Specific p53 Mutations Detected in Plasma and Tumors of Hepatocellular Carcinoma Patients by Electrospray Ionization Mass Spectrometry

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Abstract

Hepatocellular carcinoma (HCC), a common cause of cancer deaths worldwide, has several major etiological risk factors, including infection with the hepatitis viruses and exposure to aflatoxin B1. A specific missense mutation resulting from a guanine to thymine transversion at the third position of codon 249 in the p53 tumor suppressor gene has been reported in 10–70% of HCCs from areas of high dietary exposure to aflatoxin B1. Short oligonucleotide mass analysis was compared with DNA sequencing in 25 HCC samples for specific p53 mutations. Mutations were detected in 10 samples by short oligonucleotide mass analysis in agreement with DNA sequencing. Analysis of another 20 plasma and tumor pairs showed 11 tumors containing the specific mutation, and this change was detected in six of the paired plasma samples. Four of the plasma samples had detectable levels of the mutation; however, the tumors were negative, suggesting possible multiple independent HCCs. Ten plasma samples from healthy individuals were all negative. This molecular diagnostic technique has implications for prevention trials and for the early diagnosis of HCC.

Introduction

HCC1 is one of the most common cancers worldwide, and there is a striking geographical variation in incidence. In the People’s Republic of China, HCC accounts for over 250,000 deaths annually with an incidence rate in some areas of the country approaching 150 cases/100,000/year. Etiological factors that have been associated with the development of the disease include infection with the hepatitis B virus or hepatitis C virus and exposure to high levels of dietary AFB1 (1, 2).

Mutations in the p53 tumor-suppressor gene have been identified in a majority of human cancers, and distinct mutational spectra are observed within this gene across cancers of different tissues (3). The most striking example of a specific mutation in the p53 gene is a G→T transversion in the third base of codon 249, which has been detected in 10–70% of HCCs from areas with a high exposure to AFB1, whereas this mutation is absent from HCC in regions with negligible exposure to AFB1 (4–6). Studies in bacteria have shown that aflatoxin exposure causes almost exclusively G→T transversions, providing support for the implication that aflatoxin causes this specific mutation (7). Furthermore, it has been shown that the aflatoxin-epoxide can bind to codon 249 of p53 in vitro (8), and human hepatocarcinoma cells exposed to aflatoxin in the presence of rat liver microsomes had a high prevalence of G→T transversions in codon 249 of the p53 gene (9, 10).

Recently, we have developed a sensitive and specific method for detection of defined genetic variants in PCR-amplified products of the APC gene using ESI-MS (11). In this paper, we report on the application of SOMA to the analysis of p53 mutations in tumor DNA from HCC patients from Qidong and Shanghai and provide a comparison of SOMA to DNA sequencing. Additionally, tumor and plasma pairs from 20 cases of HCC have been analyzed and show for the first time a relationship between the occurrence of this mutation in tumor tissue and its presence in blood circulation.

Materials and Methods

Case Materials. Liver tumor and plasma samples were obtained as part of an ongoing prospective cohort investigation of liver cancer in high-risk areas of China. This collaboration between the Shanghai Cancer Institute, the Qidong Liver Cancer Institute, and Johns Hopkins University has been approved by each of the respective Institutional Review Boards for Human Research. The p53 mutational analysis by DNA sequencing in the initial 25 tumor samples has been reported previously (12). Healthy normal plasma samples were collected from individuals in the United States in an independent study for the analysis of dietary exposures, and this was also approved by the Johns Hopkins University Institutional Review Boards for Human Research.

DNA Extraction. Genomic DNA was prepared from frozen liver tissue using a QIAamp Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer’s recommendations.

Blood samples were collected in EDTA-containing tubes and plasma was transferred to a plain tube and stored at −70°C until further processing. DNA was extracted from plasma using a QIAamp Blood Kit (Qiagen) according to the manufacturer’s protocol. A final elution volume of 50 µl was used. DNA was isolated from 100 µl of plasma for the Chinese samples and from 300 µl of plasma for the normal controls.

PCR. Primers used for PCR amplification were as follows: (a) p53–8F1: 5’-CTACAACATCATGTAACAGCTGGACGCGTGGCGCTGAAC-3’; and (b) p53–8R1: 5’-CTGGAGCTTTCACCACGTGAGTGTGGTAGTGGAGAGT-3’.

The expected size of the product was 84 bp. PCR was performed as described previously (13). Reactions were performed with 25–50 ng of genomic DNA from liver tissue or with 10 µl of DNA eluate from plasma samples. The final reaction volume was 50 µl, and thermocycling conditions were 95°C for 2 min and then 40 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s.

Preparation and Purification of Short Oligonucleotides. Digestion of the PCR products with BpmI restriction enzyme and subsequent purification of the fragments were performed as described previously (11). The size of the internal fragments after BpmI digestion was 8 bp. These fragments were then subjected to HPLC as described previously (11), with the modification that the column used was a 1 × 150 mm YMC ODS-AQ C18 reversed phase column (5 µm, 120 Å pore size; Waters Corp., Milford, MA), and the gradient used was

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3 The abbreviations used are: HCC, hepatocellular carcinoma; SOMA, short oligonucleotide mass analysis; ESI-MS, electrospray ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; AFB1, aflatoxin B1; HPLC, high-pressure liquid chromatography.
from 60% A:40% B programmed to 40% A:60% B in 5 min. Solvent A was 0.4 M 1,1,1,3,3,3-hexafluoro-2-propanol (pH6.9), and solvent B was 50:50 (v:v) 0.8 M 1,1,1,3,3,3-hexafluoro-2-propanol:methanol.

**ESI-MS.** Mass spectra were obtained with a LCQ ion-trap mass spectrometer (Thermoquest; Finnigan MAT, San Jose, CA) equipped with an ESI source operated in the negative ionization mode. The instrument was tuned daily by infusion at 3 μl/min of a synthetic oligonucleotide (10 ng/μl in 60% A:40% B) into the HPLC mobile phase through a low dead-volume tee. Typical settings for the spray voltage were −3.0 to −4.0 kV, and the heated capillary was held at 150°C. Each of the oligonucleotide ions was isolated in turn and subjected to collision-induced dissociation at 30% collision energy. Full scan spectra of the resultant fragment ions from m/z 750 to m/z 2000 were acquired, and signals from up to three specific fragment ions were summed as a function of time for each of the oligonucleotides. The mass spectrometer was programmed to acquire data in the centroid mode (1 Da) as a function of time for each of the oligonucleotides. The mass spectrometer was tuned daily by infusion at 3 μl/min of a synthetic oligonucleotide (10 ng/μl in 60% A:40% B) into the HPLC mobile phase through a low dead-volume tee. Typical settings for the spray voltage were −3.0 to −4.0 kV, and the heated capillary was held at 150°C. Each of the oligonucleotide ions was isolated in turn and subjected to collision-induced dissociation at 30% collision energy. Full scan spectra of the resultant fragment ions from m/z 750 to m/z 2000 were acquired, and signals from up to three specific fragment ions were summed as a function of time for each of the oligonucleotides. The mass spectrometer was programmed to acquire data in the centroid mode (1 μs/scan; 200 msec; isolation width, 3 Da) using six scan events monitoring each [M-2H]2− oligonucleotide individually [Scan event 1, AGG-s (5′-CGGAGGCC-3′), m/z 1256.3 → 750–2000; scan event 2, AGG-as (5′-CCTCCGGT-3′), m/z 1219.8 → 750–2000; scan event 3, AGT-s (5′-CGGAGGCC-3′), m/z 1244.3 → 750–2000; scan event 4, AGT-as (5′-ACCTCCGGT-3′), m/z 1231.8 → 750–2000; scan event 5, AGA-s (5′-CGGAGACC-3′), m/z 1248.8 → 750–2000; and scan event 6, AGA-as (5′-CTCCGGT-3′), m/z 1227.3 → 750–2000]. Analysis of tumor/plasma pairs was performed using only the first four scan events. Reconstructed ion chromatograms were generated and smoothed from this raw data using an isolation width of 1.0 Da and normalized to the largest of the six oligonucleotide ion peaks. The fragment ions used for each oligonucleotide were: AGG-s, m/z 1047.3 + 1180.7 + 1566.0; AGG-as, m/z 1268.6 + 1347.8 + 1637.2; AGT-s, m/z 1404.0 + 1693.1; and AGT-as, m/z 979.3 + 1286.0 + 1652.2. A sample was considered positive for a mutation if there was a signal in both the mutant sense and mutant antisense channels and if the signal was >3% when normalized to the AGG-as peak.

**Results**

**Comparison of SOMA and DNA Sequencing for the Detection of p53 Mutations.** Initial experiments were performed to develop a strategy for an ESI-MS/MS analysis to measure AGG→AGT and AGG→AGA mutations in codon 249 of the p53 gene. For this, SOMA was used in a masked study to analyze 25 HCC samples from patients in Shanghai and Qidong that had been analyzed previously by more traditional methods. The DNA samples were sequenced for mutations in the p53 gene, and 10 of 24 (41.7%) had a detectable G→T mutation in codon 249 with a readable sequence not generated for one sample (12). For the SOMA technique, PCR primers were designed to generate 8-mer oligonucleotides after PCR amplification and BpmI restriction digestion. These fragments were then suitable for analysis using HPLC-ESI-MS/MS. Typical chromatograms for samples with and without a mutation are shown in Fig. 1, A and B. Samples containing only the wild-type sequence had a signal in the channels monitoring masses representative of both the wild-type (AGG) sense and antisense alleles (Fig. 1A). For samples with a G→T mutation at the third base of codon 249, peaks were detected in the sense and antisense channels for both the wild-type (AGG) alleles and the G→T mutant alleles (AGT) (Fig. 1B).

A specific G→T mutation was found by SOMA in 10 of 25 (40%) of the samples, and no mutations were detected by SOMA in DNA from adjacent, histopathologically normal liver tissue. Of the samples from Shanghai, 30% (3 of 10) had a G→T mutation in codon 249 of the p53 gene, whereas this mutation was detected in 46.7% (7 of 15) of the samples from Qidong. There were no G→A mutations detected in any of the samples. This was in agreement with the results obtained by DNA sequencing, with the exception that the readable sequence was unattainable in one sample by DNA sequencing. Thus, SOMA was found to have greater sensitivity compared with DNA sequencing for the measurement of codon 249 p53 mutations as data were obtained for all 25 patients.

**p53 Mutations in Liver Tumors and Paired Plasma Samples.** A prospective cohort investigation is being conducted in Qidong, People’s Republic of China, to examine risk factors for the development of liver cancer. In this study, 2200 men and women have been followed for 8 years and plasma samples collected every 12 months. Twenty liver tumor and plasma pairs obtained at the time of cancer diagnosis arising within this cohort were analyzed for G→T mutations in codon 249 of the p53 gene. The four mass chromatograms for each sample represent the sense (s) and antisense (as) masses for the wild-type (AGG) sequence and the AGT mutation. Patient I has a mutation in both tumor and plasma DNA, whereas patient II does not have a detectable mutation in either tumor or plasma DNA.
Each sample was adjudged to be positive for the p53 mutation when a signal in both the sense and antisense AGT channel was recorded. Paired plasma samples were also analyzed, and 6 out of 11 (55%) patients with a codon 249 mutation in the tumor tissue had a positive plasma sample for the mutation. Representative data are shown in Fig. 2. Five of the nine patients lacking the p53 mutation in their tumor also had no detectable levels of this DNA in their plasma; however, four plasma samples had detectable levels of the codon 249 mutation, whereas none was detected in the liver tissue, suggesting a possible second occult HCC. Ten plasma samples from healthy, normal individuals from the United States were also analyzed, and all of these samples were negative for the p53 specific mutation. In this sample set, a 3-fold larger sample of plasma (300 μl) was used for DNA isolation to achieve a robust wild-type signal. This analysis establishes for the first time a direct link between p53 mutations in liver tumors and detection in plasma samples.

Discussion

We have used SOMA to analyze DNA and plasma samples from HCCs from patients living in Qidong and Shanghai for mutations in codon 249 of the p53 gene. A set of these samples had been analyzed previously by DNA sequencing (12), and all of the mutations detected by DNA sequencing were identified as positive for the mutation by SOMA. These results indicate that SOMA is a sensitive and accurate method for detection of specific mutations.

A higher percentage of the tumors from Qidong had a G→T mutation at the third base of codon 249 of the p53 gene than tumors from Shanghai (46.7% compared with 30%). The mutation frequency corresponds to exposure to aflatoxins because these areas have high and intermediate exposure levels, respectively. The frequency of mutations in the samples from Qidong is similar to that found in other studies (5, 14). However, for HCC cases from Shanghai, lower mutation frequencies have been reported (14).

AFB$_1$ has been observed to induce G→T and G→A mutations at approximately the same frequency in a plasmid-based system with a lacZ mutational target (15, 16), and G→A mutations have also been detected at the third base of codon 249 of the p53 gene in HepG2 cells exposed to aflatoxin in the presence of rat liver microsomes (9). It was therefore of interest to determine whether G→A mutations were present in tumor or normal tissue from HCC patients using SOMA. This could be easily achieved by monitoring masses representing alleles with a G→A transition during the same injection, and therefore did not require additional sample analysis time. No G→A mutations were detected in any of the normal or tumor samples, indicating this mutation is not common in human HCC cases. There have been no previous reports that this mutation has been found in human HCC cases. Because the substitution of a G→A at the third base of codon 249 does not change the amino acid in the protein, this mutation is, therefore, unlikely to provide a selective advantage even if it were formed and subsequent samples were not analyzed for this mutation.

Whereas the detection of specific p53 mutations in liver tumors has provided insight into the etiology of certain liver cancers, the application of these specific mutations to the early detection of cancer offers great promise for prevention (17). A recent report by Kirk et al. (18) reported for the first time the detection of codon 249 p53 mutations in the plasma of liver tumor patients from the Gambia; however, the mutational status of the tumors was not known. These authors also reported a small number of cirrhosis patients having this mutation, and given the strong relationship between cirrhosis and the future development of HCC, the possibility of this mutation being an early detection marker needs to be explored. In this study, we have reported for the first time the relation of plasma and tumor pairs for the occurrence of specific p53 mutations. The presence of a detectable mutation in the plasma when the tumor tissue had only wild-type alleles could be indicative of multiple independent HCC nodules in these patients. The absence of a mutation in the plasma sample from a patient whose tumor was found to have a mutation may be attributable to insufficient sensitivity. Thus, SOMA is a highly accurate method for detection of specific mutations in human tumor samples, and future prospective studies will determine the practical limit of detection. The signal that is measured is quantitative in nature, and we therefore envisage future applications of SOMA in the quantitation of mutations in samples containing low levels of mutant allele in a background of normal alleles. In future studies, the predictive power of this marker to assess HCC development will be investigated.

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References


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