Hepatocellular Carcinoma p53 G>T Transversions at Codon 249: The Fingerprint of Aflatoxin Exposure?

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The molecular epidemiology of p53 mutations allows the possibility of correlating particular mutations with specific environmental carcinogens and establishing one step in the causal pathway between exposure to carcinogens and the development of cancer. A striking example is the G>T transversion at the third base pair of codon 249 observed in liver cancer patients possibly exposed to high levels of aflatoxins in their agricultural products. In this paper, we describe a systematic review of the literature and assess the quality of the available data. We found methodologic limitations in the studies. In particular, the key independent variable, aflatoxin exposure, was not assessed in these studies, with the exception of one study that measured a marker of exposure. Instead, nationality, geographic residence, or geographic site of hospital were used as surrogate markers for exposure. Patients from areas with high aflatoxin levels were more likely to have p53 mutations than were patients from areas with low aflatoxin levels. In the group with p53 mutations, patients from areas with high aflatoxin levels had higher proportions of mutations with codon 249 G>T transversions. The differences in proportions with p53 mutations were significant, as were the differences in proportions of codon 249 G>T transversions among patients with p53 mutations. Aflatoxin may increase the proportion of p53 mutations by causing a single mutation, the codon 249 G>T transversion, thus explaining some of the excess liver cancer associated with aflatoxin exposure. However, it is premature to conclude that p53 mutations are established markers for environmental carcinogens.

Key words: cancer, epidemiology, gene, hepatocellular carcinoma, meta-analysis, molecular epidemiology, mutation, p53, Environ Health Perspect 105:392–397 (1997)

The molecular epidemiology of p53 mutations allows the possibility of correlating particular mutations with specific environmental carcinogens and establishing one step in the causal pathway between exposure to carcinogens and the development of cancer. Three key characteristics of p53 mutations may make this possible: 1) the p53 gene may be the most frequently mutated gene reported in human cancers; 2) the p53 gene codes for a protein that appears to control processes that play a role in carcinogenesis; and 3) considerable variation has been reported in p53 mutations by base pair site, base pair change, and mutation type (1–9). Thus, many have suggested that p53 mutations are the fingerprint of environmental carcinogens (10–13).

While molecular biologists have enthusiastically embraced this possibility, claiming that "the p53 gene has become a valuable molecular biomarker in etiologic studies..." (14), epidemiologists might take a more circumspect view of the accumulated data regarding p53 mutations and specific environmental carcinogens. One of the most striking examples of a p53 mutation associated with a specific environmental carcinogen and cancer is that of the G>T transversion at the third base pair of codon 249 observed in liver cancer patients living in areas of the world with high levels of aflatoxins in their agricultural products (15,16). This observation may be consistent with epidemiologic research showing an association between primary hepatocellular carcinoma (HCC) and aflatoxin exposure in ecological studies and in nested case–control studies measuring individual biomarkers of exposure (17–19) and with laboratory studies that have demonstrated that aflatoxin B1 induces a G>T transversion at the third position of codon 249 in human HCC cells (20).

Early studies reported a high occurrence of the mutation in tissue samples from HCC patients living in areas such as Qidong, China, where the risk of aflatoxin exposure is high (15,16), and lower occurrences in samples from countries such as Great Britain or Germany, where aflatoxin exposure levels are low (21,22). After these studies appeared, some investigators concluded that the codon 249 base 3 G>T transversion is a consequence of aflatoxin exposure and a step in the development of liver cancer in patients exposed to aflatoxin. Since then, this conclusion has been frequently and firmly expressed, but the correlation between aflatoxin exposure, the codon 249 G>T transversion, and liver cancer has not been quantified or fully described. Is liver cancer in individuals exposed to aflatoxin B1 always associated with the codon 249 G>T transversion? Does the codon 249 G>T transversion in liver cancer patients ever occur in the absence of aflatoxin exposure? Is the excess liver cancer associated with endemic aflatoxin exposure explained by the occurrence of codon 249 G>T transversions? What variables modify the occurrence of codon 249 G>T transversions? From an epidemiologic perspective, it is desirable to assess the strength of association between the presumed causal variable, aflatoxin exposure, the intermediate variable, codon 249 G>T transversion at the third base pair, and the outcome variable, liver cancer. Quantifying these relationships is a step toward making predictive statements with implications for reducing liver cancer incidence in populations exposed to high aflatoxin levels and using codon 249 G>T transversions as markers of aflatoxin exposure.

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In this paper, we describe a systematic review of the literature and assess the quality of the data with respect to the above questions. More than 20 studies in the past 5 years have described the occurrence of codon 249 G>T transversions in HCC patients living in geographic areas with varying levels of aflatoxin exposures. We describe an analysis of the data from multiple small studies that relied primarily on ethnicity and/or nationality as a surrogate indicator of possible aflatoxin exposure.

Methods
A Medline search for the years 1991–1995 produced a data set of 123 abstracts of articles referring to p53 gene mutations or protein expression in hepatocellular carcinoma. References were obtained and included in the analysis if the article described p53 mutations in a sample of patients with hepatocellular carcinoma. Research was excluded that measured p53 protein expression as markers for genetic mutation, but did not describe gene sequencing. Review articles, letters, and animal studies were also excluded. Twenty-seven studies were retrieved and reviewed for inclusion in the analysis (14–16,21–44). Studies were excluded if they did not provide sufficient information on all patients for analysis (33,41,45) or if they measured mutations at codon 249 without reporting the total number of p53 mutations in the sample population (15,16,32,35). These included some of the first studies to have noted a high occurrence of mutations at codon 249. The final sample included 20 studies published between 1991 and 1995 that describe p53 mutations in patients with hepatocellular carcinoma. The study samples sizes ranged from 12 to 140 with an average sample size of 31.

In early studies, it was common to sequence small fragments of the p53 gene or to measure specific mutations, but in the last few years it has become standard to sequence exons 5–8, if not the entire p53 gene. This may mean that early studies underestimated the occurrence of mutations in the p53 gene. The first studies quickly identified the frequent occurrence of the G>T transversion at the third base pair of codon 249 in liver cancer patients, which lead to subsequent studies measuring this particular mutation only. This research focus may have led to an overestimate of the proportion of p53 mutations occurring at codon 249 in liver cancer. In the last few years, the trend has been toward more extensive sequencing of the gene. Variation in the extent of sequencing may bias estimates of the prevalence of p53 mutations, as well as estimates of the proportion of codon 249 mutations, although the degree of bias has not been assessed. Of the studies analyzed below, 12 reported sequencing exons 5–8, 5 reported sequencing more exons 5–8, and 3 sequenced exons 6–8, 7–8, and 6–7 only.

The studies relied on the patient's nationality or the country that supplied the tumor samples as surrogate markers for exposure. The patients were not interviewed to determine their diets, food sources, or previous residences; no study measured actual agricultural exposure, and only one study reported measuring biomarkers of aflatoxin exposure. Hollstein and colleagues measured DNA adducts in the liver and serum albumin of their 15 HCC patients in Thailand (39). For the purpose of this review, patients were categorized as having high or low presumed aflatoxin exposure based on the authors' assessment of aflatoxin exposure and the International Agency for Research on Cancer monograph designating countries as areas of high or low aflatoxin exposure (19). Thus, exposure was not actually measured, but nationality or geographic residence were used as surrogates for exposure.

The assignment of nationality was itself a problem in some studies, many of which relied on stored tumor specimens at university hospitals where patients may have come from wide geographic areas. For example, the study by Oda and colleagues relied on tumor samples obtained from 140 patients at the National Cancer Center Hospital, in Tokyo, Japan (4). Of the patients, 128 were Japanese, 6 were Korean, 4 were Indonesian, and 2 were Taiwanese. The authors considered the group of patients to have been "mainly Japanese" and to have had low exposure to aflatoxin B1. Although the authors described nationalities of the patients, it was not clear whether the non-Japanese patients were immigrants living in Japan or foreigners coming to Japan for treatment. The data were not presented by nationality and there was no way for a reader to remove the non-Japanese patients from the study sample. Thus, the entire group collected by Oda and colleagues was categorized as Japanese and as having low aflatoxin exposure, which leaves open the possibility that some patients in the group may have had high aflatoxin exposure but were misclassified as low exposure. Misclassification was a possibility in all these studies, a fact that is of concern because even nondifferential misclassification can bias the measure of effect, usually towards the null hypothesis (46,47).

Despite the limitations of the exposure data, we proceeded with the analyses because the literature so frequently cites the example of aflatoxin, codon 249 G>T

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| Table 1. Proportions of HCC patients with p53 mutations, by categorization of aflatoxin exposure |
|-----------------|-----------------|-----------------|-----------------|
|                 | Exons sequenced | Gender | Age range | p53 mutations* |
| References      |                 |       |           |                |
| Low aflatoxin exposure |                 |       |           |                |
| Shi et al. (14) | 5–8             | NI    | NI        | 0.31 (13/42)   |
| Bourdon et al. (29) | 2–11           | 16 m, 4 | 28–68    | 0.30 (6/20)    |
| De Benedetti et al. (29) | 5–8           | 9 m, 3 | 9–75     | 0.0 (0/12)     |
| Fujimoto (27) | 6–8             | NI    | NI        | 0.29 (4/14)    |
| Diamantis et al. (28) | 5–6             | NI    | NI        | 0.42 (16/38)   |
| Ng et al. (28) | 5–9             | 28 m, 3 | 38–72   | 0.22 (6/26)    |
| Volkmann et al. (24) | 5–8             | NI    | 2–68     | 0.32 (6/19)    |
| Nose et al. (23) | 2–9             | 18 m, 2 | 28–67    | 0.15 (3/20)    |
| Hosono et al. (30) | 5–8             | 20 m  | 30–71    | 0.15 (3/20)    |
| Nishida et al. (31) | 5–10           | 40 m, 13 | 30–71 | 0.32 (17/53)   |
| Shieh et al. (32) | 5–8             | 12 m, 6 | 2–74    | 0.06 (1/18)    |
| Tanaka et al. (37) | 5–8             | NI    | NI        | 0.29 (10/34)   |
| Li et al. (38)  | 7–8             | NI    | 16 (3/10) | 0.33 (6/19)    |
| Hollstein et al. (39) | 5–8           | 12 m, 3 | 17–73   | 0.13 (2/15)    |
| Challen et al. (27) | 6–7             | 9 m, 10 | 21–76    | 0.11 (2/19)    |
| Oda et al. (40) | 5–8             | NI    | NI        | 0.33 (4/100)   |
| Sheu et al. (42) | 5–8             | 40 m, 2 | 35–72   | 0.33 (20/61)   |
| Li et al. (40)  | 7–8             | NI    | 16 (3/10) | 0.33 (6/19)    |
| Bressac et al. (43) | 5–8             | NI    | 50 (5/10) | 0.80 (4/5)     |
| Hsu et al. (42) | 5–9             | 12 m, 4 | 31–65   | 0.50 (8/16)    |

Abbreviations: HCC, hepatocellular carcinoma; NI, no information; m, male; f, female.  
*Proportion of patients; number in parenthesis is number of patients.  
**Ethnicity, nationality, or geographic location.
transversions, and liver cancer as demonstrating that p53 mutations are markers for exposure. In our analyses, we relied on nationality as a surrogate of exposure and found that studies reported data for a total of 628 HCC patients, 46 of whom were categorized as high aflatoxin exposure, and 582 of whom were categorized as low aflatoxin exposure. The high aflatoxin group included patients from Qidong, China, and southern Africa. The low aflatoxin group included patients from Singapore, Italy, Alaska, Japan, Taiwan, Europe, Shanghai, Thailand, Great Britain, and Germany.

The studies provided little information on variables that may be associated with the causal, intervening, or outcome variables, or variables that may modify the effect of aflatoxin exposure on the occurrence of codon 249 G>T transversions. Seven of the 20 studies provided no information on the gender or age of the patients. Information on gender was available for 317 patients, 242 of whom were males (76%) and 75 of whom were females (Table 1). In most studies, p53 data were not cross-tabulated with gender, thus precluding the analysis of data by gender. In studies that presented tables of raw data, the reader had the option of reanalyzing the data by age; in most studies, information about the age of the patient was presented as a range describing the entire patient group. Four studies included patients under 10 as well as patients over 70; 7 studies included patients in their 20s through 70s. Two studies reported patients in their 30s through 70s. It would be desirable to restrict patients to those with onset in adulthood. The inclusion of patients whose cancer may have had strong hereditary components rather than environmental etiologies would bias the measure of effect by underestimating the proportion of codon 249 G>T transversions in a group of liver cancer patients. In our analyses it was not possible to remove childhood cancer patients from the patient groups.

Thirteen studies provided information about hepatitis B serology that was cross-tabulated with p53 data. Hepatitis B surface antigens were measured in 11 of the studies and hepatitis B viral DNA was measured in 2 of the studies. Data were available describing hepatitis B serology for 449 patients; thus it was possible to assess the role of hepatitis B exposure as a potential confounder of the relationship between aflatoxin exposure and p53 codon 249 G>T transversions. Because ecologic data show a correlation between hepatitis B surface antigen positivity and the incidence of liver cancer and there is some geographic overlap with the areas of high aflatoxin exposure, it would be useful to separate the associations of hepatitis B and aflatoxin with p53 mutations, and the codon 249 G>T mutation in particular.

Only one study of the 20 reviewed measured p53 mutations in two groups of patients—one with high probability of exposure to aflatoxin and the second with a lower probability of exposure to aflatoxin. The remaining 19 studies measured p53 mutations in patient groups that were either high or low exposure and compared their results to those obtained in other studies. Thus, it was not possible to calculate measures of effect within studies, and approaches involving weighted averages of stratum specific effects (e.g., Mantel–Haenszel) could not be used.

Results

Aflatoxin and p53 mutations. The data are summarized in Table 1. Eighteen studies described patients with presumed low levels of aflatoxin exposure and three studies described patients with high levels of aflatoxin exposure (one study described two groups of patients). Data were reported from a total of 628 patients: 582 with low aflatoxin exposure and 46 with high aflatoxin exposure. The proportions of HCC patients with p53 mutations ranged from 0 to 0.42 in the low-exposure group and from 0.45 to 0.50 in the high-exposure group (p = 0.0077, Wilcoxon rank test), indicating an association between p53 mutations and higher presumed aflatoxin exposure.

p53 mutations were reported in 183 HCC patients, 36 of whom had G>T transversions at the third base of codon 249. In the low aflatoxin exposure group, the proportion of codon 249 G>T transversions out of all p53 mutations ranged from 0 to 0.50 in the 18 studies. In the high aflatoxin exposure group the proportion ranged from 0.80 to 1.0 (p = 0.0055, Wilcoxon rank test). These data suggest that high presumed aflatoxin exposure is associated with higher proportions of codon 249 G>T transversions in HCC patients with p53 mutations.

Hepatitis B and aflatoxin. Aflatoxin exposure is often seen in regions with high hepatitis B exposure, and it would be important to distinguish the separate effects of these two independent exposures. Thirteen studies collected data on the occurrence of p53 mutations by hepatitis B serology in liver cancer patients (Table 2). Data were available on a total of 449 patients, 201 of whom had positive hepatitis B serology and 248 of whom had negative hepatitis B serology. To determine whether hepatitis B exposure was associated with aflatoxin exposure, we compared the distribution of positive hepatitis B serology in the presumed high and low aflatoxin groups. The propor-

| References | Hepatitis B measure | p53 mutations by hepatitis B exposure | RR p53 mutations, hepatitis B + and B− | Codon 249 mutations among patients with p53 mutations, by hepatitis B exposure |
|------------|---------------------|--------------------------------------|----------------------------------------|------------------------------------------|
| Bourdon et al. (25) | HbsAg, anti-HBc, or anti-HBs | 2/5 (0.4) | 3/15 (0.2) | 2 | 0/2 | 0/3 |
| De Benedetti et al. (26) | HbsAg | 0/10 | 0/2 | – | – | – |
| Ng et al. (29) | HbsAg | 6/24 (0.25) | 0/2 | – | 2/6 (0.33) | 0/0 |
| Volkman et al. (24) | HBV | 2/7 (0.29) | 4/12 (0.33) | 0.86 | 1/2 (0.5) | 0/4 |
| Nose et al. (23) | HbsAg | 1/8 (0.13) | 2/12 (0.17) | 0.75 | 0/1 | 0/2 |
| Hosoda et al. (30) | HbsAg | 3/17 (0.17) | 0/3 | – | 0/3 | 0/0 |
| Shieh et al. (34) | HbsAg | 3/17 (0.17) | 14/46 (0.30) | 1.41 | 0/3 | 0/14 |
| Li et al. (38) | HBV DNA | 3/15 (0.2) | 0/3 | – | 1/3 (0.33) | 0/4 |
| Holstein et al. (39) | HbsAg | 2/7 (0.29) | 6/20 (0.30) | 0.62 | 1/2 (0.5) | 0/0 |
| Oda et al. (40) | HbsAg | 10/30 (0.33) | 36/98 (0.36) | 0.92 | 1/10 (0.1) | 1/36 (0.03) | 0/0 |
| Sheu et al. (42) | HbsAg | 15/41 (0.37) | 5/20 (0.25) | 1.46 | 3/15 (0.2) | 0/5 |

| Categorized as high aflatoxin exposure |
| Li et al. (38) | HBV DNA | 8/16 (0.5) | 1/4 (0.25) | 2 | 8/8 (1.0) | 1/1 (1.0) |
| Brassac et al. (43) | HbsAg or Ab and/or anti-HBc | 5/8 (0.63) | 0/1 | – | 4/5 (0.8) | 0/0 |

Abbreviations: HCC, hepatocellular carcinoma; RR, relative risk; B+, positive for hepatitis B; B−, negative for hepatitis B; HbsAg, hepatitis B surface antigen; anti-HBs, anti-hepatitis B surface antigen; HBV, hepatitis B virus; HbsAb, hepatitis B surface antibody; anti-HBc, anti-hepatitis B core antigen.

*pNumber of patients; number in parenthesis is proportion of patients.
Aflatoxin and p53 mutations, controlling for hepatitis B. The association between high aflatoxin and p53 mutations was still observed even after restricting the analysis to hepatitis B positive patients. The proportions of p53 mutations were lower in patients with presumed low aflatoxin exposure compared to the high exposure group (Table 2; 0–0.43 in the low group, 0.5–0.63 in the high group; \( p = 0.04 \), Wilcoxon rank test). We could not make a similar comparison for hepatitis B negative patients because the numbers of hepatitis B negative patients with high aflatoxin exposure were so small.

The association between aflatoxin and codon 249 G>T transversions also was observed after restricting the analysis to hepatitis B positive patients. The proportions of codon 249 G>T transversions were lower in patients with presumed low aflatoxin exposure compared to the high exposure group (Table 2; 0–0.5 in the low group, 0.8–1.0 in the high group; \( p = 0.04 \), Wilcoxon rank test). Again, we could not make a similar comparison for hepatitis B negative patients because the numbers of hepatitis B negative patients with high aflatoxin exposure were so small. Thus, it appears that the association between aflatoxin and p53 mutations is not explained by any confounding introduced by possible associations between aflatoxin and hepatitis B.

Hepatitis B and p53 mutations, controlling for aflatoxin. We then attempted to assess the independent association of hepatitis B positive serology with p53 mutations. p53 mutations were reported in 60 out of 201 (29.9%) patients with positive hepatitis B serology and in 66 out of 248 (26.6%) patients with negative hepatitis B serology. The Mantel–Haenszel estimate of the relative risk was 1.3 (CI, 0.88–1.82) for the effect of hepatitis B exposure on the occurrence of p53 mutations, indicating the possibility of a small increase in p53 mutations associated with hepatitis B exposure, but the confidence interval of the estimate included 1. In seven of these studies, the small sample sizes resulted in zero cells, and relative risks were not calculated. In six studies of patients with presumed low aflatoxin exposure, the estimates of relative risk for the effect of hepatitis B exposure on the occurrence of p53 mutations were as follows: 0.75, 0.86, 0.92, 1.41, 1.46, and 2.0. Two studies showed a protective effect of hepatitis B exposure, one study showed no effect, and three studies showed increases in p53 mutations associated with hepatitis B exposure. In the two studies describing patients with presumed high aflatoxin exposure, one study had zero in one of its cells and no relative risk was calculated. The second study (38) reported twice as many hepatitis B positive patients (8/16) with p53 mutations compared to hepatitis B negative patients (1/4). The data suggest that hepatitis B positive individuals may have an increased occurrence of p53 mutations, independent of the effect of aflatoxin exposure, but the increase was not statistically significant in these data. Given the occurrence of a p53 mutation, it was then of interest to determine whether hepatitis B positive serology was associated with an increase in the occurrence of codon 249 G>T transversions.

Of the 130 p53 mutations in HCC patients for whom hepatitis B serology was measured, 26 (20%) were codon 249 base 3 G>T transversions. Of the 11 studies that reported hepatitis B serology and codon 249 G>T transversions in patients with presumed low aflatoxin exposure, 6 studies showed a higher proportion of codon 249 G>T transversions in the hepatitis B positive group than in the hepatitis B negative group. In five of these six studies, relative risks could not be calculated in the studies with zero cells, but the one study with data in all four cells showed a relative risk of 3.3 (40). The other five studies found no codon 249 G>T transversions in either group (Table 2). The data seem to suggest an association between hepatitis B positive serology and the codon 249 G>T transversion in patients with low aflatoxin exposure, but statistical tests were not significant.

It would be interesting to know whether hepatitis B positive serology was associated with an increase in codon 249 G>T transversions in patients with high aflatoxin exposure. Two studies reported hepatitis B serology and codon 249 G>T transversions in patients with presumed high aflatoxin exposure. Almost all the patients had hepatitis B positive serology and almost all the patients had codon 249 G>T transversions; thus, it was not possible to separate the effects of aflatoxin and hepatitis B in this group of patients.

Discussion
As early as 1991, Ozturk (15) stated that "a codon 249 mutation of the p53 gene identi-
group did not have any p53 mutations. It was also unclear whether the codon 249 G>T transversion occurred in the absence of aflatoxin exposure. The mutation was reported in 16 individuals living in areas with low aflatoxin levels. Perhaps these individuals had migrated from other areas and had, in fact, been exposed to aflatoxin; national residence is a poor surrogate for lifetime exposure to aflatoxin. Alternatively, aflatoxin may not be the only carcinogen to cause the codon 249 G>T transversion.

Our analysis of published data suggests that hepatitis B positive serology may be independently associated with the codon 249 G>T transversion. That is, in patients with presumed low aflatoxin exposure, patients positive for hepatitis B seemed to have higher occurrences of the mutation. Such a finding may be biologically plausible, although the exact role of hepatitis B viral infection in promoting codon 249 G>T transversions is debated. The complex interrelationships between aflatoxin exposure, hepatitis B history, hepatocellular carcinoma, and p53 mutations could not be sorted out with the data from these studies. However, they suggest the possibility of independent effects of aflatoxin and hepatitis B on the occurrence of the codon 249 G>T transversion in p53. While the associations between hepatitis B and p53 mutations (and the codon 249 G>T transversion) were not statistically significant, they may warrant further investigation. The 1993 IARC monograph (19) stated, “what evidence is available does not strongly suggest a direct relationship between codon 249 mutation and HBV status” and cited three studies comprised of observations from 40 patients; two of the studies reported no codon 249 mutations in any patient (positive or negative for hepatitis B). Based on our review of the data, we would be inclined to examine this question further.

Several examples have been cited in the literature that illustrate the possibility that p53 mutations serve as the fingerprint of environmental carcinogens. Of these, the association between aflatoxin, codon 249 G>T transversions, and hepatocellular carcinoma may be one of the clearest examples. We systematically reviewed the literature regarding this example to determine whether the data support the concept that p53 mutations are the markers of aflatoxin B1, an environmental carcinogen. We concluded that the data do not yet permit quantification of the association between aflatoxin and codon 249 mutations and that it is premature to state definitively that the codon 249 G>T transversion is the fingerprint of aflatoxin B1. Epidemiologic methods and principles can be applied to this newly emerging and fascinating area of research. In particular, we suggest a more complete characterization of exposure, larger sample sizes, use of control groups within studies, data reporting that permits cross-tabulation of relevant characteristics, information pertaining to potential confounders, and appropriate statistical analyses. From an epidemiologic point of view, fundamental research has yet to be done. The data regarding p53 mutations in hepatocellular carcinoma are intriguing and suggestive, but conclusions that p53 mutations are established markers for environmental carcinogens are premature.

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