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Molecular Epidemiology of p53 Protein Mutations in Workers Exposed to Vinyl Chloride

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The production of mutations in cellular tumor suppressor genes such as p53 is involved in the development of many human cancers. These mutations result in the expression of mutant forms of the encoded p53 protein which can potentially serve as a biomarker for this carcinogenic process. Workers exposed to vinyl chloride who are at risk for the development of the sentinel neoplasm angiosarcoma of the liver represent a model population for the study of such a mutant p53 biomarker, since vinyl chloride is known to cause specific p53 mutations in persons with angiosarcoma of the liver. To determine the relation between vinyl chloride exposure and this p53 biomarker, the authors examined serum samples collected between 1987 and 1992 from a cohort of 225 French vinyl chloride workers and 111 unexposed controls (matched according to age, sex, race, smoking, and alcohol drinking) for the presence of mutant p53 protein, using an enzyme-linked immunosorbent assay. Stratification of the exposed workers by quartile of vinyl chloride exposure (in estimated ppm-years) yielded a statistically significant trend of increasing odds ratios for p53 biomarker seropositivity with increasing exposure. These results suggest that this serum biomarker for mutant p53 protein is related to vinyl chloride exposure and may be an early indicator of carcinogenic risk in exposed individuals. *Am J Epidemiol* 1998;147:302–8.

epidemiology, molecular; genes, p53; hemangiosarcoma; liver neoplasms; occupational exposure; protein p53; vinyl chloride

The production of specific mutations in cellular oncogenes and tumor suppressor genes is believed to be involved in the development of certain cancers related to exposure to chemical carcinogens (1). The occurrence of such mutations in oncogenes and tumor suppressor genes results in the expression of mutant forms of their encoded protein products which participate in the process of cellular transformation and hence the development of cancer (1). Detection of the expression of such mutant protein products in vivo could therefore result in their serving as potential biomarkers for the molecular epidemiologic study of chemical carcinogenesis in human populations exposed to carcinogens (1). A model population for such study is provided by workers who have been occupationally exposed to vinyl chloride and are at risk for the development of a sentinel neoplasm, angiosarcoma of the liver (2).

Vinyl chloride is a known animal and human carcinogen which is rapidly absorbed following respiratory exposure and is primarily metabolized in the liver by the cytochrome P450 2E1 system to the electrophilic metabolites chloroethylene oxide and chloroacetaldehyde (3). These metabolites react with DNA bases to form adducts that are known to be mutagenic in bacterial and mammalian cells, including 7-(2′-oxoethyl)guanine, 1,N^6-ethenoadenine, 3,N^4-ethenocytosine, and N^2-3-ethenoguanine (3). In animal experiments, the oxoethyl- adduct is the major liver DNA adduct formed, representing 98 percent of all adducts, but it is the least persistent, with a half-life of approximately 62 hours (4). On the other hand, the less common etheno- adducts are highly persistent, with half-lives of more than 30 days, which suggests that they are poorly recognized by the liver DNA repair system, and the ethenoadenine adduct is apparently not repaired at all (5).

The production of such ethenoadenine adducts could account for the occurrence of adenine→thymine transversions identified in the p53 tumor suppressor gene in vinyl chloride-associated angiosarcomas of the

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Abbreviation: CI, confidence interval.

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liver (figure 1). For example, in a study of p53 gene mutations in tumors or cell lines from vinyl chloride-exposed workers, three of five (60 percent) were found to contain an adenine→thymine transversion at codon 179, 249, or 255 of the p53 protein (6, 7), and similar mutations have not been identified in liver angiosarcomas not induced by vinyl chloride (8). These mutations result in the substitution of leucine for the normal arginine at amino acid residue 179, the substitution of tryptophan for the normal arginine at amino acid residue 249, or the substitution of phenylalanine for the normal leucine at amino acid residue 255 in the encoded p53 protein product. These amino acid substitutions occur in regions of the p53 protein that have been highly conserved throughout evolution, which suggests that they are critical for determining the structure and function of the protein (9).

In fact, using a mouse monoclonal antibody that is specific for the mutant conformations of this protein, we have shown that these amino acid substitutions alter the protein's normal structure and result in accumulation of the mutant protein in liver angiosarcoma tumor cells and in the serum of individuals with these tumors (9). To date, we have identified accumulations of mutant p53 protein via immunohistochemistry in 11 of 15 (73 percent) angiosarcomas of the liver from vinyl chloride-exposed workers (2, 9, 10). In seven of these cases, serum samples were available; five of them were tissue-positive and two were tissue-negative for mutant p53 protein. The tissue-negative

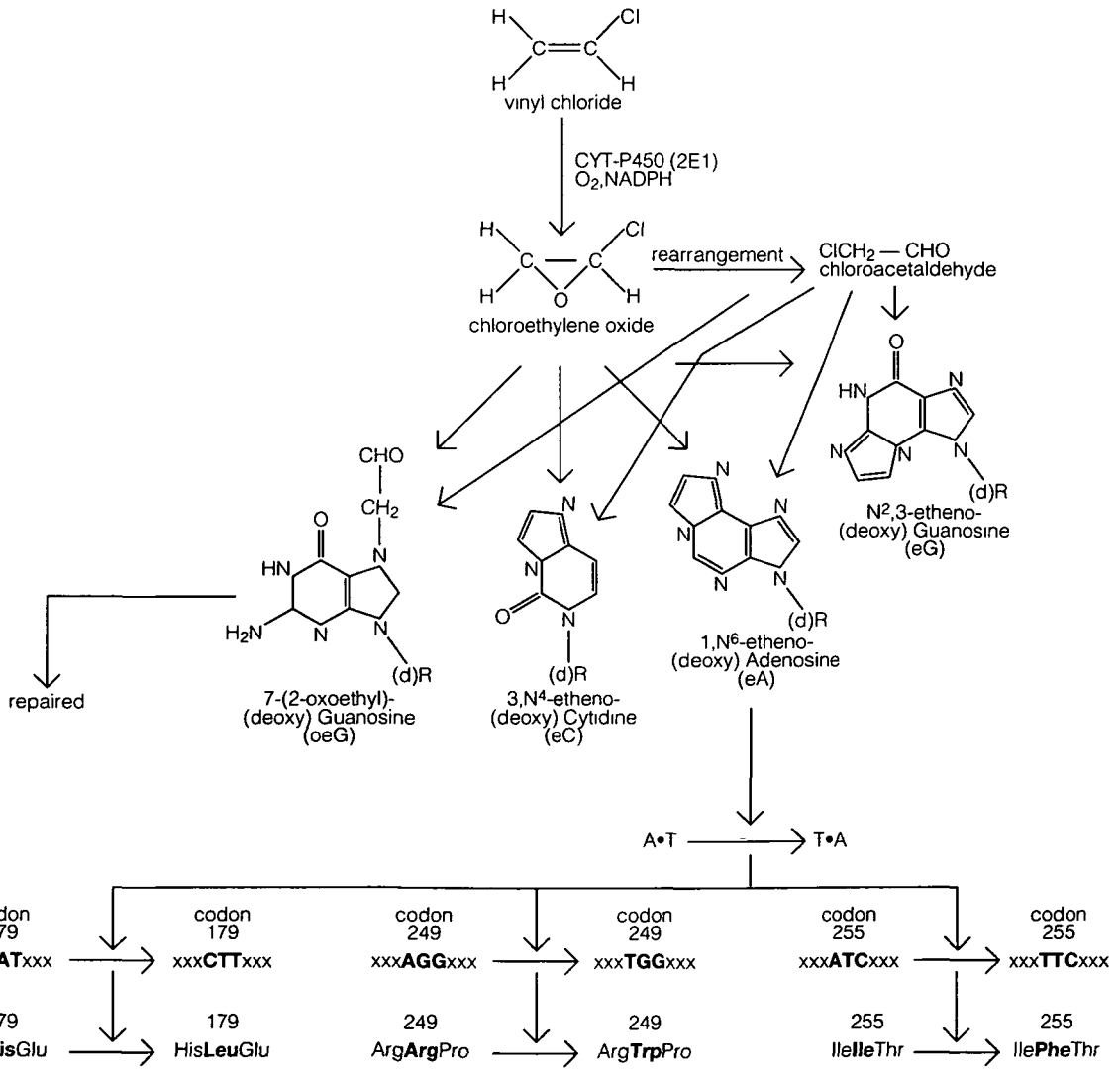

FIGURE 1. Proposed carcinogenic mechanism of vinyl chloride involving the production of mutations in the p53 protein. CYT-P450, cytochrome P450; NADPH, nicotinamide adenine dinucleotide phosphate; C, cytosine; A, adenine; T, thymine; G, guanine; His, histidine; Glu, glutamic acid; Leu, leucine; Arg, arginine; Pro, proline; Trp, tryptophan; Ile; isoleucine; Thr, threonine; Phe, phenylalanine.

cases were also seronegative for mutant p53, whereas the tissue-positive cases were seropositive for mutant p53. Thus, the detection of mutant p53 protein in serum in this group accurately reflected the occurrence of p53 mutations in these individuals' tumor tissue.

In addition, in a preliminary study, we examined the serum expression of mutant p53 protein in 21 vinyl chloride-exposed workers without angiosarcomas of the liver and in 18 matched unexposed controls (2). Only one of the unexposed controls (6 percent) was seropositive, but three of the exposed workers (14 percent) were, which suggests that detection of the mutant p53 protein in serum may indicate the mutagenic effect of vinyl chloride exposure prior to the development of malignancy. The present study extends these observations by examining the presence of the mutant p53 biomarker in the serum of 225 vinyl chloride-exposed workers and 111 matched unexposed controls, to test the hypothesis that this biomarker is directly related to vinyl chloride exposure.

MATERIALS AND METHODS

Subjects and samples

Subjects for study were selected from a previously described population of vinyl chloride-exposed workers and unexposed controls (11). Briefly, a cohort of more than 650 workers employed in vinyl chloride polymerization plants in France since 1950 have been followed by INSERM (Lyon, France). A group of 225 workers with available serum samples was randomly selected from job categories with likely vinyl chloride exposure (such as autoclave cleaner/operator, vinyl chloride production, packing/drying, and maintenance); selection was made among four estimated exposure levels to obtain quartiles of exposed workers of approximately the same size. Estimates of vinyl chloride exposure in ppm-years were based on years worked in a given job category, weighted by the presumed ppm level of exposure as defined by the exposure matrix of Heldaas et al. (12) for vinyl chloride workers. Thus, the 225 workers were divided into exposure strata as follows: ≤500 ppm-years (i.e., 4–500 ppm-years), $n = 54$; 501–2,500 ppm-years, $n = 62$; 2,501–5,000 ppm-years, $n = 51$; and >5,000 ppm-years (i.e., 5,001–46,702 ppm-years), $n = 58$. With this stratification, the average exposure level in each of the groups was approximately four times that of the preceding group. For these individuals, information was also available on age, sex, race, smoking status, and alcohol consumption. All of the exposed workers were white males, with an average age of 53.8 years (range, 35–78 years); 42.2 percent had ever smoked cigarettes, 25.3 percent consumed alcohol daily on a regular basis, and the average vinyl chloride exposure level was 3,735 ppm-years (range, 4–46,702 ppm-years). For these workers, serum samples had been collected between 1987 and 1992 by routine venipuncture techniques and had been stored frozen at −20°C until the time of analysis.

Controls ($n = 111$) were selected from hospitalized patients with noncancer diagnoses who had stored serum samples and no known exposure to vinyl chloride or other known carcinogens (except for cigarette smoke), so as to be group-matched to the exposed workers according to age (±5 years), sex, race, smoking status (ever vs. never), and alcohol consumption (daily vs. not daily). All of the unexposed controls were white males, with an average age of 57.7 years (range, 28–83); 37.8 percent had ever smoked cigarettes, and 28.8 percent regularly consumed alcohol daily. On the basis of their clinical and exposure histories, these controls were assumed to have no a priori reason to be positive for p53 mutations—other than those individuals exposed to cigarette smoke, smoking being known to cause p53 mutations (13). For these unexposed controls, serum samples had been similarly collected between 1987 and 1989 by routine venipuncture techniques and had been stored frozen until the time of analysis.

Sample analysis

All samples were analyzed for the presence of mutant p53 protein by laboratory workers blinded to exposure status, as described previously (2). The analysis was performed with a sandwich enzyme-linked immunosorbent assay based on the mouse monoclonal antibody PAb240, which is specific for the mutant conformations of the protein. The serum samples were analyzed directly without any pretreatment, so that any p53 protein present would presumably be in a nondenatured state, and thus the assay would detect only mutant p53 and no wild-type protein. Briefly, microtiter wells were precoated with PAb240, and then 100 μl of serum was added to each well and incubated overnight at 4°C. After washing, 100 μl of a rabbit polyclonal reporter antibody for p53 was added to each well and allowed to incubate at room temperature for 2 hours. After additional washing, the remaining reporter antibody was bound to horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G, and color was developed by incubation with the chromogenic substrate 2,2′-azino-di-[3-ethylbenzthiazoline sulfate]. The absorbance of each well was read on a spectrophotometric plate reader at 405 nm, and the concentration of mutant p53 was determined by comparison with the absorbance of standard solutions of purified, recombinant, mutant human p53. On the ba-

sis of previous results obtained with serum from mutation-positive cases of liver angiosarcoma (9), a positive serum result for mutant p53 protein was considered to be any level greater than 0.25 ng/ml.

Data analysis

For the initial analysis, results of biomarker status determination (positive or negative) were stratified by degree of vinyl chloride exposure (no exposure or one of the four quartiles of exposure), and, using the unexposed controls as the reference group, crude Mantel-Haenszel odds ratios and 95 percent confidence intervals were calculated for each stratum along with χ^2 and p values for trend. Using logistic regression analysis, adjusted odds ratios and related trends were similarly calculated adjusting for age (in decades), smoking status (ever vs. never), and alcohol consumption (daily vs. not daily). Finally, the crude and adjusted analyses were repeated for the vinyl chloride-exposed groups alone, using the lowest exposure quartile (≤500 ppm-years) as the reference category.

RESULTS

Among the vinyl chloride-exposed workers, 91 (40 percent) were seropositive for mutant p53 protein. Among the unexposed controls, nine (8 percent) were seropositive for mutant p53 protein. This difference in the proportion of seropositives between the unexposed controls and the vinyl chloride-exposed workers was statistically significant ($\chi^2 = 35.65, p < 0.00001$). No significant difference in seropositivity was noted by job category among the exposed workers ($\chi^2 = 0.76$, $p = 0.38$). In addition, no significant difference in seropositivity was noted by the presence of clinical signs or symptoms associated with vinyl chloride exposure (e.g., Raynaud's syndrome, osteolysis, hepatomegaly, hyperechogenicity of the liver, etc.) among the exposed workers ($\chi^2 = 0.03, p > 0.50$).

When the biomarker results were stratified by quartile of vinyl chloride exposure with an odds ratio of 1 assigned to the unexposed controls (table 1), the crude odds ratio for the presence of biomarker increased from 4.77 (95 percent confidence interval (CI) 2.04–11.16) in the lowest exposure quartile (≤500 ppm-years) to 5.80 (95 percent CI 2.59–12.99) in the second quartile (501–2,500 ppm-years), 10.07 (95 percent CI 4.55–22.30) in the third quartile (2,501–5,000 ppm-years), and 12.14 (95 percent CI 5.63–26.18) in the highest quartile (>5,000 ppm-years). Thus, the trend for increasing biomarker positivity with increasing exposure was statistically significant ($\chi^2 = 43.47, p < 0.00001$).

Because of potential problems in terms of comparability with the control group (see "Discussion" section), the analysis was repeated excluding the control group and using men in the lowest exposure quartile (≤500 ppm-years) as the reference group, with an assigned odds ratio of 1 (table 2). In this case, the crude odds ratio for the presence of biomarker increased from 1.22 (95 percent CI 0.55–2.68) in the second quartile (501–2,500 ppm-years) to 2.11 (95 percent CI 0.95–4.70) in the third quartile (2,501–5,000 ppm-years) and 2.54 (95 percent CI 1.17–5.52) in the highest quartile (>5,000 ppm-years). The trend for increasing biomarker positivity with increasing exposure remained statistically significant ($\chi^2 = 7.33$, $p = 0.007$).

Although the unexposed controls were group-matched to the exposed workers for possible confounding variables, several of these could still have potentially influenced the observed exposure-biomarker trend. For example, individuals with greater exposure are likely to be of greater age, and if the occurrence of biomarker varies with age, this could account at least in part for the observed trend. However, no significant variation in the occurrence of biomarker with age (by decade) was present among the unexposed controls

TABLE 1. Dose-response relationship between serum biomarker evidence of mutant p53 protein and vinyl chloride exposure in 225 French workers, using unexposed controls as the reference group

Exposure (ppm-years)	Biomarker findings		Crude odds ratio*	95% CI†	Adjusted odds ratio‡	95% CI
	Negative	Positive				
0 (n = 111)	102	9	1.00		1.00	
≤500 (n = 54)	38	16	4.77	2.04–11.16	4.16	1.63–10.64
501–2,500 (n = 62)	41	21	5.80	2.59–12.99	5.76	2.39–13.85
2,501–5,000 (n = 51)	27	24	10.07	4.55–22.30	10.24	4.20–24.95
>5,000 (n = 58)	28	30	12.14	5.63–26.18	13.26	5.52–31.88

* For trend, $p < 0.00001$.
† CI, confidence interval.
‡ For trend, $p < 0.0001$. Adjusted for age, smoking status, and alcohol consumption.

TABLE 2. Dose-response relationship between serum biomarker evidence of mutant p53 protein and vinyl chloride exposure in 225 French workers, using the group with the lowest exposure as the reference group

Exposure (ppm-years)	Biomarker findings		Crude odds ratio*	95% CI†	Adjusted odds ratio‡	95% CI
	Negative	Positive				
≤500 (n = 54)	38	16	1.00		1.00	
501–2,500 (n = 62)	41	21	1.22	0.55–2.68	1.21	0.54–2.72
2,501–5,000 (n = 51)	27	24	2.11	0.95–4.70	2.08	0.87–5.00
>5,000 (n = 58)	28	30	2.54	1.17–5.52	2.36	0.96–5.84

* For trend, $p = 0.007$.
† CI, confidence interval.
‡ For trend, $p = 0.03$. Adjusted for age, smoking status, and alcohol consumption.

($\chi^2 = 4.48, p = 0.35$). Cigarette smoking has not been associated with angiosarcoma of the liver, but smoking is known to be capable of producing p53 mutations in other tissues (13), and it is theoretically possible that smoking could have an influence on the mutagenic effects of vinyl chloride by altering respiratory exposure, absorption, and/or metabolism. Among the unexposed controls, all nine of the seropositive individuals were smokers, so seropositivity was significantly related to smoking status in these men ($\chi^2 = 16.09, p < 0.0001$). No such significant relation was found among the exposed workers (approximately equal proportions of smokers and nonsmokers were seropositive), although a trend for higher seropositivity among smokers was present in the two lowest exposure groups. Furthermore, animal studies have suggested that ethanol may positively interact with vinyl chloride in the production of angiosarcoma of the liver (14). Among the unexposed controls, biomarker was present in a higher proportion of drinkers (12.5 percent seropositive) than of nondrinkers (6.3 percent seropositive), although this difference did not achieve statistical significance ($\chi^2 = 1.16, p = 0.28$). Similarly, among the exposed workers, biomarker was detected in a higher proportion of drinkers (47.4 percent seropositive) than of nondrinkers (38.1 percent seropositive), but this difference did not achieve statistical significance ($\chi^2 = 1.52, p = 0.22$).

To account for any potential interaction between age, smoking, and drinking and the exposure-biomarker relation, we adjusted the odds ratios for these variables (table 1), but the effect of adjustment was minimal. The adjusted odds ratio for the presence of biomarker still increased from 4.16 (95 percent CI 1.63–10.64) in the lowest exposure quartile (≤500 ppm-years) to 5.76 (95 percent CI 2.39–13.85) in the second quartile (501–2,500 ppm-years), 10.24 (95 percent CI 4.20–24.95) in the third quartile (2,501–5,000 ppm-years), and 13.26 (95 percent CI 5.52–31.88) in the highest quartile (>5,000 ppm-years). The trend for increasing biomarker positivity with increasing exposure remained significant ($p < 0.0001$).

Similarly, when the analysis was restricted to the exposed workers only, using the lowest exposure group as the reference category, the effect of adjustment was also negligible (table 2). The adjusted odds ratio for the presence of biomarker increased from 1.21 (95 percent CI 0.54–2.72) in the second quartile (501–2,500 ppm-years) to 2.08 (95 percent CI 0.87–5.00) in the third quartile (2,501–5,000 ppm-years) and 2.36 (95 percent CI 0.96–5.84) in the highest quartile (>5,000 ppm-years). The trend for increasing biomarker positivity with increasing exposure remained significant ($p = 0.03$).

DISCUSSION

The main purpose of this study was to determine whether the expression of a serum biomarker for mutant p53 protein was related to vinyl chloride exposure. The finding of a significant dose-response relationship strongly supports this hypothesis, and thus provides corroboration for the proposed carcinogenic pathway of vinyl chloride via p53 protein mutation in humans (figure 1). These results parallel previous findings of a strong dose-response relationship between vinyl chloride exposure and the serum expression of mutant *ras* oncogene-encoded p21 protein, presumably produced by guanine→adenine transitions in codon 13 of the K-*ras* gene caused by the N^2,3-ethenoguanine vinyl chloride–DNA adduct (11). Together these results suggest that the production of mutant p21 oncogene protein and mutant p53 tumor suppressor gene protein are important steps in the development of angiosarcoma of the liver in vinyl chloride-exposed individuals. Future research should focus on the use of such combinations of biomarkers for the prediction of subsequent development of cancer.

The obvious biologic significance of these biomarkers stems from the fact that they apparently represent

important steps on the causal pathway toward disease and thus may have significant predictive value. The results of this study do not provide any estimation of the predictive value of the p53 biomarker in determining which individuals will subsequently develop cancer and which will not, but follow-up of this cohort should provide this information. There is evidence from other studies that serum markers of mutant p53 protein may have predictive value. For example, in a study of banked serum samples from asbestos-exposed workers, some of whom subsequently developed asbestos-related malignancies such as lung cancer and mesothelioma, mutant p53 protein was detectable in serum up to 7 years prior to the time of diagnosis of p53-positive cancer; in that study, the positive predictive value of serum mutant p53 protein was 0.75, and the negative predictive value was 0.57 (15–17). Therefore, it seems likely that seropositivity for mutant p53 protein will have reasonable predictive value in the present study.

If the predictive value of this biomarker is borne out by follow-up, the information will be of little practical clinical utility in the absence of effective interventions to abort the carcinogenic mechanisms. In the future, the development of specific chemopreventive interventions for seropositive individuals may be feasible. For example, it may be possible to utilize p53 peptides or antibodies that cause mutant p53 proteins in vitro to revert to normal conformation and function in vivo (18, 19) for treatment of seropositive individuals and prevention of cancer.

Besides the scientific advantage of documenting carcinogenic pathways in vivo in humans via biomarkers and the clinical advantage of exploiting the predictive value of biomarkers for purposes of prevention, molecular epidemiologic studies such as this may have relevance for risk assessment. For example, the presence or absence of biomarkers among subgroups of workers with varying levels of exposure could provide intermediary evidence for a potentially protective exposure level. In most Western countries, the workplace exposure limit for vinyl chloride has been 1 ppm since 1974 (3). However, if some individuals with such low exposure levels (e.g., <40 ppm-years or 1 ppm for 40 working years) tested positive for these biomarkers, this could suggest that current permissible exposure limits may not be adequately protective. It is interesting to note that in the current study, 14 individuals had estimated exposures below 40 ppm-years (average = 11 ppm-years; range, 4–18 ppm-years), and five of them (35.7 percent) were seropositive. The adjusted odds ratio for seropositivity in this subgroup in comparison with the unexposed controls was just statistically significant (odds ratio = 4.16, 95 percent CI 1.01–17.18). Further study of these biomarkers in low exposure cohorts appears warranted.

Several limitations of this study should be emphasized. First, no direct vinyl chloride exposure data for this cohort were available. As noted above, exposure was estimated on the basis of job category, years worked, and exposures extrapolated from historical evaluations of similar work situations (12). The INSERM cohort tends to be a stable worker population that spends long periods of time in job categories generally recognized as having a high likelihood of vinyl chloride exposure and minimal exposure to other potential workplace carcinogens, but the exact vinyl chloride exposure of the cohort remains unknown. The amount of error that this uncertainty in exposure classification introduces into analysis is similarly unknown, although the strength of the dose-response relationship demonstrated provides some reassurance as to the reasonableness of the estimation used.

Second, the estimation of data on potentially confounding factors, particularly amounts of smoking and drinking, was also somewhat crude. This may partially explain the lack of statistical significance for an alcohol interaction with vinyl chloride exposure in relation to the biomarker, which was anticipated on the basis of animal data suggesting a synergistic effect of ethanol and vinyl chloride in the induction of angiosarcoma of the liver (14). In terms of smoking, it was not surprising to note that all of the seropositive individuals among the controls were smokers, since smoking is another known carcinogenic exposure that can produce p53 mutations (13). Although complete data on smoking were unavailable, further investigation established that most (eight of the nine) of these seropositive controls were current smokers with significant tobacco exposure (i.e., more than two packs per day for many years). A similar effect of p53 seropositivity in unexposed controls who are heavy smokers has been noted in other studies (15–17). The fact that this effect of smoking in the exposed group was not significant may be partially attributable to the fact that smoking was estimated to be much lighter (i.e., less than one pack per day) in the exposed group. Further study with more precise delineation of the timing, patterns, and quantity of smoking and drinking will be necessary in order to better define these potentially confounding effects.

Lastly, as noted above, the controls were drawn from a population of hospital patients, who may not be the same as healthy controls drawn from the general working population in terms of risk for this biomarker. Attempts to collect samples from a more appropriate control population are under way. Nevertheless, analysis of the data in the exposed group alone, omitting

the control group, still demonstrated a significant trend for expression of the biomarker with exposure. Thus, despite the possible limitations, these results strongly support the presence of a dose-response relationship between vinyl chloride exposure and serum mutant p53 biomarker status.

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