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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 restriction fragment length polymorphism. *TP53R249S* mutation was detected in 3 out 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

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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%–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.

Keywords

Hepatocellular carcinoma; Hispanics; Aflatoxin

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 (U.S.), 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 have 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, non-alcoholic 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 manuscript, we explored the possibility that aflatoxin exposure could be a contributing risk factor for HCC in Hispanics in South Texas. Aflatoxin is a mycotoxin 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 35–61% of HCC tumors (12–14). In moderate aflatoxin exposure regions such as Thailand, *TP53R249S* mutation is present in 8–27% 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 U.S., *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 36–53% of HCC patients in Gambia and from 26–34% 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 U.S. (24). Aflatoxin exposure is not believed to play any role in hepatocarcinogenesis in the U.S. 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). While 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. 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 non-tumoral 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. 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 previously described (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=2606) 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. Cell free DNA (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 (ddPCR)

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 μl of DNA was used in each reaction. Thermocycling conditions were: 95°C for 10 min, followed by 40 cycles of 94°C for 30 sec and 55°C for 1 min, followed by 98°C for 10 min. 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 ≥ 0.1% and with at least 2 visible mutant signals were considered positive for the mutation as previously described (29, 30).

Restriction Fragment Length Polymorphism (RFLP)

Exon 7 of TP53 was amplified with 2 rounds of PCR as previously described (24). Both PCR reactions involved a 15-min hotstart DNA polymerase (KAPA Biosystems) activation at 95°C, 40 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a final 5-min 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 (250ng) 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 False Discovery Rate (FDR)

The Cancer Genomic Atlas (TCGA) HCC database analysis

Liver Hepatocellular Carcinoma (TCGA) was chosen on the cBioPortal online platform (33, 34) (http://www.cbioportal.org/). 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 to those without that mutation. The results are displayed as Kaplan-Meier plots with *P* values from a logrank test. Using the cBioPortal Enrichment module, we retrieved mRNA expression data from all HCC tumors and compared genes from tumors with *TP53R249S* mutant to non-mutant tumors using Student's t-tests.

Statistical analysis of clinical variables

Statistical difference between each group was assessed by Student's t tests for continuous variables and by Fisher's exact tests for categorical variables using Graphpad™ 6.0 software and R Version 3.3. A value of $p<0.05$ was considered 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 droplet digital PCR (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 Figure 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 (Figure 1A–C). *TP53R249S* mutation was detected in 3 out of the 41 HCCs (7.3%) [HCC-T1, HCC-T17 and HCC-T41], with 33.5%, 66.5% and 62.5% mutant

allele fractions, respectively (Figure 1D–F). *TP53R249S* mutation was not detected in non-tumor adjacent liver. The presence of *TP53R249S* mutation in these 3 tumors was further confirmed by restriction fragment length polymorphism (RFLP) (Supplementary Figure S1). The assay was again validated using HepG2 and PLC/PRF/5 cell lines (Supplementary Figure S1). After digestion by restriction enzyme HaeIII, two bands (92bp and 66bp) 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 158bp as observed for PLC/PRF/5 (Supplementary Figure 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 158bp fragment and cleaved 92 and 66bp fragments, confirming the presence of *TP53R249S* mutation in the HCC-T1, HCC-T17 and HCC-T41 DNA samples (Supplementary Figure 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 to 33% for those subjects without *TP53R249S* mutation and 66.7% of them had multiple tumors compared to 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 cell free DNA in HCC patients, seeking care at MD Anderson Cancer Center

We then measured *TP53R249S* mutation in cell free DNA (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% non-alcoholic 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 (Figure 2). The presence of the *TP53R249S* mutation in these 4 cfDNA samples was further confirmed by RFLP (Supplementary Figure 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 (AFP) levels, degree of differentiation, presence of cirrhosis, child score or tumor stage among patients with *TP53R249S* mutation compared to 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 Figure 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* mutation, one was born in Mexico while the other was born in the US. 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 Aspartate transaminase (AST) to Platelet Ratio Index (APRI) ≥1, predictive of the presence of cirrhosis or advanced fibrosis. To that end, we interrogated a community-based Hispanic cohort at the US-Mexico Border, the Cameron County Hispanic Cohort (CCHC). We previously identified 102 CCHC subjects with APRI ≥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 previously published (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-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 ≥1.5, we identified 239 upregulated and 194 downregulated genes in HCCs with *TP53R249S* mutation compared to 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 Interleukin 17 receptor E (IL17RE), all overexpressed in samples with *TP53R249S* mutation compared to 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 ($p=1.10\times10^{-9}$ and $p=8.85\times10^{-5}$, respectively) and E2f, E2F4, as the top upstream regulators affected by *TP53R249S* mutation status ($p=1.62\times10^{-12}$ and 4.39×10^{-11}, respectively) (Table 3).

TP53R249S mutation in HCC in The Cancer Genomic Atlas (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 out 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 to those negative for this mutation (Supplementary Table S4). Patients with *TP53R249S* mutation had early onset of disease with an average age of diagnosis at 48.8 compared to 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-Meir 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 Figure 4A). 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* ($p=3.55\times10^{-26}$ and 3.09×10^{-14}) and the same top upstream regulators, namely E2F4 and E2F1 ($p=6.12\times10^{-61}$ and 4.29×10^{-41}) (Supplementary Figure 4B).

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 Cameron County Hispanic Cohort (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 US-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). While 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% vs 20% consuming corn tortillas at least once a day, respectively. 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 restriction fragment length polymorphisms (RFLP) and droplet digital PCR (ddPCR). While 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), digital droplet PCR (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 2 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. While indeed, such studies have been done in other countries (13, 22, 41), this has never been done in the HCC population in the U.S. 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 U.S suggesting that while 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 under-expressed 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 pro-apoptotic and anti-apoptotic 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%–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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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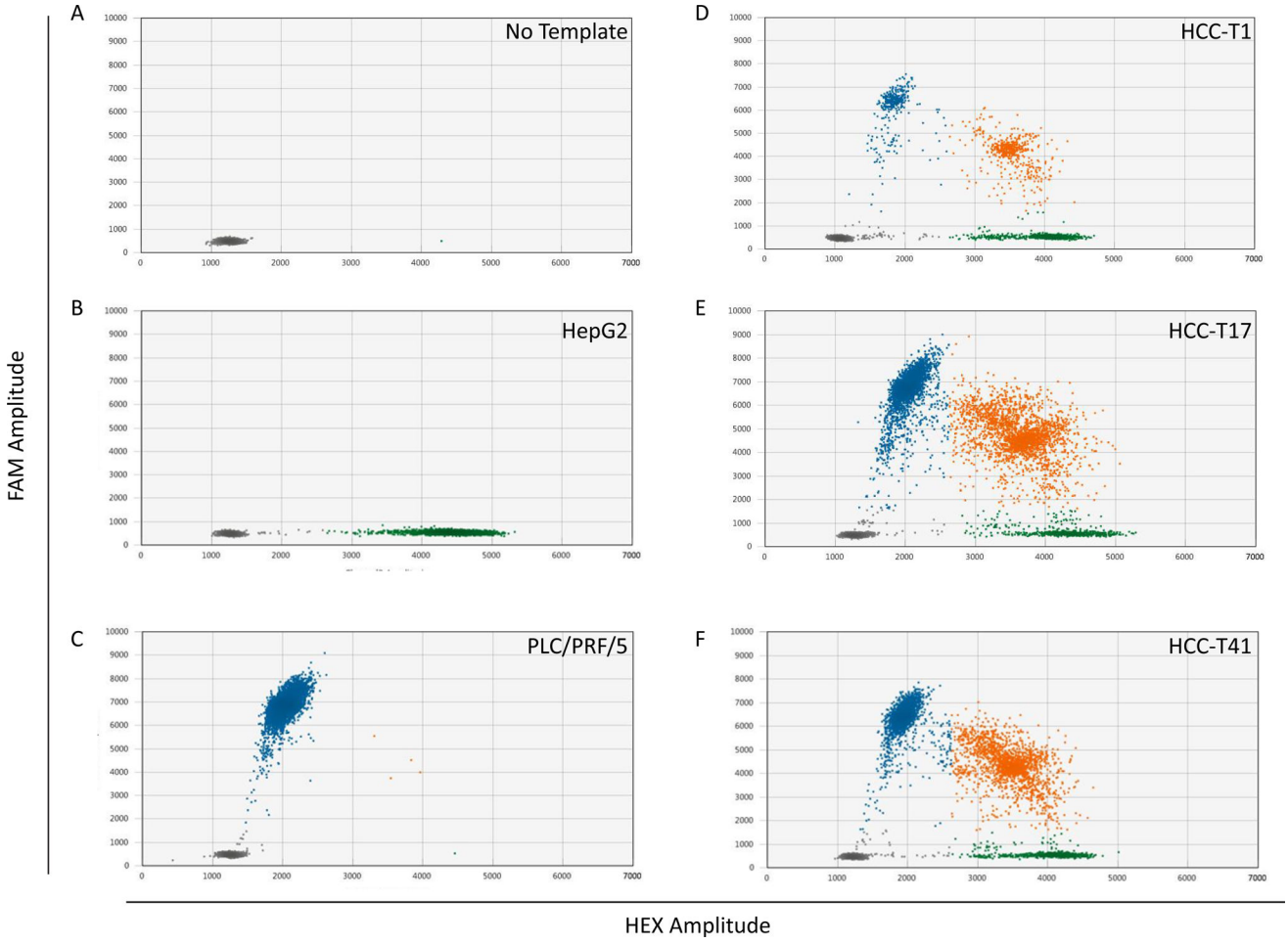

Figure 1. *TP53R249S* mutation detection in HCC tumors by droplet digital PCR

Droplet population observed for *TP53R249S* assay in **(A)** no template control, **(B)** HepG2 cell line, not harboring the *TP53R249S* mutation, **(C)** PLC/PRF/5 cell line, harboring the *TP53R249S* mutation, and **(D–F)** HCC samples positive for *TP53R249S* mutation. HEX amplitude is up to 7000 on x-axis and FAM amplitude up to 10000 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.

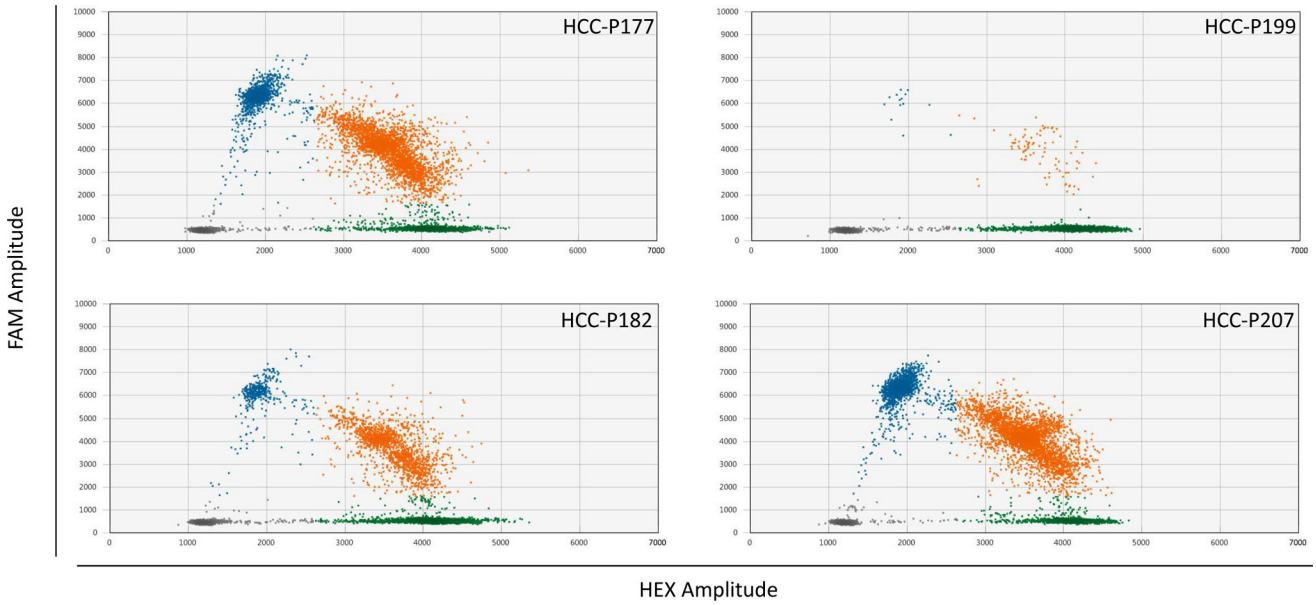

Figure 2. *TP53R249S* mutation detection in cfDNA by droplet digital PCR

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 1

Demographic and clinical characteristic 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 (1.4–12700)	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%	

data are presented as mean (range) or frequency %

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≥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)	13638.5 (1–660959.3)	742.9 (4.3–1808.3)	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			
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%	

data are presented as mean (range) or frequency %

Table 3

Ingenuity Pathways Analysis (IPA) for differentially expressed genes in HCCs with *TP53R249S* mutation

	***p* value**
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}