Prospective detection of codon 249 mutations in plasma of hepatocellular carcinoma patients

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A specific missense mutation in the p53 tumor gene at codon 249 has been reported in over 50% of hepatocellular carcinoma (HCC) tumors and in paired blood samples from areas of high dietary exposure to aflatoxin B₁, including Qidong, People’s Republic of China. Using a combination of pre-digestion with HaeIII, PCR and mass spectrometry, the temporality of this mutation in plasma before and after the clinical diagnosis of HCC was examined. Sixteen liver cancer cases, diagnosed between 1997 and 2001, were selected from a prospective cohort of 1638 high-risk individuals in Qidong on the basis of available annual plasma samples spanning the years before and after diagnosis. The codon 249 mutation was detected in plasma samples obtained after diagnosis in seven of the 15 cases (46.7%) with PCR amplifiable DNA, which is in accord with the reported prevalence of this mutation in HCC. The persistent detection of this mutation in plasma collected annually following diagnosis was statistically significant (P = 0.024, two-tailed) in repetitive samples following diagnosis. Moreover, the mutation was detected in the plasma of four of eight cases positive at the time of diagnosis at least 1 year and in one case 5 years prior to diagnosis. Tracking of the marker in pre-diagnostic samples was borderline statistically significant (P = 0.066). None of the 18 healthy US control plasma samples had any detectable mutations. We have therefore found that pre-diagnosis biomarkers of specific p53 mutations can be measured in plasma and this suggests a paradigm for developing these markers for use in prevention and intervention trials.

Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer morbidity and mortality in many parts of the world, including Asia and sub-Saharan Africa, where there are upwards of 500,000 new cases each year and over 200,000 deaths in the People’s Republic of China alone (1). The major etiological factors associated with development of HCC in these regions are infection with hepatitis B and/or hepatitis C virus and exposure to high levels of aflatoxin B₁ (AFB₁) in the diet (2,3). Detailed knowledge of the etiology of HCC has spurred many mechanistic studies to understand the pathogenesis of this nearly always fatal disease.

Mutations in the p53 tumor-suppressor gene are found in a majority of human cancers and distinct mutational spectra have been observed for different cancer types (4). One of the most striking examples of a ‘molecular fingerprint’ in the p53 gene is a guanine to thymine (G to T) transversion at the third base of codon 249, resulting in an amino acid change of arginine to serine, that is found in 50% of HCCs from regions with high exposure to AFB₁, including Qidong, People’s Republic of China (5–8). In contrast, this mutation is absent from HCCs in regions with negligible levels of AFB₁ exposure (9,10). In vitro evidence also indicates that exposure to AFB₁ induces a guanine to thymine transversion at codon 249 of the p53 gene (11–14).

Several studies have now demonstrated that DNA isolated from serum and plasma of cancer patients contains the same genetic aberrations as DNA isolated from an individual’s tumor (15–18). The process by which tumor DNA is released into circulating blood is unclear but may result from accelerated necrosis, apoptosis or other processes (19). Recently, p53 mutations have been detected in DNA isolated from the plasma of patients with HCC (20,21). As the specific G to T mutation at codon 249 results in the loss of a restriction enzyme site present in the wild-type sequence, Kirk et al. (20) were able to use restriction length fragment polymorphism (RFLP) to detect the mutations in the plasma of liver cancer patients in The Gambia, West Africa. We used an electrospray ionization mass spectrometry (ESI-MS)-based method called short oligonucleotide mass analysis (SOMA) (22), to detect p53 mutations in tumor and plasma pairs from Qidong, People’s Republic of China (21). Recently, we found that combining SOMA with pre-digestion using HaeIII resulted in an improved detection limit of 0.4% mutant alleles in the presence of wild-type alleles for the codon 249 mutation in p53 when compared with RFLP; 6% mutant alleles in presence of wild-type alleles (23). In the present study we have employed this more sensitive method to examine the temporality of detecting this mutation in annual plasma samples obtained before and after the clinical diagnosis of HCC.

Materials and methods

Case materials

Plasma samples were obtained as part of an ongoing prospective cohort investigation of liver cancer in Qidong City, Jiangsu Province, People’s Republic of China. At the start of the study in 1992, 852 healthy hepatitis B virus surface antigen (HBsAg)-positive individuals (776 males and 76 females) and 786 HBsAg-negative individuals (723 males and 63 females) residing in six rural townships within Qidong City were recruited. Their ages ranged from 20 to 65 years at the time of enrollment. For the study presented here, 16 cases of liver cancer diagnosed between 1997 and 2001 were selected on the basis of

Abbreviations: AFB₁, aflatoxin B₁; ESI-MS, electrospray mass spectrometry; HCC, hepatocellular carcinoma; RFLP, restriction fragment length polymorphism; SOMA, short oligonucleotide mass analysis.
DNA extraction

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, Valencia, CA) 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 300 μl of plasma for the US samples as described previously (21,23).

Mutation detection by SOMA

SOMA was performed as described previously (21,23). Samples were pre-digested by incubating DNA with 5 U of HaelIII in a volume of 10 μl at 37°C for 2 h. DNA was purified from this reaction mix. The thermocycling conditions were 95°C for 2 min, then 40 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 2 min. Negative controls (no DNA added) were included for each set of PCR reactions. PCR product was purified by ethanol precipitation and digested with 8 U BgIII (New England BioLabs, Beverly, MA) overnight at 37°C in a volume of 20 μl to release the internal fragments. A phenol±chloroform extraction followed by an ethanol precipitation and the presence of SeeDNA (Amersham Pharmacia, Piscataway, NJ) was performed to purify samples for analysis by ESI-MS. Positive and negative controls were obtained from tumor samples as described previously (21,23).

The digested fragments were resuspended in 10 μl of the HPLC mobile phase (70:30:0.1 (v/v) solvent A solvent B, where solvent A was 0.1 M, 1.1,1,3,3,3-hexafluoro-2-propanol (pH 6.9) and solvent B was 50:50 (v/v) 0.8 M 1,1,1,3,3,3-hexafluoro-2-propanol:methanol) and 8 μl was introduced into the HPLC coupled to the ESI-MS. HPLC was carried out at 30 ml/min using a l × 150 mm Luna C18, 5 μm reversed phase column (Phenomenex, Torrance, CA) and Surveyor pumps (ThermoFinnigan Corp, San Jose, CA). The gradient conditions were 70% A:30% B programmed to 100% B in 5 min, where it was held for 10 min. Mass spectra were obtained with a QLC Deca ion-trap mass spectrometer (ThermoFinnigan Corp, San Jose, CA) equipped with an ESI source operated at 10% and 45% of the capillary voltage at 300°C and 2400°C, respectively. The capillary was held at 240°C and the sheath gas flow was 10 l/min using helium as the sheath gas. Each of the oligonucleotide ions was fragmented by collision-induced dissociation at 30% collision energy. Full scan spectra of the resultant fragment ions from m/z 600 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 mscan; 200 ms; isolation width 3 Da) using four scan events monitoring each [M-2H]^2- fragment ion.

Data analysis

The availability of annual plasma samples spanning the time before and after HCC diagnosis. All the liver cancer cases were in men who were HBsAg positive and the median age at diagnosis was 40.5 years (range 25–56 years). Liver cancer diagnosis was made by one or more of the following means: (i) surgical biopsy, (ii) elevated serum γ-glutamyltranspeptidase (levels >100 ng/ml) with consistent clinical and radiological history, (iii) positive computerized axial tomography scan and (iv) ultrasonography with consistent clinical history.

This collaboration between the Shanghai Cancer Institute, the Qidong Liver Cancer Institute and Johns Hopkins University has been approved by each respective Institutional Review Board for Human Research. Healthy normal plasma samples were collected from individuals in a study of dietary exposures and was approved by the Johns Hopkins School of Public Health Committee for Human Research.

DNA and were not counted in the analysis of the data. Samples were scored as a positive or negative for mutation by at least two separate individuals and the data were then used for non-parametric statistical analysis. All of the mutant positive and negative data were then dichotomized to the date of liver cancer diagnosis resulting in scores for positive and negative mutant status before and after clinical diagnosis. The longitudinal data on each individual were summarized by the number of positive samples rB and rA out of the nA amplified samples before and nA amplified samples after the diagnosis, respectively. In order not to only provide a measure of the likelihood of mutation in p53 before and after HCC diagnosis, but to also quantify the level of persistence, we used a beta-binomial model. Specifically, if P denotes the probability of a sample showing a codon 249 mutation in p53, we modeled the belief about P as a beta distribution with parameter (1−P)/P and (1−P)/(1−P); and we modeled r given P as binomial (n, P) with n representing the number of amplified samples. It follows that the marginal distribution of r is beta-binomial with mean nP and variance nP(1−P)(1−n−1). The parameter P represents the likelihood of mutation in p53, and p is the correlation of the presence of mutation in within individual samples (i.e. persistence). Excess frequencies of low (e.g. 0) and/or high (e.g. 1) values of r/n are consonant with p > 0 (i.e. over-dispersion due to persistence). We summarized the inferences by providing the confidence intervals for P and two-sided P-values for testing the null hypothesis of lack of persistence (H0: p = 0). We implemented maximum likelihood methods using the EGRET statistical package (Cytel, Cambridge, MA). Methods used here to quantify persistence of mutation have proven previously to be useful for the assessment of persistence of human papilloma virus infection (24) and for the evaluation of clustering of inactive days in stays of patients in hospitals (25).

Results

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 over 50% of HCCs in Qidong, People’s Republic of China (8). This mutation has only been detected in tumor samples, but has also been measured in DNA isolated from paired blood samples obtained from patients at the time of surgery (20,21). Recently, we found that a combination of SOMA, an electrospray ionization mass spectrometry technique, with pre-digestion using HaeIII resulted in an improved detection limit of mutant alleles in the presence of wild-type alleles for the codon 249 mutation in p53 (23). In this current investigation, we have explored the temporality of the detection of this mutation in plasma before and after the clinical diagnosis of HCC in the same patient. This study was facilitated by the availability of longitudinally collected plasma samples from a cohort of 1638 high-risk individuals in Qidong, People’s Republic of China that have been followed since 1992.

Sixteen liver cancer cases diagnosed between 1997 and 2001 were selected for study on the basis of having available plasma samples that spanned the years before and after HCC diagnosis. Eighteen additional plasma samples from healthy US adults were used as controls and all samples were coded and randomized for analysis. Consistent with previous findings (21), DNA from some of the plasma samples could not be amplified by PCR. While amplification problems tended to be sporadic, the inability to isolate amplifiable DNA from some plasma samples was consistent despite using different lots of QIAgen isolation columns. Table I lists the details of the date of diagnosis, death, sample collection by year and month, ability to PCR amplify DNA from a plasma sample and p53 mutation status. Of the 16 liver cancer cases listed, the maximum number of potential plasma samples was 75 (1996 to time of death) and we collected 66 blood samples (88%) from this nested cohort. The nine missing samples were all due to the subjects being unavailable at the time of collection. These individuals were either out of town or, in the case of some
the HCC patients, unable to provide samples. Of the 66 plasma samples collected, DNA was isolated and could be PCR amplified in 60 of the samples (91%). It was noted that individual F had three plasma samples collected from 1996 to 1998, yet none of these samples could be PCR amplified despite numerous attempts. Thus, meaningful data on $p53$ mutations were obtained for the 15 remaining subjects. Two of the remaining three unamplifiable samples were the 1997 and 1998 collections from individual J. Of the 18 plasma samples obtained from health US controls, we were able to isolate and PCR amplify DNA from every sample.

All of the plasma DNA samples were analysed for the codon 249 mutation on at least 2 separate days. Samples were judged to be positive for the mutation if there was a detectable signal in a wild-type sense or anti-sense channel and at least one or both of the mutant sense and anti-sense channels. As described in the Materials and methods section, the specificity of the signal was dependent upon the detection of unique fragment ions arising from the MS/MS fragmentation pattern. The smallest signal to be obtained in this analysis was in the mutant anti-sense channel as there was only one fragment ion used.

Table II summarizes the detection of the codon 249 mutation in the pre- and post-diagnosis plasma samples. At the top of Table II, we described the frequency of the mutation (i.e. $100 r_A/n_A$ and $100 r_B/n_B$), in the $n_A$ and $n_B$ samples provided by individuals before and after diagnosis, respectively. Although the majority of individuals did not show mutation before the diagnosis, the descriptive statistics at the top of Table II are

<table>
<thead>
<tr>
<th>Individual</th>
<th>Date of diagnosis</th>
<th>Date of death</th>
<th>Year collected</th>
<th>p53 results</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Oct 1999</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Mar 2000 - Apr 2000</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Apr 1998</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Oct 1998</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Sep 1999 - Nov 1999</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Oct 1999</td>
<td>1996</td>
<td>Mutation</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Oct 1999</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Sep 1997 - Mar 2001</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>May 2001</td>
<td>1996</td>
<td>No mutation</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Oct 1999</td>
<td>1996</td>
<td>Mutation</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Oct 2001</td>
<td>1996</td>
<td>Mutation</td>
<td></td>
</tr>
</tbody>
</table>

Table II. Detection of $p53$ mutations at codon 249: pre- and post-liver cancer diagnosis

<table>
<thead>
<tr>
<th>Percent of individuals with mutation</th>
<th>Pre-diagnosis $n = 15^a$</th>
<th>Post-diagnosis $n = 11^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of individuals (%)</td>
<td>No. of individuals (%)</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>10 (67%)</td>
<td>5 (45%)</td>
</tr>
<tr>
<td>0 to 50%</td>
<td>3 (20%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>51 to &lt;100%</td>
<td>1 (7%)</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>100%</td>
<td>Pre-diagnosis</td>
<td>Post-diagnosis</td>
</tr>
<tr>
<td>Persistence of mutation (P value)</td>
<td>0.277 (P = 0.066)</td>
<td>0.549 (P = 0.024)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Percent of mutant samples (95% CI)</th>
<th>Percent of mutant samples (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>21.7% (9.7, 41.9)</td>
</tr>
<tr>
<td>0% to 50%</td>
<td>44.6% (21.6, 70.2)</td>
</tr>
</tbody>
</table>

*a Individuals with amplifiable DNA. 
*b Codon 249 mutations in liver tumors in Qidong (8) = 52%.

The HCC patients, unable to provide samples. Of the 66 plasma samples collected, DNA was isolated and could be PCR amplified in 60 of the samples (91%). It was noted that individual F had three plasma samples collected from 1996 to 1998, yet none of these samples could be PCR amplified despite numerous attempts. Thus, meaningful data on $p53$ mutations were obtained for the 15 remaining subjects. Two of the remaining three unamplifiable samples were the 1997 and 1998 collections from individual J. Of the 18 plasma samples obtained from health US controls, we were able to isolate and PCR amplify DNA from every sample.

All of the plasma DNA samples were analysed for the codon 249 mutation on at least 2 separate days. Samples were judged to be positive for the mutation if there was a detectable signal in a wild-type sense or anti-sense channel and at least one or both of the mutant sense and anti-sense channels. As described in the Materials and methods section, the specificity of the signal was dependent upon the detection of unique fragment ions arising from the MS/MS fragmentation pattern. The smallest signal to be obtained in this analysis was in the mutant anti-sense channel as there was only one fragment ion used.
consistent with over-dispersion (particularly for samples post-diagnosis) due to persistence of the mutation for which beta-binomial models are appropriate. Results from the analysis using a beta-binomial distribution are shown at the bottom of Table II. In the samples collected prior to liver cancer diagnosis, 21.7% of the plasma samples had detectable levels of the codon 249 mutation, with a 95% confidence interval (CI) of 9.7–41.9%. The persistence of this pre-diagnosis marker was borderline statistically significant ($P = 0.066$, two-tailed). None of the 18 healthy US control plasma samples had any detectable mutations. The data indicate that nearly one-half of the potential patients with this marker can be detected at least 1 year and in one case 5 years prior to cancer diagnosis. The codon 249 mutation in $p53$ was detected in 44.6% of all plasma samples following the diagnosis of liver cancer with 95% CI from 21.6 to 70.2%. This level of positive samples following liver cancer diagnosis compares with ~50% of all liver tumors in Qidong (8), suggesting a nearly 90% concordance between plasma and tumor $p53$ codon 249 mutation outcome. Further, the persistence of this mutation for detection in plasma once it became measurable was statistically significant ($P = 0.024$, two-tailed) in repetitive samples following diagnosis.

Representative SOMA chromatograms of wild-type $p53$ codon 249 sense (AGGs) and antisense (AGGas) alleles and mutant sense (AGTs) and antisense (AGTas) alleles are shown in Figure 1. These samples represent a spectrum of the plasma samples from HCC patients and two controls; a plasma control from a US healthy individual and a DNA sample obtained from a liver tumor that had been shown previously to contain the codon 249 $p53$ mutation (23,26). Panel A depicts a plasma sample with a codon 249 mutation detected in both the mutant sense and antisense channels. This sample was obtained several months before the individual was diagnosed with HCC. For qualitative comparative purposes within a given sample, the integrated mass areas of the chromatographic peaks for the wild-type sense, wild-type antisense, mutant sense and mutant antisense were calculated. In Panel A the mass areas for AGGs, AGGas, AGTs and AGTas were 553 600, 506 600, 707 900 and 245 660, respectively. Panel B depicts a plasma sample containing the codon 249 mutation in the AGTs channel (mass area = 30150), no detectable signal in the antisense mutant channel and wild-type sense and antisense mass areas of 627 500 and 960 920, respectively. This individual was diagnosed with HCC 5 years after this sample was collected. Panel C depicts the plasma sample, which had the highest mutant signal in the sense (AGTs mass area = 11 841 330) and antisense (AGTas mass area = 5 339 103) among all that were examined in this study. Parenthetically, this patient was diagnosed with HCC immediately after this sample was obtained and succumbed to the disease 2 months later. Panels D and E represent a plasma control sample from the US samples and a liver tumor positive control, respectively. Collectively the data suggest that nearly one-half of the potential patients with this marker can be detected at least 1 year and in one case 5 years prior to diagnosis. We have therefore found that pre-diagnosis biomarkers of specific $p53$ mutations can be measured in plasma and this suggests a paradigm for developing these markers for use in prevention and intervention trials.

**Discussion**

The development and validation of biomarkers, particularly those associated with exposure, biologically effective dose, early biological effects and risk and the utilization of these tools in the conduct of etiologic investigations and preventive interventions in high-risk populations has been an area of active research over the past decade (27–29). Molecular biomarker studies provide insight to address the relationships between exposure to toxic environmental chemicals and development of chronic human diseases and to identify those individuals at higher risk for disease. Many of the investigations in environmental carcinogenesis have focused upon specific chemical exposures and genetic susceptibility factors, such as metabolic polymorphisms, that confer increased risk in exposed individuals. Complementary to these investigations has been the rapidly evolving elucidation of the molecular mechanisms of carcinogenesis and the identification of critical genes that control cancer development and progression (30).

Recently, the discovery that DNA from cells undergoing apoptosis and other turnover processes was found in the blood has resulted in the ability to non-invasively measure mutations in target genes such as $p53$ (19). Early detection of disease biomarkers offers the potential to discern the genetic consequences of environmental exposures in high-risk individuals (31). Thus, a combined use of exposure and genetic biomarkers might reveal the subset of high-risk people within a population who would most benefit from targeted, mechanism-based interventions.

The recognition that there are critical genetic targets in cells, particularly oncogenes and tumor-suppressor genes, for environmental carcinogens is perhaps best exemplified by the linkage of aflatoxin exposures and specific mutations in the $p53$ tumor-suppressor gene (5–10,12–14). A recent report by Kirk et al. (20) demonstrated the detection of codon 249 $p53$ mutations in the plasma of liver tumor patients from The Gambia, West Africa. While tumor tissue was not available in this study to correlate the presence of this mutation in plasma sera with the tumor, the prevalence of this mutation in HCC patients in West Africa was consistent with previously described data (7). Significantly, these researchers also reported a small number of sera in cirrhosis patients having this mutation and given the strong relation between cirrhosis and future development of HCC, the possibility of this mutation being an early detection marker was implied. In Jackson et al. (21), we reported for the first time the relation of plasma and tumor pairs obtained in Qidong for the occurrence of specific $p53$ mutations. Thus, the initial reports of the presence of a highly specific $p53$ mutation in the sera of HCC patients provide the rationale for further validation of this biomarker for detection of cancer.

The next stage in the validation process of the codon 249 $p53$ mutation in serum was to compare the two reported methods for detection of this specific mutation; RFLP and SOMA (23). RFLP has an advantage that it is a relatively simple technique that relies on the fortuitous presence of a restriction endonuclease site that is either created or destroyed by the mutation of interest. For the aflatoxin-specific $p53$ mutation, a $Hae$III site was present in the wild-type sequence that was lost when there was a mutation at the third base of codon 249. In order to avoid a false positive result, there must be complete digestion of the wild-type sequence and optimization of the digest conditions was required to achieve reliable digestion. SOMA relies upon the molecular mass determination of the PCR amplified fragment and therefore was not subject to the RFLP problem of restriction enzyme-mediated false positives due to incomplete digestion. The sensitivity to detect the presence of mutant in a background of wild-type DNA was
performed using an identical set of serially diluted samples. The sensitivity of each method was determined by the sample with the lowest percentage of mutant allele in the serially diluted series in which a \( p53 \) mutation was consistently detected. The findings indicated that the electrospray mass spectrometry method, SOMA, was ~2.5 times more sensitive than RFLP and this was increased to 15-fold more sensitive if the wild-type alleles were pre-digested with \( Hae \)III prior to PCR amplification during SOMA (23).

In this current investigation we were able for the first time to examine the temporality of the detection of the \( p53 \) mutation in plasma before and after the clinical diagnosis of liver cancer. This study was facilitated by the availability of annually collected plasma samples from a cohort of high-risk individuals in

**Fig. 1.** SOMA analysis of codon 249 mutations in the \( p53 \) tumor-suppressor gene. AGGs and AGGas represent the wild-type sense and antisense allele and the AGTs and AGTas segments are the mutant sense and antisense fragments in codon 249, respectively. (A) A plasma sample with a codon 249 mutation from individual J from an individual N who later that same year was diagnosed with HCC. (B) A plasma sample containing the mutation from individual E whose HCC diagnosis occurred 5 years later. (C) A sample that had the largest mutant signal among all tested in this study. (D) A control plasma sample from a US individual. (E) A chromatogram from a liver tumor specimen obtained in Qidong that had been analyzed previously for mutations (22). All of the scales are in relative abundance of 100% for the largest ion.
Qidong, People’s Republic of China. The selection of the HCC cases was framed by the availability of plasma samples that bracketed the years before and after diagnosis. At the present time there are no data from experimental models or human investigations that provide information on the pharmacokinetics of these mutant DNA fragments in circulation. Future studies need to characterize the accumulation and time-course of this DNA to determine appropriate sampling intervals, nonetheless the availability of samples collected in yearly intervals begins to provide information that can be used to determine the predictive value of these biomarkers. The controls for this study were drawn from healthy US controls where both HCC is low and this p53 mutation has yet to be found in any tumor. The results of this current study found that the codon 249 mutation in p53 was detected in 44.6% of all plasma samples following the diagnosis of liver cancer, compared with ~50% of all liver tumors in Qidong (8), suggesting a nearly 90% concordance between plasma and tumor outcome. Further, the persistence of this mutation for detection in plasma was statistically significant (P = 0.024, two-tailed) in repetitive samples following diagnosis. Prior to liver cancer diagnosis, 21.7% of the plasma samples had detectable levels of the codon 249 mutation. This suggests that nearly one-half of the potential patients with this marker can be detected at least 1 year and in one case 5 years prior to diagnosis. None of the 18 healthy US control plasma samples had any detectable mutations. These findings bolster the previous results of this mutation occurring in plasma samples of liver cirrhosis patients (20) who are at much greater risk of HCC and with the demonstration that this marker presages HCC, the use of this biomarker must continue to be validated.

The use of molecular biomarkers in blood for the early detection of cancer is well established for many forms of the disease and in the case of HCC serum alpha-fetoprotein has become a well-established marker (32). While the use of alpha-fetoprotein as a diagnostic marker for liver cancer is widely used in high-risk areas because of its ease of use and low cost, this marker does suffer from low specificity due to its occurrence in diseases other than liver cancer (33,34). This lack of specificity has contributed to the identification of other molecular biomarkers that are possibly more mechanistically associated with HCC development including hypermethylation of the p16, p15 and GSTP1 promoter regions (35–38). Results from these investigations of the p16, p15 and GSTP1 promoter hypermethylation indicate that these markers are prevalent in HCC, but there is as of yet limited information on the temporality of these genetic changes prior to clinical diagnosis. Thus, the data reported here on p53 mutations prior to diagnosis are among the first evidence pointing to the use of these genetic alterations showing increased risk for an individual.

The data reported in this study, further supports the use of mass spectrometry as a sensitive and specific method for the detection of genetic changes at specific sites in DNA. In addition to the better sensitivity of SOMA compared with RFLP for the detection of codon 249 mutations in p53, the use of mass spectrometry has the potential to develop SOMA as a quantitative method once a method of normalizing levels of wild-type and mutant alleles is characterized. The rapid expansion of our knowledge on the kinetics of these molecular markers in blood will contribute to the enhanced utilization of these markers in future studies. Further, a quantitative approach would have important applications in using the p53 codon 249 mutation as a biomarker for aflatoxin exposure and HCC development, underpinning its use for early detection of HCC and/or as an intermediate endpoint in intervention trials.

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References


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