Geographic Variation of *p53* Mutational Profile in Nonmalignant Human Liver

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Fifty-eight percent of hepatocellular carcinomas (HCCs) from Qidong, China, contain an AGG to AGT mutation at codon 249 of the *p53* tumor suppressor gene, a mutation that is rarely seen in HCCs from Western countries. The population of Qidong is exposed to high levels of aflatoxin B_1 (AFB_1), a fungal toxin that has been shown to induce the same mutation in cultured human HCC cells. To investigate the role of AFB_1 and of these *p53* mutations in hepatocarcinogenesis, normal liver samples from the United States, Thailand, and Qidong (where AFB_1 exposures are negligible, low, and high, respectively) were examined for *p53* mutations. The frequency of the AGG to AGT mutation at codon 249 paralleled the level of AFB_1 exposure, which supports the hypothesis that this toxin has a causative—and probably early—role in hepatocarcinogenesis.

Hepatocellular carcinoma is the predominant cause of cancer mortality in sub-Saharan Africa and southern China. Epidemiological studies have identified infection with hepatitis B virus (HBV) and food contamination with AFB_1 as major and possibly synergistic risk factors (*1*). The hypothesis that AFB_1 has a causative role in the etiology of these cancers is supported by the finding that approximately 55% of the HCCs from these areas contain an AGG to AGT mutation at codon 249 of the *p53* tumor suppressor gene (*2*), a mutation that is preferentially induced in cultured Hep G2 human hepatocytes exposed to AFB_1 (*3*). In contrast, less than 4% of HCCs from most developed countries, in which exposure to AFB_1 is low, contain this mutation (*4*, *5*).

To further investigate the mutagenic action of AFB_1 and to better define the timing and role of codon 249 AGT mutations in hepatocarcinogenesis, we determined the abundance of this mutation in nonmalignant liver samples from HCC patients. We compared samples from the United States, urban Thailand, and Qidong, China, where AFB_1 exposures are negligible, low, and high, respectively. Positions 2 and 3 of codon 249 (AGG) and positions 1 and 2 of codon 250 (CCC) are part of a Hae III restriction site, and changes in this recognition sequence have been measured by the restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR) method (*6*). Absolute mutation frequencies per allele were estimated in several samples with the use of an internal mutant standard, as described (*3*, *6*). The mutant standard, which is added at the outset to the cellular DNA, consists of a *p53* construct with several nucleotide changes relative to wild-type *p53*; this standard is co-amplified with bona fide mutated sequences from the tissue specimens.

The relative abundance of all possible point mutations in codons 249 and 250 was determined for nonmalignant liver tissue obtained from three HCC patients, one from the United States, one from Thailand, and one from Qidong (Fig. 1). In contrast to the U.S. sample (Fig. 1A), in which there was a low incidence of several different mutations, the samples from Thailand (Fig. 1B) and Qidong (Fig. 1C) had a particularly high incidence of the codon 249 AGT mutation. Malignant liver tissue from the same Chinese patient (Fig. 1D) had an even higher incidence of this mutation. This patient also had moderately elevated incidences of the codon 249 AGC mutation (in both nonmalignant and malignant samples) and of the codon 249 AGA mutation (in nonmalignant tissue only).

We then determined the relative abundance of the major mutations detected in these samples—that is, codon 249 AGC, AGT, and AGA, and codon 250 ACC—in a larger group of nonmalignant liver specimens from the United States, Thailand, and Qidong (Table 1). In the U.S. samples and in two samples from Thailand, no consistent pattern of mutation was apparent, and the relative abundance of the codon 249 AGT mutation was low, ranging from 10 to 24%. In contrast, a third sample from Thailand and all samples from Qidong had moderately to strongly elevated incidences of the AGT mutation, ranging from 31 to 77%. A slightly elevated relative abundance of the

Table 1. Relative abundance of *p53* codon 249 and 250 mutations in nonmalignant liver tissue from donors in the United States, Thailand, and Qidong. The experimental conditions were as in (*3*). The relative abundance of a mutation corresponds to the percentage of λ plaques containing a particular base pair change relative to a total of 800 to 1200 plaques on five petri dishes; 10^6 initial copies of *p53* were analyzed for the U.S. samples and 2×10^7 copies were analyzed for all other samples. Samples marked with an asterisk were from healthy U.S. donors; all other samples were of nonmalignant tissue from HCC patients. Samples from healthy donors in Qidong were not available. ND, not determined; wt, wild-type.

Specimen	Source	Relative abundance (%) of mutation at				*p53* genotype of HCC
		Codon 249			Codon 250	
		AGC	AGT	AGA	ACC	
91–19*	U.S.A.	9	16	7	23	ND
91–22*	U.S.A.	10	23	7	19	ND
91–23*	U.S.A.	2	15	3	10	ND
89–56	U.S.A.	8	11	3	9	wt
89–64	U.S.A.	7	24	0	12	wt
S1	Thailand	1	14	8	1	Codon 254 AAG
S4	Thailand	3	39	1	4	ND
S6	Thailand	1	22	0	7	Codon 249 AGT
S16	Qidong	ND	48	0	0	Codon 249 AGT
S17	Qidong	6	31	0	0	Codon 249 AGT
S18	Qidong	5	34	2	1	wt
S19	Qidong	9	67	18	0	Splice site mutation
S31	Qidong	15	77	10	0	Codon 248 CTG
S32	Qidong	9	49	4	4	wt
S33	Qidong	15	72	4	0	Codon 245 GAC
S34	Qidong	9	61	3	0	wt
S35	Qidong	13	53	5	2	wt
S36	Qidong	12	45	9	1	Codon 249 AGT
S37	Qidong	11	60	20	0	Codon 249 AGT

*From healthy U.S. donors.

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codon 249 AGC mutation that had no relation to the geographical origin of the donor was observed in several samples. The exon VII *p53* genotype of the corresponding tumor DNA from patients with HCC was determined by direct sequencing (*7*) (Table 1). There was no correlation between the presence or absence of a codon 249 AGT mutation in the tumor and the relative abundance of this mutation in the corresponding nonmalignant tissue.

Absolute frequencies of the four major codon 249 and 250 mutations in *p53* were estimated by calibration of the data for relative abundance with an internal mutant standard in three samples from Thailand and four samples from Qidong (Table 2). The frequencies represent the ratios of a specific mutated DNA sequence to total wild-type sequences contained in the original tissue specimen. All mutations occurred at low frequencies from <2 to 13 × 10^{-7} in the samples from Thailand. A similar mutational profile was seen in earlier studies of cultured Hep G2 cells (*3*). In contrast, in the samples from Qidong, the codon 249 AGT mutation was always present at high frequencies, in the range of 0.45 × 10^{-5} to 1.84 × 10^{-5}. All four samples from Qidong also contained elevated incidences of the codon 249 AGC mutation, which has been observed only rarely in HCC. Again, there was no consistent relation between the frequency of the codon 249 AGT mutation in the nonmalignant tissue and its presence in the corresponding HCC.

The values for absolute frequencies have to be considered estimates rather than precise measurements. Although we have demonstrated that the DNA fragment containing *p53* exon VII and the mutant standard DNA are amplified at comparable efficiencies (*3*), we cannot be certain that the copy number of the standard molecules remains exactly proportional to that of the bona fide mutated sequences throughout the many steps of the RFLP-PCR protocol (*3*, *6*).

Because the frequencies of codon 249 AGT mutations in nonmalignant tissues

Table 2. Absolute frequencies of *p53* codon 249 and 250 mutations in nonmalignant liver tissues from east Asian HCC patients. Before gel electrophoresis, we added 10 or 25 copies of an internal mutant standard to Hae III–restricted DNA containing 2 × 10^7 copies of the *p53* gene (*3*). The values were calculated from the mutant standard content of the RFLP-PCR products (determined by λ plaque hybridization with a specific probe for the mutant standard), from the initial number of mutant standard copies, and from the number of copies of the *p53* gene present in the Hae III–digested cellular DNA (*3*). The *p53* genotype of the tumor DNA was determined by direct sequencing after amplification of exon VII by PCR. The result was highly reproducible (±3%), as shown by recloning the RFLP-PCR product. ND, not determined; wt, wild-type.

Specimen	Source	Absolute mutation frequency (× 10^{-7})				*p53* genotype of HCC
		Codon 249			Codon 250	
		AGC	AGT	AGA	ACC	
S1	Thailand	0	5	3	0	Codon 254 AAG
S4	Thailand	10	13	0	0	ND
S6	Thailand	0	3	0	0	Codon 249 AGT
S16	Qidong	ND	184	0	0	Codon 249 AGT
S18	Qidong	6	45	3	0	wt
S32	Qidong	17	100	8	7	wt
S36	Qidong	50	180	35	6	Codon 249 AGT

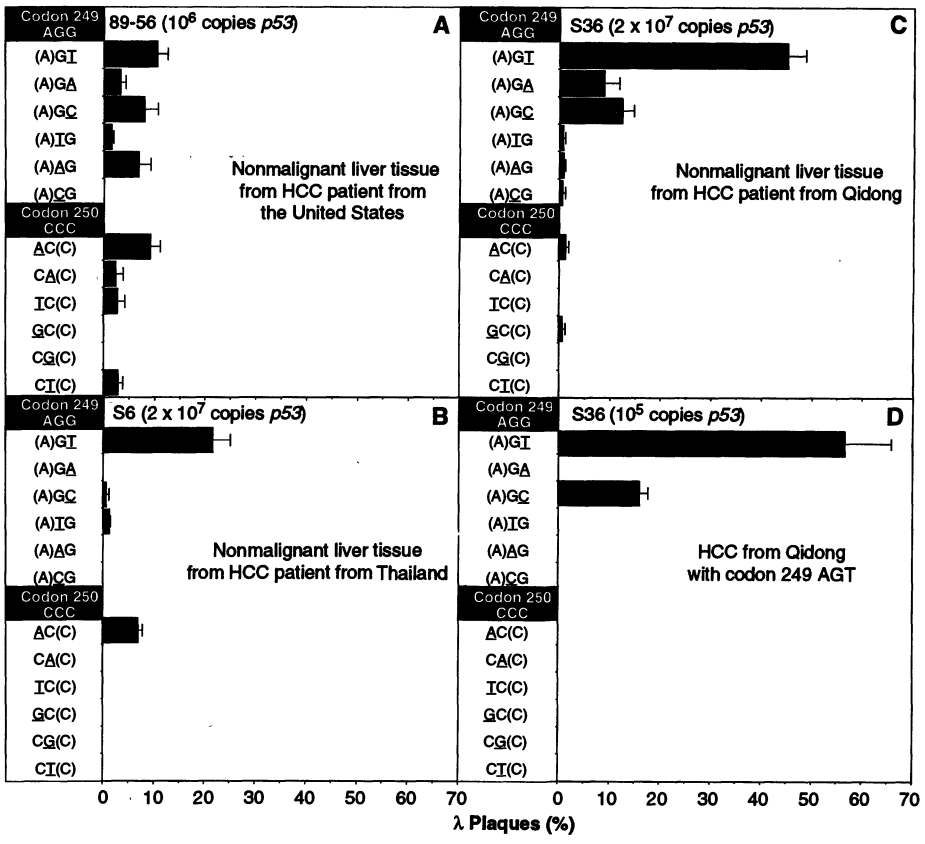

Fig. 1. Relative abundance of *p53* codon 249 and 250 mutations in four specimens of liver tissue from the United States and east Asia [specimen numbers 89-56, S6, S36 (nonmalignant), and S36 (malignant)]. Values in parentheses indicate number of copies of *p53* present in specimen. Liver specimens were collected at the time of surgery, minced, homogenized, and the DNA extracted as in (*3*). DNA containing the indicated number of copies of the *p53* gene was exhaustively digested with Hae III. Using gel electrophoresis, we purified a DNA fragment population containing a mutated 159–base pair (bp) *p53* segment that extends from the 5′ flanking Hae III site (nucleotide 13981) to the 3′ flanking Hae III site (nucleotide 14139). High-fidelity polymerase chain reaction (PCR) amplification of a 101-bp exon VII fragment of *p53*, cloning of the purified RFLP-PCR product into λgt10, and λ plaque analysis by hybridization with a set of mutant-specific oligonucleotide probes were done as in (*3*). The relative abundance of a mutation corresponds to the percentage of λ plaques containing a particular base pair change relative to a total of 800 to 1200 λ plaques on five petri dishes. Error bars denote SD for the data derived from individual petri dishes. The SD of data obtained by recloning the same RFLP-PCR product was less than ±3%. Similar mutation spectra were obtained in four additional U.S. specimens and in two additional Qidong specimens. The *p53* genotype of the tumor DNA was determined by direct sequencing after amplification of exon VII by PCR. (**A**) Nonmalignant specimen from HCC patient from the United States, (**B**) nonmalignant specimen from HCC patient from Thailand, (**C**) nonmalignant specimen from HCC patient from Qidong, and (**D**) malignant specimen from HCC patient in (C).

from HCC patients generally paralleled the level of AFB_1 exposure, our results support the hypothesis that AFB_1 is a causative mutagen in liver cancer. Indeed, this hypothesis is strengthened by laboratory studies showing preferential (but not exclusive) induction of the codon 249 AGT mutation by AFB_1 in cultured Hep G2 cells (*3*). The predominance of this mutation could be, at least in part, a consequence of preferential induction by AFB_1, as a result of sequence context and inefficient repair of pre-mutagenic lesions (*8*). However, we cannot yet rule out a role for other carcinogens. For example, bulky heterocyclic amines in cooked foods and oxidants released by inflammatory leukocytes (*9*) possess the same specificity for G to T transversions and HBV infection is associated with inflammation. All of the liver specimens from Qidong and Thailand were from HBV-infected individuals. However, no correlation has been reported between the incidence of *p53* mutations in HCC and in HBV infection alone (*10*), but HBV infection and exposure to AFB_1 may be synergistic risk factors (*11*).

The abundance of *p53* codon 249 AGT mutations in nonmalignant specimens from Qidong suggests that cells containing these mutations have undergone early, albeit limited, expansion in the histologically normal tissue. Because there was no consistent relation between the mutation frequency in the nonmalignant tissue and the genotype of the tumor from the same patient (Tables 1 and 2), it appears that additional factors, for example, loss of the remaining wild-type allele, are required for the further expansion of mutant cells during tumor progression. The presence of this mutation may merely increase the probability that a cell will progress along the pathway of hepatocarcinogenesis. Two tumors with the *p53* codon 249 AGT genotype also had elevated frequencies of codon 249 AGC mutations, which indicates that the tumors have a multifocal composition. Both codon 249 AGT and AGC mutations result in the replacement of arginine by serine in the mutant protein, which suggests that *p53* codon 249 serine has acquired an oncogenic function.

The presence of elevated frequencies of codon 249 AGT mutations in the nonmalignant tissue of HCC patients from Qidong suggests that the mutagenic event occurred early in hepatocarcinogenesis. In contrast, *p53* mutations in HCCs from geographic areas with low exposure to AFB_1 could be late events. For example, *p53* mutations have been observed more frequently in large tumors and in advanced grades of malignancy in HCCs from Japan (*12*). Similarly, in other organs such as the colon (*13*) and the bladder (*14*), *p53* mutations are thought to occur late in tumorigenesis. However, the methods used in previous work may not have been sensitive enough to detect mutations at early stages of tumorigenesis, before substantial expansion of the mutant cells had occurred.

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Prevention of Lipopolysaccharide-Induced Lethal Toxicity by Tyrosine Kinase Inhibitors

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Septic shock results from excessive stimulation of the host immune system, especially macrophages, by lipopolysaccharide (LPS), or endotoxin, which resides on the outer membrane of bacteria. Protein tyrosine kinase inhibitors of the tyrphostin AG 126 family protect mice against LPS-induced lethal toxicity. The protection correlates with the ability of these agents to block LPS-induced production of tumor necrosis factor α (TNF-α) and nitric oxide in macrophages as well as LPS-induced production of TNF-α in vivo. Furthermore, this inhibitory effect correlated with the potency of AG 126 to block LPS-induced tyrosine phosphorylation of a $p42^{MAPK}$ protein substrate in the murine macrophage.

Septic shock is a major cause of death among patients in intensive care units and ranks 13th in the causes of deaths overall in the United States (*1*). Septic shock results from a systemic infection by Gram-negative bacteria that causes hypotension and multiorgan dysfunction. Except for surgical and supportive care, no specific therapy is known, although very limited success has been reported recently through the use of antibodies against endotoxin (*2*).

Sepsis and septic shock result primarily, if not exclusively, from excessive stimulation of the host immune system, especially macrophages, by the complex glycolipid LPS (endotoxin), which resides in the outer membrane of the bacteria (*3*). Lipopolysaccharide stimulates immunocytes, mainly macrophages, to generate tumor necrosis

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