Aflatoxin levels and prevalence of *TP53* aflatoxin-mutations in hepatocellular carcinomas in Mexico

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Abstract

Objective. To determine the exposure to aflatoxin B_1 (AFB_1) in southern Mexico and the presence of the aflatoxin signature mutation in hepatocellular carcinoma (HCC) tissue from patients from a cancer referral center. **Materials and methods.** We estimated the prevalence and distribution of AFB_1 in a representative sample of 100 women and men from Chiapas using the National Health and Nutrition Survey 2018-19**.** We also examined the presence of the aflatoxin signature mutation in codon 249 (R249S), and other relevant mutations of the *TP53* gene in HCC tissue blocks from 24 women and 26 men treated in a national cancer referral center. **Results.** The prevalence of AFB_1 in serum samples was 85.5% (95%CI 72.1-93.1) and the median AFB_1 was 0.117 pg/µL (IQR, 0.050–0.350). We detected *TP53* R249S in

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Niveles de aflatoxina y prevalencia de mutaciones del gen *TP53* por aflatoxina en carcinoma hepatocelular en México.
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Resumen

Objetivo. Determinar la exposición a aflatoxina_B_1 (AFB_1) en el sur de México y la presencia de la mutación característica de AFB_1 en tejido de carcinoma hepatocelular (CHC) de pacientes de un centro oncológico. **Material y métodos.** Se estimó la prevalencia y distribución de AFB_1 en una muestra representativa de 100 mujeres y hombres de Chiapas a partir de la Encuesta Nacional de Salud y Nutrición 2018-19. También se observó la presencia de la mutación característica de AFB_1 en el codón 249 (R249S), y otras mutaciones relevantes del gen *TP53* en bloques de tejido de CHC de 24 mujeres y 26 hombres estudiados en un centro de referencia nacional de oncología. **Resultados.** La prevalencia de AFB_1 en las muestras de suero fue de 85.5% (IC95% 72.1-93.1) y la mediana de la concentración 0.117 pg/

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three of the 50 HCCs (6.0%) and observed four other G>T transversions potentially induced by AFB_1. **Conclusion.** Our analysis provides evidence that AFB_1 may have a relevant role on HCC etiology in Mexico.

Keywords: aflatoxin B1; mycotoxin; environmental biomarkers; mutation; liver neoplasia; epidemiology

µL (IQR, 0.050-0.350). Se detectó *TP53* R249S en tres de 50 casos de CHC (6.0%) y se observaron cuatro transversiones G>T potencialmente inducidas por AFB_1. **Conclusión.** El presente análisis proporciona evidencia de que la AFB_1 puede tener un papel relevante en la etiología del CHC en México.

Palabras clave: aflatoxina B1; micotoxinas; biomarcadores ambientales; mutación; neoplasias hepáticas; epidemiología

In Mexico, hepatocellular carcinoma (HCC), follows a unique epidemiologic pattern. Male-to-female mortality ratios are close to one.[1] And Hepatitis B virus (HBV; 0.2%)[2] and hepatitis C virus (HCV; 0.4%)[3] seroprevalence are low. However, contamination of maize tortilla, a staple food in Mexico, with aflatoxin B_1 (AFB_1), a potent liver carcinogen, has been shown to be as high as 95% in store samples in Veracruz.[4] And Chiapas and Guatemala, together represent the region with the highest HCC burden in the Americas. We aimed to estimate AFB_1 exposure in a representative sample of adults from Chiapas.[1] To further characterize the relevance of aflatoxin in Mexico, we determined the presence of the aflatoxin signature mutation in codon 249 (R249S) of the *TP53* gene[5] in HCC tissue from patients from a cancer referral center.

Materials and methods

National Health and Nutrition Survey, 2018-2019 (Ensanut 2018-19)

This nationally representative probabilistic multistage stratified cluster sampling survey (representative at the regional, state, urban/rural, and socioeconomic strata-level) obtained information from 44 069 households.[6] A sample of 899 households from the state of Chiapas was probabilistically selected. Among 869 respondents (96.6%), we identified 308 individuals between 40 and 59 years of age (considered to be at highest risk for HCC)[7] and randomly selected 100 serum samples from the 202 (65.5%) persons who donated a blood sample. Age, sex, education and rural residence distribution of selected participants was comparable to survey participants.

Measurement of AFB_1-lysine (AFB_1-lys)

We assessed AFB_1-lys (pg/uL serum) by taking 250µL of samples which were stored at -70°C. We then added 250µL of a Pronase-PBS (13mg mL-1) solution and incubated in water-bath at 37-40°C for 4.5h. Solid-phase extraction (SPE) was conducted on a Waters Oasis MAX 1cc 30mg extraction cartridge with a Phenomenex 24-port vacuum manifold. We conducted analyses using UPLC-TOF-MS/MS Waters, model Synapt G2 SI, equipped with a BEH C18 column (2.1x50µm, 1.7µm), with electrospray ionization in positive ion mode as previously described.[8] Quantitation was performed using an 8-point, serially diluted, isotope dilution calibration curve in 25% aqueous methanol (v/v). The limits of detection and quantification were both 0.010 pg/µL.

Hepatocellular carcinoma tissue samples

We identified 61 women and 85 men from mostly Mexico City and surrounding areas treated in 2005-2015 for HCC at the *Instituto Nacional de Cancerología* for whom formalin-fixed paraffin-embedded HCC tissue blocks were available. We extracted date and place of birth, residence, HBV/HCV serostatus, regular alcohol use, ever smoking, diabetes diagnosis, and anthopometry from medical records. We confirmed the HCC diagnosis by central review. We included all women (n=26) and a random sample of 26 men (out of 40) with HCC risk factor information. We excluded two women born outside of Mexico.

DNA extraction and sequencing

Chilled blocks were sectioned until the entire tissue was accessed. Duplicate samples of two 10uM sections were collected, and the blade and microtome cleaned before the next sample was processed. FFPE (formalin-fixed, paraffin-embedded) sections were incubated with lysis buffer for 15 min at 80°C. Proteinase K was added, and samples incubated at 70°C overnight. The clear solution under the wax was removed and the DNA was extracted using Mag-Bind FFPE DNA kit (Omega Biotek, Norcross, GA) and eluted in 40 µl of ddH2O. DNA was quantitated using the Qubit Fluorometer dsDNA high sensitivity assay kit (ThermoFisher Scientific).

Targeted capture was performed on all exons of 245 known cancer-related genes using a NimbleGen capture array. Libraries were prepared with the Kapa HyperPlus

kit, quantified using the PicoGreen dsDNA Reagent, normalized, and pooled. The pooled samples were captured with the custom NimbleGen Roche SeqCap EZ Choice custom panel, and 2x150bp sequencing was performed on either an Illumina HiSeq4000, or NovaSeq. Sequences were aligned, and *TP53* mutations were identified. Variants passing quality control and filtering were visually confirmed using the Integrative Genomics Viewer (IGV). Variants observed 1 or 2 times, and likely to be enriched in somatic mutations were analyzed for mutational signatures using Mutagene.*

Statistical analysis

Median AFB_1-lys and interquartile range (IQR) were calculated prior to log10-transformation for other analyses. We estimated AFB_1 exposure prevalence and geometric means and 95% confidence intervals (95%CI) using non-response-adjusted sampling weights based on probabilities of selection of households, individuals, and blood sample collection participants. We used linear regression to estimate the ratio of geometric means and 95%CIs across age, sex, habitual residence (urban/rural), ethnicity, and education and Poisson regression to estimate prevalence ratios (and 95%CIs) using Stata (StataCorp. 2015. Release 14. College Station, TX). The study was approved by the institutional review boards at Mexico's National Institutes of Public Health and Cancer.

Results

In a representative sample of adults aged 40-59 from Chiapas (mean age, 48.3 years; SD ± 6.0;) nearly 50% were from rural areas and approximately one third indigenous. The overall prevalence of detectable AFB_1-lys was 85.5% (95%CI 72.1, 93.1) representing 970 702 urban and rural individuals in Chiapas (table I). Prevalence appeared to be higher in younger, indigenous adults who live in rural areas. Median AFB_1 was 0.117 pg/μL (IQR, 0.300) and the geometric mean 2.03 pg AFB_1-lys /μL (95%CI 1.11, 3.72; table II). Adduct levels were three-fold higher in men relative to women (3.68 vs.1.20 pg AFB_1-lys /μL) and in participants living in rural as compared to those in urban areas (3.67 vs.1.22 pg AFB_1-lys /μL).

Characteristics of the 50 HCC patients are shown in table III. The median age was 63 years (interquartile range, 20). The prevalence of chronic HBV infection (HBsAg+) was 4.0% and the prevalence of HCV infection (anti-HCV+) was 14.0%. Overweight and obesity were common in both women and men. Cirrhosis was present in 25% of the women and 42.3% of the men. We detected the *TP53* R249S mutation in three patients (all from mostly rural regions) of the 50 HCCs (6.0%). Two of them were from Veracruz and one was born in Oaxaca (but lived in Mexico City). Four patients with other *TP53* G>T transversions (two samples with *TP53* V157F, one with *TP53* V203L, and one with *TP53* G245V). In total, seven (14%) of patients had mutations that could be related to AFB_1 exposure. Within this group, three tumors were from women and evidence of chronic HCV infection was present in only one patient. A preliminary analysis of mutational signatures revealed that up to 10% of somatic mutations in these tumors may be due to aflatoxin (figure 1).

Table I
PREVALENCE AND PREVALENCE RATIOS OF DETECTED SERUM AFB1-LYS ACCORDING TO PARTICIPANT CHARACTERISTICS. CHIAPAS, MEXICO, 2018-2019

	Unadjusted	
	Prevalence (95%CI)	Prevalence ratio (95%CI)
Overall	85.5 (72.1, 93.1)	-
Age		
40-49	89.7 (75.5, 96.1)	Ref.
50-59	80.2 (59.8, 91.7)	0.89 (0.73, 1.08)
Sex		
Male	86.5 (69.7, 94.7)	Ref.
Female	84.6 (66.2, 93.9)	0.97 (0.81, 1.16)
Residence		
Rural	88.4 (75.6, 94.9)	Ref.
Urban	83.1 (57.5, 94.7)	0.94 (0.74, 1.18)
Indigenous		
No	88.6 (75.1, 95.3)	Ref.
Yes	77.4 (46.2, 93.1)	0.87 (0.62, 1.21)
Education		
0-6 years	83.5 (66.9, 92.6)	Ref.
≥7 years	89.7 (69.8, 97.0)	1.07 (0.89, 1.29)

Weighted n=1 135 324. Indigenous, participants who reported speaking an indigenous language. Sampling weights used for all estimates.

* https://www.ncbi.nlm.nih.gov/research/mutagene/identify

Table II
Geometric means and geometric mean ratios of serum AFB1-lys according to participant characteristics. Chiapas, Mexico, 2018-2019

		Unadjusted	
	N=100	*Geometric mean (95%CI)**	*Ratio of geometric mean (95%CI)*
Overall		2.03 (1.11, 3.72)	-
Age			
40-49	49	2.74 (0.95, 7.86)	Ref.
50-59	51	1.38 (1.06, 1.79)	0.50 (0.16, 1.51)
Sex			
Male	56	3.50 (1.00, 12.25)	Ref.
Female	44	1.25 (1.10, 1.42)	0.35 (0.10, 1.25)
Residence			
Rural	54	3.87 (1.03, 14.57)	Ref.
Urban	46	1.17 (1.09, 1.25)	0.30 (0.08, 1.13)
Indigenous			
No	72	1.67 (1.04, 2.66)	Ref.
Yes	28	3.41 (0.50, 23.05)	2.04 (0.27, 15.14)
Education			
0-6 years	68	2.53 (1.03, 6.23)	Ref.
≥7 years	32	1.28 (1.13, 1.45)	0.50 (0.20, 1.25)

Weighted n=1 135 324. Indigenous, participants who reported speaking an indigenous language.
* In pg AFB1-lys/μL. Sampling weights used for all estimates.

Table III
Characteristics of HCC patients included in the study (n=50) diagnosed between 2005-2015 in Mexico City by gender

	Women n= 24	*Men n= 26*
Median age, years (IQR)	64.5 (27)	63.0 (6)
Hepatitis B virus +	1 (4.2)	1 (3.8)
Hepatitis C virus +	3 (12.5)	4 (15.4)
Regular alcohol intake	3 (12.5)	17 (65.4)
Ever smoker	5 (20.8)	12 (46.1)
Mean BMI, kg/m² (± SD)	24.9 (5.4)	25.5 (3.3)
BMI categories		
Normal weight, <25	13 (54.2)	15 (57.7)
Overweight, 25-29.9	7 (29.2)	7 (26.9)
Obese, ≥30	4 (16.6)	4 (15.4)
Diabetes	7 (29.2)	9 (34.6)
Missing information	6 (25.0)	3 (11.5)
Cirrhosis	6 (25.0)	11 (42.31)
Fatty liver	7 (29.2)	6 (23.1)
Differentiation		
Well	8 (33.4)	11 (42.3)
Moderate	11 (45.8)	8 (30.8)
Poor	5 (20.8)	7 (26.9)
Mutations		
TP53 R249S	0 (0)	3 (11.5)
Other TP53 G>T*	3 (12.5)	1 (7.7)

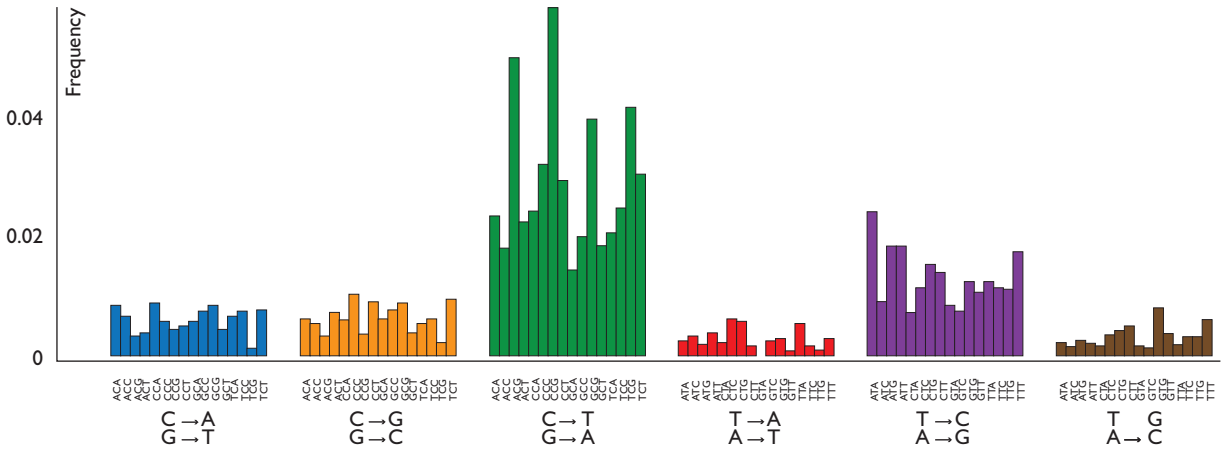
IQR: interquartile range; BMI: body-mass index=weight in kilograms divided by height in meters squared; SD: standard deviation; HCC: hepatocellular carcinoma. Data presented as number (%) unless otherwise specified.
* includes TP53 V157F, V203L, and G245V.

Discussion

We found a high prevalence of exposure to aflatoxin in urban and rural Chiapas. However, circulating AFB_1 levels were moderate. Also, aflatoxin-associated *TP53* R249S mutation prevalence was moderate relative to high aflatoxin exposure regions in the world.

In Chiapas, AFB_1 exposure was >85%. While the magnitude of the exposure was important (0.117 pg/μL; 2.8 pg/mg), it was not as high as in Guatemala (in the Southern border of Chiapas) where AFB_1 was detectable in all participants and the median AFB_1-lys was 8.4 pg/mg (23.8 conversion factor from pg/uL to pg/mg).[9] An initial AFB_1 estimate in adult indigenous women in Mexico found widespread exposure but at a more moderate level relative to very high HCC burden areas.[10] While the study included women mostly in their early thirties, our results may be partly consistent with that observation. However, the exposure levels reported in our study in adults currently living in Chiapas confer increased risk of HCC.[11]

AFB_1 undergoes enzymatic conversion in the liver by CYP3A4 to the AFB_1-8,9-epoxide, the active metabolite that reacts with DNA to form an AFB-N7-guanine adduct. These adducts principally cause G:T/C:A mutations on the transcribed strand through the transcription-coupled pathway of nucleotide excision repair.[12] Almost two decades ago a small study in



* Mutations observed 1 or 2 times and likely enriched in somatic mutations were analyzed using Mutagene Identify (https://www.ncbi.nlm.nih.gov/research/mutagene/identify).

FIGURE 1. FREQUENCY OF MUTATIONS* OBSERVED 1 OR 2 TIMES ON 50 WOMEN AND MEN WITH HCC DIAGNOSED BETWEEN 2005-2015 IN MEXICO CITY

Northern Mexico documented the presence of the AFB_1 codon 249 mutations in HCC tissue (19% prevalence).[13] In another study, this mutation was not present in any of the 69 HCC tissue samples from highly selected patient population in Mexico City most of whom with cirrhosis.[14] The prevalence of this mutation in our study was lower that what was observed then and in Guatemala (24% prevalence) but higher than the 1-3% seen in low aflatoxin regions (the US, Europe, Korea).[5,15] While *R249S* is the dominant *TP53* aflatoxin-induced mutation, this mutation has been studied almost exclusively in high HBV prevalence regions. We also saw several examples of mutation G T / C:A in *V157F*. This mutation is another hotspot in *TP53*, but has not been causally linked to aflatoxin exposure in addition, *R249S* and *V157F* have been reported to be associated with a higher stem-cell-like gene expression and poorer survival.[16]

Major strengths of our analyses are generalizability of AFB_1 exposure estimates to adults in Chiapas and detailed sequencing of *TP53* mutations. However, AFB_1-lys analyses were done in a limited number of participants affecting confidence on subgroup analyses. The examination of the *TP53* mutations was performed in HCC cases that may not be generalizable to all patients seen in Mexico. Currently most HCC cases are diagnosed using imaging without pathology confirmation and, similar to other countries, only a fraction of HCCs were biopsied. Also, the patient population included in our study may not adequately represent the source population of HCC patients in Mexico. In conclusion as the HCC burden remains understudied, our analysis provides evidence that in Mexico AFB_1 may have a potentially important role in the burden of HCC. The five areas in Mexico with the highest HCC burden in order are Chiapas, Veracruz, Yucatán, Tabasco, and Campeche. Future research should focus in these geographic areas and accurately estimate aflatoxin exposure, identify subpopulations at risk and characterize sources of exposures.

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Declaration of conflict of interests. The authors declare that they have no conflict of interests.

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