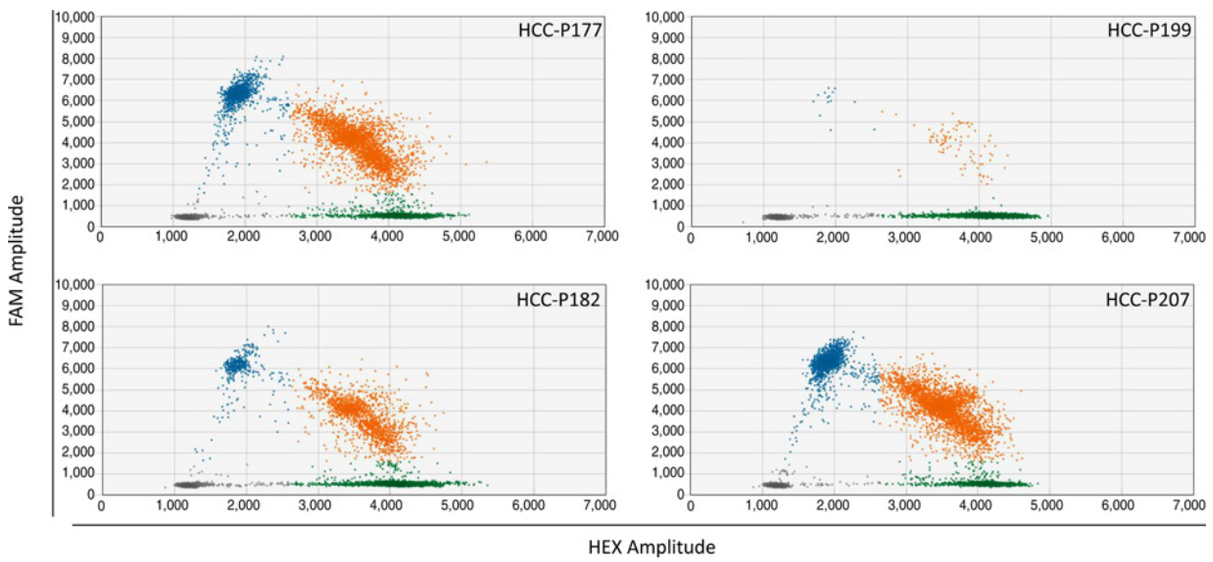

Figure 2.
TP53R249S mutation detection in cfDNA by ddPCR. Droplet population observed for *TP53R249S* assay in cfDNA samples positive for *TP53R249S* mutation. Key for dots: black, empty droplets; blue, mutant DNA FAM-positive droplet; green, wild-type DNA HEX-positive droplets; orange, wild-type and mutant DNA double-positive droplets.

Table 2. Demographic and clinical characterization of HCC patients with *TP53R249S* mutation in cfDNA

	Without *TP53R249S* mutation	With *TP53R249S* mutation	*P*
Age	65.1 (30–88)	55.8 (42–65)	0.101
Male (%)	71.5%	100%	0.578
Ethnicity/race			0.002
Hispanic	15.4%	50%	
Asian	3.7%	50%	
Black	7.9%	0%	
Non-Hispanic White	55.6%	0%	
unknown	17.3%	0%	
Obese (BMI $\geq$ 30)	37.7%	50%	0.635
Diabetes (%)	40.7%	50%	1.000
NAFLD/NASH			0.295
Yes	10.3%	33.3%	
No	89.7%	66.7%	
HCV			0.306
Yes	33.2%	0.0%	
No	66.8%	100.0%	
HBV			0.193
Yes	20.3%	50.0%	
No	79.7%	50.0%	
Cirrhosis			
Cirrhosis-alcoholic	19.5%	0.0%	0.407
Cirrhosis-not alcoholic	34.6%	75.0%	
No cirrhosis	45.9%	25%	
AFP (ng/mL)	13,638.5 (1–660,959.3)	742.9 (4.3–1,808.3)	0.691
Multiple tumors	63.4%	50%	0.627
Cirrhosis (%)	54.15%	75%	0.628
Differentiation			1.000
Well	39.2%	50%	
Moderate	34.6%	25%	
Poor	26.2%	25%	
Child score			1.000
A	80.4%	100%	
B	17.8%	0%	
C	1.9%	0%	
Tumor stage			0.912
I	15.1%	25%	
II	16.5%	0%	
III	38.7%	50%	
IV	29.7%	25%	

NOTE: Data are presented as mean (range) or frequency %.

mutation, one was born in Mexico, while the other was born in the United States. The other patients harboring the *TP53R249S* mutation were born in Asia.

Prevalence of *TP53R249S* mutation in plasma cfDNA in subjects with cirrhosis/advanced fibrosis in Hispanics in South Texas

Because *TP53R249S* mutation has been reported in subjects with cirrhosis in regions of high AFB1 exposure, we also measured *TP53R249S* mutation in plasma cfDNA of Hispanics in South Texas, with AST to APRI $\geq$1, predictive of the presence of cirrhosis or advanced fibrosis. To that end, we interrogated a community-based Hispanic cohort at the U.S.–Mexico border, the CCHC. We previously identified 102 CCHC subjects with APRI $\geq$1. These subjects were more likely to have diabetes and HCV, to present with higher body mass index (BMI), waist circumference, fasting triglyceride, glucose, and insulin levels than controls. The detailed clinical and demographic features of these subjects have been published previously (7). *TP53R249S* mutation was measured in cfDNA of 96 of these 102 subjects but was not detected in any of these samples.

Transcriptomic signature associated with *TP53R249S* mutation

To determine whether *TP53R249S* mutation is associated with a specific tumor transcriptomic signature, we generated gene expression profiles using RNA sequencing (RNA-Seq) on the same HCC tumors from Hispanics analyzed for *TP53R249S* mutation. We then analyzed the gene expression profiles according to the presence or absence of *TP53R249S* mutation. Using $P < 0.05$ and fold change $\geq$1.5, we identified 239 upregulated and 194 downregulated genes in HCCs with *TP53R249S* mutation compared with HCCs without *TP53R249S* mutation (Supplementary Table S3). The largest expression changes were observed for solute carrier family 6 member 11 (SLC6A11), seizure-related 6 homolog-like 2 (SEZ6L2), epiplakin 1 (EPPK1), solute carrier family 2 member 14 (SLC2A14), transmembrane protein 82 (TMEM82) and IL17 receptor E (IL17RE), all overexpressed in samples with *TP53R249S* mutation compared with samples without mutation, and for eukaryotic translation elongation factor 1, alpha-2 (EE1A2), homolog of odd Oz 2 (ODZ2), serum amyloid A2 (SAA2), and glutathione S-transferase theta 1 (GSTT1), all underexpressed in samples with *TP53R249S* mutation. Ingenuity Pathway Analysis (IPA) of all 433 genes identified cell-cycle control of chromosomal replication and role of BRCA1 in DNA damage response, as the top canonical pathways affected by *TP53R249S* mutation status ($P = 1.10 \times 10^{-9}$ and $P = 8.85 \times 10^{-5}$, respectively) and E2f, E2F4, as the top upstream regulators affected by *TP53R249S* mutation status ($P = 1.62 \times 10^{-12}$ and 4.39×10^{-11}, respectively; Table 3).

TP53R249S mutation in HCC in TCGA

To compare our results of *TP53R249S* mutation in HCC in Hispanics in South Texas to other HCC population cohorts, we interrogated TCGA data using the cBioPortal for Cancer Genomics (http://www.cbioportal.org/). *TP53R249S* mutation was detected in 11 of the 373 HCC tumors in TCGA (2.9%). There was no difference in gender, presence of cirrhosis, viral hepatitis or NAFLD/NASH, or child score among patients with *TP53R249S* mutation compared with those negative for this mutation

Table 3. IPA for differentially expressed genes in HCCs with *TP53R249S* mutation

	P
Top canonical pathway	
Cell-cycle control of chromosomal replication	1.10×10^{-9}
Role of BRCA1 in DNA damage response	8.85×10^{-5}
Upstream regulators	
E2F	1.62×10^{-12}
E2F4	4.39×10^{-11}

(Supplementary Table S4). Patients with *TP53R249S* mutation had early onset of disease with an average age of diagnosis at 48.8 compared with 59.9 for those without this mutation ($P = 0.006$; Supplementary Table S4). A significant difference in ethnicity/race distribution in patients harboring *TP53R249S* mutation was also observed, with *TP53R249S* mutation detected in Asians (72.7%), Blacks (18.2%), and Hispanics patients (9.1%) but not in non-Hispanic Whites ($P = 0.004$). Patients harboring *TP53R249S* mutation were more likely to have stage II (40%) and III (50%) disease than those not harboring the mutation (24.1% and 23.8%, respectively, $P = 0.036$). Kaplan–Meier plot analysis showed that the presence of *TP53R249S* mutations was significantly associated with shorter overall survival and short disease-free survival ($P = 0.008$ and 0.001, respectively; Supplementary Fig. S4A). We also used the cBioPortal for Cancer Genomics (http://www.cbioportal.org/) to further assess the impact of *TP53R249S* mutation on gene expression in HCCs in TCGA. IPA analysis of genes differentially expressed in tumors with *TP53R249S* mutation, identified as for the HCCs in Hispanics in South Texas, the same top canonical pathways, namely cell-cycle control of chromosomal replication and role of BRCA1 in DNA damage response ($P = 3.55 \times 10^{-26}$ and 3.09×10^{-14}) and the same top upstream regulators, namely E2F4 and E2F1 ($P = 6.12 \times 10^{-61}$ and 4.29×10^{-41}; Supplementary Fig. S4B).

Discussion

The aim of this study was to determine whether aflatoxin dietary exposure plays a role in the etiology of HCC in Hispanics in South Texas. To that end, we measured a hallmark mutation induced by aflatoxin exposure in HCC, namely *TP53R249S*. Because this mutation was previously detected in both tumor tissue and plasma cfDNA of patients with HCC but also patients with liver cirrhosis (23, 35), we measured this mutation in HCC tumors from Hispanics in South Texas, in plasma cfDNA from patients with HCC, seeking care at MD Anderson Cancer Center and in plasma cfDNA from Hispanics with advanced fibrosis or cirrhosis, participants of the community-based CCHC. All subjects were enrolled in Texas counties with very high rates of liver cancer (36, 37). The national mortality rate of liver cancer in 2014 was $6.81_{/100,000}$ but reached $8.74_{/100,000}$ in Texas. The samples used in our study were collected in counties along the U.S.–Mexico border (Cameron and Webb counties) and along the Gulf Coast (Galveston and Harris counties). The 2014 liver cancer mortality rates in all 4 counties were significantly higher than the statewide rate, ranging from $9.51_{/100,000}$ to $14.30_{/100,000}$. All 4 counties were among the top 17% of Texas counties with the highest rates of liver cancer. Therefore, the tumor samples and other biological materials analyzed in this study were indeed from hotspots for liver cancer in Texas. From the dietary surveys in CCHC and in the National Health and Nutrition Examination Survey (NHANES) 2005–2006 (38), we extracted data on daily consumption of grain and of corn tortillas in Mexican Americans in CCHC ($n = 2606$) and in Mexican Americans nationwide ($n = 1397$). Although the overall consumption of grain products was similar for both groups (87% and 94%, respectively), Mexican Americans in Cameron/Webb counties in Texas consumed significantly more corn tortillas than Mexican American nationwide, with 56% versus 20% consuming corn tortillas at least once a day, respectively. A total of 19.1% of Mexican Americans in Cameron/Webb counties consumed 3 or more corn tortillas daily.

The technologies we used for the detection of *TP53R249S* mutation were RFLPs and ddPCR. Although RFLP is a wildly used technique for the detection of *TP53R249S* mutation based on the modification of a restriction site by the mutation (14, 24, 35), ddPCR is a novel highly sensitive and robust technology for detection of rare mutations using massive sample partitioning and fluorescence-based detection (39, 40). We observed a good agreement between RFLP and ddPCR results.

Overall, we found *TP53R249S* mutation present in 7.3% (3/41) of Hispanic HCC tumors collected from two different sites in South Texas. This is higher, although not significantly, than the overall prevalence found in TCGA (11/373, 2.9%), even among Hispanics (1/18, 5.5%). We also reported for the first time, the detection of *TP53R249S* mutation in plasma cfDNA in 218 HCC patients. Although, indeed, such studies have been done in other countries (13, 22, 41), this has never been done in the HCC population in the United States. We found a significant difference in ethnicity/race distribution of patients with *TP53R249S* mutation ($P < 0.0001$). This mutation was only detected in Hispanics and Asians. The frequency of *TP53R249S* mutation in cfDNA from Hispanic HCC patients was 5.7%. Of importance, *TP53R249S* mutation was found in a Hispanic born in the United States, suggesting that although the role of aflatoxin exposure in HCC among Hispanic in South Texas is low, we cannot exclude it.

Patients harboring *TP53R249S* mutation were likely to be younger (55.7 vs. 65.0, $P = 0.039$). This result was further confirmed in HCCs from TCGA and is in agreement with a study performed in Thailand, a country with moderate aflatoxin exposure (15). Patients with *TP53R249S* mutation also presented with a significantly reduced overall survival and disease-free survival. *TP53R249S* mutation has been shown to be a prognostic marker in a Chinese cohort with HCC patients from high and moderate aflatoxin exposure areas (19).

A limitation of the study is the lack of examination of the correlation with actual aflatoxin dietary exposure in those individuals with *Tp53R249S*. Future studies should include the measurements in the same

individuals, of plasma biomarkers of covalent adduction to DNA or protein or urinary AFB1-DNA repair products together with the measurement of *Tp53R249S* in plasma cfDNA. Such studies would be particularly valuable in prospective cohorts with serial biospecimens, allowing for a time-to-event correlation analysis between these biomarkers.

Integrative analysis of *TP53R249S* mutation status and RNA-Seq transcriptomic data from both the HCC tumors from Hispanics in South Texas and the TCGA HCCs, identified cell-cycle control of chromosome replication and role of BRCA1 in DNA damage response as the top two canonical pathways in HCCs with *TP53R249S* mutation. Several genes overexpressed in HCCs with *TP53R249S* mutation are known to be associated with poor survival in HCC and other human cancers. These include SLC2A14 and IL17RE (42). EPPK1 serves as a useful marker for hepatic and pancreatic progenitor cells (43, 44). Among the genes underexpressed in HCCs with *TP53R249S* mutation, null genotype of GSTT1 increases the risk of lung cancer, prostate cancer, and HCC (45). BRCA1 and E2F1 mRNA expression was increased in tumors bearing *TP53R249S* mutation in both datasets. Overexpression of BRCA1 has been reported in HCC and shown to correlate with mesenchymal-like feature and chemoresistance (46). Members of the E2F family, E2F1 and E2F4, were identified as upstream regulators of the genes associated with *TP53R249S* mutation. E2F family of transcription factors plays vital roles in cell proliferation, apoptosis, differentiation, senescence, DNA damage response, and DNA repair (47). In HCC, E2F1 has both been shown to have proapoptotic and antiapoptotic roles, in addition to proliferative effects (48). Copy number gain of E2F1 resulted in dosage-dependent spontaneous HCC in mice, suggesting a direct and cell-autonomous role for E2F in HCC (49). Microsatellite instability and mutations of E2F4 commonly occur in HCC and may play an important role in hepatocarcinogenesis (50). Our results demonstrate for the first time an association between *TP53R249S* mutation with E2F network, suggesting that aflatoxin exposure could promote HCC onset through E2F. Our results also demonstrate for the first time an association between *TP53R249S* mutation with BRCA1 pathway. Whether BRCA1/BRCA2 alterations could increase susceptibility to HCC onset in the context of aflatoxin dietary exposure should be further evaluated.

In conclusion, *TP53R249S* mutation was detected in 5.7% to 7.3% of Hispanic patients with HCC in South Texas. This mutation was associated with development of HCC at a young age and worse prognosis. *TP53R249S* was however not detected in cfDNA from Hispanics in South Texas with cirrhosis or advanced fibrosis. Therefore, aflatoxin exposure may contribute to a small number of HCCs in Hispanics in South Texas, but the detection of *TP53R249S* mutation in plasma cfDNA is not a promising risk predictor marker in subjects with cirrhosis or advanced fibrosis in this population.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359–86.
2. Global Burden of Disease Study Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for

the Global Burden of Disease Study 2013. Lancet 2015; 386:743–800.
3. Ryerson AB, Eheman CR, Altekruse SF, Ward JW, Jemal A, Sherman RL, et al. Annual Report to the Nation on the Status of Cancer, 1975–2012, featuring the increasing incidence of liver cancer. Cancer 2016;122:1312–37.
4. White DL, Thrift AP, Kanwal F, Davila J, El-Serag HB. Incidence of hepatocellular carcinoma in all 50 United States, from 2000 through 2012. Gastroenterology 2017;152:812–20.
5. Garza A, Vatcheva KP, Pan JJ, Rahbar MH, Fallon MB, McCormick JB, et al. Liver and other gastrointestinal cancers are frequent in Mexican Americans. J Racial Ethn Health Disparities 2016;3:1–10.
6. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, et al. Hepatocellular carcinoma. Nat Rev Dis Primers 2016;2:16018.
7. Jiao J, Watt GP, Lee M, Rahbar MH, Vatcheva KP, Pan JJ, et al. Cirrhosis and advanced fibrosis in hispanics in Texas: the dominant contribution of central obesity. PLoS One 2016;11: e0150978.
8. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monogr Eval Carcinog Risks Hum 2002;82:1–556.
9. Wild CP, Gong YY. Mycotoxins and human disease: a largely ignored global health issue. Carcinogenesis 2010;31:71–82.
10. Smela ME, Hamm ML, Henderson PT, Harris CM, Harris TM, Essigmann JM. The aflatoxin B(1) formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. Proc Natl Acad Sci U S A 2002;99:6655–60.
11. Gouas D, Shi H, Hainaut P. The aflatoxin-induced TP53 mutation at codon 249 (R249S): biomarker of exposure, early detection and target for therapy. Cancer Lett 2009;286:29–37.
12. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 1991;350:427–8.
13. Szymanska K, Lesi OA, Kirk GD, Sam O, Taniere P, Scoazec JY, et al. Ser-249TP53 mutation in tumour and plasma DNA of hepatocellular carcinoma patients from a high incidence area in the Gambia, West Africa. Int J Cancer 2004;110:374–9.
14. Szymanska K, Chen JG, Cui Y, Gong YY, Turner PC, Villar S, et al. TP53 R249S mutations, exposure to aflatoxin, and occurrence of hepatocellular carcinoma in a cohort of chronic hepatitis B virus carriers from Qidong, China. Cancer Epidemiol Biomarkers Prev 2009;18:1638–43.
15. Chittmittrapap S, Chieochansin T, Chaiteerakij R, Treeprasertsuk S, Klaikaew N, Tangkijvanich P, et al. Prevalence of aflatoxin induced p53 mutation at codon 249 (R249s) in hepatocellular carcinoma patients with and without hepatitis B surface antigen (HBsAg). Asian Pac J Cancer Prev 2013;14:7675–9.
16. Galy O, Chemin I, Le Roux E, Villar S, Le Calvez-Kelm F, Lereau M, et al. Mutations in TP53 and CTNNB1 in relation to hepatitis B and C infections in hepatocellular carcinomas from Thailand. Hepat Res Treat 2011;2011:697162.
17. Nogueira JA, Ono-Nita SK, Nita ME, de Souza MM, do Carmo EP, Mello ES, et al. 249 TP53 mutation has high prevalence and is correlated with larger and poorly differentiated HCC in Brazilian patients. BMC Cancer 2009;9:204.
18. Soini Y, Chia SC, Bennett WP, Groopman JD, Wang JS, DeBenedetti VM, et al. An aflatoxin-associated mutational hotspot at codon 249 in the p53 tumor suppressor gene occurs in hepatocellular carcinomas from Mexico. Carcinogenesis 1996;17: 1007–12.
19. Woo HG, Wang XW, Budhu A, Kim YH, Kwon SM, Tang ZY, et al. Association of TP53 mutations with stem cell-like gene expression and survival of patients with hepatocellular carcinoma. Gastroenterology 2011;140:1063–70.
20. Ortiz-Cuaran S, Villar S, Gouas D, Ferro G, Plymoth A, Khuhaprema T, et al. Association between HBX status, aflatoxin-induced R249S TP53 mutation and risk of hepatocellular carcinoma in a case-control study from Thailand. Cancer Lett 2013;331:46–51.
21. Kuang SY, Lekawanvijit S, Maneekarn N, Thongsawat S, Brodovicz K, Nelson K, et al. Hepatitis B 1762T/1764A mutations, hepatitis C infection, and codon 249 p53 mutations in hepatocellular carcinomas from Thailand. Cancer Epidemiol Biomarkers Prev 2005;14:380–4.
22. Huang XH, Sun LH, Lu DD, Sun Y, Ma LJ, Zhang XR, et al. Codon 249 mutation in exon 7 of p53 gene in plasma DNA: maybe a new early diagnostic marker of hepatocellular carcinoma in Qidong risk area, China. World J Gastroenterol 2003;9:692–5.
23. Gouas DA, Villar S, Ortiz-Cuaran S, Legros P, Ferro G, Kirk GD, et al. TP53 R249S mutation, genetic variations in HBX and risk of hepatocellular carcinoma in The Gambia. Carcinogenesis 2012; 33:1219–24.
24. Kirk GD, Camus-Randon AM, Mendy M, Goedert JJ, Merle P, Trepo C, et al. Ser-249 p53 mutations in plasma DNA of patients with hepatocellular carcinoma from The Gambia. J Natl Cancer Inst 2000;92:148–53.
25. Wood GE. Mycotoxins in foods and feeds in the United States. J Anim Sci 1992;70:3941–9.
26. Hoque A, Patt YZ, Yoffe B, Groopman JD, Greenblatt MS, Zhang YJ, et al. Does aflatoxin B1 play a role in the etiology of hepatocellular carcinoma in the United States? Nutr Cancer 1999; 35:27–33.
27. Johnson NM, Qian G, Xu L, Tietze D, Marroquin-Cardona A, Robinson A, et al. Aflatoxin and PAH exposure biomarkers in a U.S. population with a high incidence of hepatocellular carcinoma. Sci Total Environ 2010;408:6027–31.
28. Ramirez AG, Munoz E, Parma DL, Michalek JE, Holden AEC, Phillips TD, et al. Lifestyle and clinical correlates of hepatocellular carcinoma in South Texas: a matched case-control study. Clin Gastroenterol Hepatol 2017;15:1311–2.
29. Riva F, Bidard FC, Houy A, Saliou A, Madic J, Rampanou A, et al. Patient-specific circulating tumor DNA detection during neoadjuvant chemotherapy in triple-negative breast cancer. Clin Chem 2017;63:691–9.
30. Beije N, Helmijr JC, Weerts MJ, Beaufort CM, Wiggin M, Marziali A, et al. Somatic mutation detection using various targeted detection assays in paired samples of circulating tumor DNA, primary tumor and metastases from patients undergoing resection of colorectal liver metastases. Mol Oncol 2016 Oct 10. pii: S1574-7891(16)30110-7. doi: 10.1016/j.molonc.2016.10.001. [Epub ahead of print].
31. Gouas DA, Shi H, Hautefeuille AH, Ortiz-Cuaran SL, Legros PC, Szymanska KJ, et al. Effects of the TP53 p.R249S mutant on proliferation and clonogenic properties in human hepatocellular carcinoma cell lines: interaction with hepatitis B virus X protein. Carcinogenesis 2010;31:1475–82.
32. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 2009;25:1105–11.
33. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6:pl1.
34. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–4.

35. Kirk GD, Lesi OA, Mendy M, Szymanska K, Whittle H, Goedert JJ, et al. 249(ser) TP53 mutation in plasma DNA, hepatitis B viral infection, and risk of hepatocellular carcinoma. Oncogene 2005;24:5858–67.
36. Mokdad AH, Dwyer-Lindgren L, Fitzmaurice C, Stubbs RW, Bertozzi-Villa A, Morozoff C, et al. Trends and patterns of disparities in cancer mortality among US counties, 1980–2014. JAMA 2017;317:388–406.
37. Howlader N, Noone AM, Krapcho M, Miller D, Bishop K, Kosary CL, et al. , eds SEER Cancer Statistics Review, 1975–2014. Bethesda, MD: NCI; 2017. Available from: https://seer.cancer.gov/csr/1975_2014/.
38. Centers for Disease Control and PreventionQuestionnaires, Datasets, and Related Documentation. Available from: cdc.gov/nchs/nhanes/nhanes_questionnaires.htm.
39. Diaz LA Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 2014;32:579–86.
40. Vogelstein B, Kinzler KW. Digital PCR. Proc Natl Acad Sci U S A 1999;96:9236–41.
41. Jackson PE, Qian GS, Friesen MD, Zhu YR, Lu P, Wang JB, et al. Specific p53 mutations detected in plasma and tumors of hepatocellular carcinoma patients by electrospray ionization mass spectrometry. Cancer Res 2001;61:33–5.
42. Liao R, Sun J, Wu H, Yi Y, Wang JX, He HW, et al. High expression of IL-17 and IL-17RE associate with poor prognosis of hepatocellular carcinoma. J Exp Clin Cancer Res 2013;32:3.
43. Matsuo A, Yoshida T, Yasukawa T, Miki R, Kume K, Kume S. Epiplakin1 is expressed in the cholangiocyte lineage cells in normal liver and adult progenitor cells in injured liver. Gene Expr Patterns 2011;11:255–62.
44. Yoshida T, Shiraki N, Baba H, Goto M, Fujiwara S, Kume K, et al. Expression patterns of epiplakin1 in pancreas, pancreatic cancer and regenerating pancreas. Genes Cells 2008;13:667–78.
45. Liu K, Zhang L, Lin X, Chen L, Shi H, Magaye R, et al. Association of GST genetic polymorphisms with the susceptibility to hepatocellular carcinoma (HCC) in Chinese population evaluated by an updated systematic meta-analysis. PLoS One 2013;8:e57043.
46. Ferroudj S, Yildiz G, Bouras M, Iscan E, Ekin U, Ozturk M. Role of Fanconi anemia/BRCA pathway genes in hepatocellular carcinoma chemoresistance. Hepatol Res 2016;46:1264–74.
47. Polager S, Ginsberg D. p53 and E2f: partners in life and death. Nat Rev Cancer 2009;9:738–48.
48. Farra R, Grassi G, Tonon F, Abrami M, Grassi M, Pozzato G, et al. The role of the transcription factor E2F1 in hepatocellular carcinoma. Curr Drug Deliv 2017;14:272–81.
49. Kent LN, Bae S, Tsai SY, Tang X, Srivastava A, Koivisto C, et al. Dosage-dependent copy number gains in E2f1 and E2f3 drive hepatocellular carcinoma. J Clin Invest 2017;127:830–42.
50. Park YM, Choi JY, Bae SH, Byun BH, Ahn BM, Kim BS, et al. Microsatellite instability and mutations of E2F-4 in hepatocellular carcinoma from Korea. Hepatol Res 2000;17:102–11.