BIOMARKERS, 1998, VOL. 3, NO. 6, 433–439

Mutant p21ras in vinyl chloride-exposed workers

YONGLIANG LI[1], MARIE-JEANNE MARION[2], MARINA ASHEROVA[1], DRISSA COULIBALY[1], STEVEN J. SMITH[1], TAMARA DO[1], WALTER P. CARNEY[3] and PAUL W. BRANDT-RAUF[1,*]

[1] Division of Environmental Health Sciences, Columbia University School of Public Health, 60 Haven Avenue B-1, New York, NY 10032, USA

[2] Unite de Recherche sur les Hepatites, le Sida et les Retrovirus Humains, INSERM, 69424 Lyon, France

[3] Oncogene Science, 80 Rogers Street, Cambridge, MA 02142, USA

Received 5 January 1998, revised form accepted 23 April 1998

The production of mutations in cellular oncogenes such as *ras* is involved in the development of many human cancers. These mutations result in the expression of mutant forms of the encoded p21 protein which can potentially serve as a biomarker for this carcinogenic process. Workers exposed to vinyl chloride (VC) who are at risk for the development of the sentinel neoplasm angiosarcoma of the liver (ASL) represent a model population for the study of such a mutant p21ras biomarker, since VC is known to cause a specific *ras* mutation in ASL. In order to determine the relationship between VC exposure and this biomarker, serum samples from a cohort of 225 French VC workers and 111 age–sex–race–smoking–drinking matched unexposed controls were examined for the presence of mutant p21ras by immunoblotting with a mouse monoclonal antibody specific for the mutant protein. Stratifying the exposed workers by degree of VC exposure in estimated ppm-years by quartiles yielded a statistically significant trend for increasing odds ratio for sero-positivity of the p21ras biomarker with increasing exposure. These results suggest that this serum biomarker is related to VC exposure and may be an early indicator of carcinogenic risk in exposed individuals.

Keywords: angiosarcoma of the liver, immunoblotting, p21ras, serum biomarker, vinyl chloride.

Introduction

The production of specific mutations in cellular oncogenes is believed to be involved in the development of certain cancers related to exposures to chemical carcinogens (Bishop 1991). The occurrence of such mutations results in the expression of mutant forms of the encoded protein products which participate in the process of cellular transformation and hence the development of cancer. The detection of the expression of such mutant protein products *in vivo* could therefore serve as potential biomarkers for the molecular epidemiological study of chemical carcinogenesis in human populations exposed to carcinogens (Brandt-Rauf *et al.* 1995a). A model population for such study is provided by workers who have been occupationally exposed to vinyl chloride (VC) and are at risk for the development of a sentinel neoplasm, angio- sarcoma of the liver (ASL).

VC is a known animal and human carcinogen capable of producing N^2–3-ethenoguanine DNA adducts in mammalian livers, which can result in G –> A transitions at the second base of codon 13 of the Ki-*ras* oncogene and the

* To whom correspondence should be addressed.

1354-750X/98 $12.00

expression of a mutant Asp 13 p21 encoded protein in resultant ASLs (Brandt-Rauf *et al.* 1995b). For example, 15 of 18 (83%) ASLs from VC-exposed workers have been found to be positive for this mutation. The mutant Asp 13 p21ras protein can be distinguished from wild-type p21 and other mutant p21s immunologically with a mouse monoclonal antibody that is specific for this mutant protein (LaVecchio *et al.* 1990). We have previously shown that it is possible to use this antibody to detect mutant p21 expression in cells in culture that contain the mutant *ras* gene by immunocytochemistry and in the extracellular supernatant by immunoblotting. Similarly, we have shown that mutant p21 expression can be detected in the tumour tissue by immunohistochemistry and in the serum by immunoblotting of VC-exposed workers with ASLs known to contain the mutant *ras* gene but not in VC-exposed workers with ASLs that do not contain the mutation (DeVivo *et al.* 1994). Thus, the detection in serum of mutant p21 seems to accurately reflect the occurrence of *ras* gene mutations in the target tissue in VC-exposed workers. We have also examined the serum expression of mutant p21 protein in 45 VC-exposed workers without ASLs and in 28 matched unexposed controls (DeVivo *et al.* 1994). There were no sero-positive individuals among the controls (0%) compared with 22 sero-positives among the exposed workers (49%), suggesting that detection of the mutant p21 protein in serum may indicate the mutagenic effect of VC exposure prior to the development of malignancy. The present study extends these observations by examining the presence of the mutant p21 biomarker in the serum of 225 VC-exposed workers and 111 matched unexposed controls to test the hypothesis that this biomarker is directly related to VC exposure.

Methods

Subjects for study were selected from a previously described population of VC-exposed workers and unexposed controls (DeVivo *et al.* 1994). Briefly, from a cohort of more than 650 workers employed in VC polymerization plants in France since 1950 and followed at INSERM, a sample of 225 workers with available serum samples was randomly selected from among the job categories with likely VC exposure (such as autoclave cleaner/operator, VC production, packing/drying and maintenance) among four groups of estimated exposure levels to obtain quartiles of exposed workers of approximately the same size. Estimates of VC 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.* (1984) for VC workers. Thus, the 225 workers divided into strata of exposure as follows: < 500 ppm-years, $N = 54$; 501–2500 ppm-years, $N = 62$; 2501–5000 ppm-years, $N = 51$; > 5000 ppm-years, $N = 58$. For these individuals, information was also available on age, gender, 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% with a history of ever having smoked cigarettes, 25·3% with regular daily alcohol consumption, and with an average VC exposure level of 3735 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 stored frozen at –20 °C until the time of analysis. Controls ($N = 111$) included all suitable subjects selected from hospitalized patients with stored serum samples with non-cancer diagnoses and no known exposure to VC so as to be group-matched to the exposed workers for age (± 5 years), gender, race, smoking status (ever versus never) and alcohol consumption (daily versus not). All of the unexposed controls were white males with an average age of 57·7 years (range = 28–83), 37·8% with a history of ever having smoked cigarettes and 28·8% with regular daily alcohol consumption. For these unexposed controls, serum samples had been similarly collected between 1987 and 1989 by routine venipuncture techniques and stored frozen until the time of analysis. The unexposed controls were not individually matched to the exposed workers for date of blood collection, but, as noted, the dates of collection of the samples for both groups overall were comparable to account for any possible differential degradation of the samples over time, even though it is known that the p21ras protein is stable for years with storage at –20 °C.

All samples were analysed for the presence of Asp 13 p21 protein blinded to the exposure status by immunoblotting using the primary mouse monoclonal antibody D146 specific for the mutant protein as

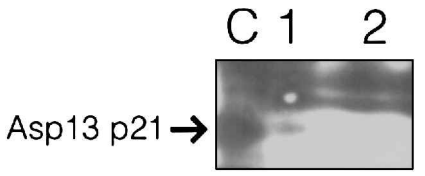

Figure 1. Representative immunoblot for Asp 13 p21ras of positive control HCT 116 cell lysate (Lane C) and serum from a positive VC-exposed worker (Lane 1) and a negative unexposed control (Lane 2) probed with mouse monoclonal antibody D146. The positive control band and all positive serum bands for p21ras migrated at the same location as the 21 kDa molecular weight markers.

described previously (DeVivo *et al.* 1994), except that the colorimetric detection system was replaced by the more sensitive enhanced chemiluminescence method (ECL, RPN 2106; Amersham, Buckinghamshire, UK), used according to manufacturer's instructions. The reliability of this assay has been demonstrated previously by the reproducibility of results on triplicate repeats of the same serum sample run on separate gels as well as consistency over time on repeat serum samples taken from the same individuals 1–2 years apart (DeVivo *et al.* 1994, Brandt-Rauf *et al.* 1995b). Also, as previously, proportionate combinations of coded serum samples from exposed workers and unexposed controls were run on each gel and results were recorded blinded to their exposure status (DeVivo *et al.* 1994). Results of mutant p21 status (positive or negative) were stratified by degree of VC exposure (unexposed or one of the four quartiles of exposure), and, using the unexposed controls as reference, crude Mantel–Haenzel odds ratios and 95 % confidence intervals were calculated for each strata along with the χ^2 and p value for trend. Using logistic regression analysis, adjusted odds ratios, 95 % confidence intervals, and trend were similarly calculated adjusting for age (in decades), smoking status (ever versus never) and alcohol consumption (daily versus not). Finally, the crude and adjusted analyses were repeated for the VC-exposed groups alone, using the lowest exposure group (<500 ppm-years) as reference.

Results

Results are presented in figure 1 and tables 1 and 2. Overall, 76 (34%) of the VC-exposed workers were seropositive for mutant p21ras protein compared with 4 (4%) of the unexposed controls, a statistically significant difference ($\chi^2 = 37{\cdot}31$, $p < 0{\cdot}00001$).

When the biomarker results are stratified in quartiles of VC exposure with an odds ratio of one assigned to the unexposed controls (table 1), the crude odds ratio for the presence of biomarker increases from 8·48 (95 % CI 3·01–23·89) in the lowest exposure group (<500 ppm-years) to 11·82 (95 % CI 4·50–31·07) in the next highest group (501–2500 ppm-years) to 14·59 (95 % CI 5·57–38·25) in the next highest group (2501–5000 ppm-years) to 21·73 (95 % CI 8·76–53·94) in the highest group (> 5000 ppm-years). Thus, the trend for increasing biomarker positivity with increasing exposure is statistically significant ($\chi^2 = 41{\cdot}41$, $p < 0{\cdot}00001$).

Although the unexposed controls were group-matched to the VC-exposed workers for potential confounders, several of these could still possibly influence the observed exposure–biomarker trend. For example, individuals with greater exposure are likely to be older, and, if the occurrence of the biomarker varies with age, this could account at least in part for the observed trend. In fact, occurrence of biomarker with age (in decades) was found to demonstrate a statistically significant trend ($\chi^2 = 8{\cdot}68$, $p = 0{\cdot}003$). In addition, animal studies have suggested that ethanol may positively interact with VC in the production of ASL (Radicke *et al.* 1981). Among the VC-exposed workers, the presence of the biomarker did occur in a higher proportion of drinkers (40·4% sero-positive) compared with non-drinkers

Table 1. Dose-response relationship between serum biomarker for mutant p21 and VC exposure using unexposed controls as reference

Exposure, ppm-years	Biomarker		Crude odds ratio (95 % CI)[a]	Adjusted odds ratio (95 % CI)[b]
	Negative	Positive		
0 ($N = 111$)	107	4	1	1
< 500 ($N = 54$)	41	13	8·48 (3·01–23·89)	10·18 (2·94–35·25)
501–2500 ($N = 62$)	43	19	11·82 (4·50–13·07)	13·61 (4·26–43·46)
2501–5000 ($N = 51$)	33	18	14·59 (5·57–38·25)	15·43 (4·83–49·28)
> 5000 ($N = 58$)	32	26	21·73 (8·76–53·94)	21·55 (6·99–66·44)

[a] For trend, $p < 0{\cdot}00001$.
[b] For trend, $p < 0{\cdot}0001$; adjusted for age, smoking status and alcohol consumption.

Table 2. Dose-response relationship between serum biomarker for mutant p21 and VC exposure using lowest exposure group as reference

Exposure, ppm-years	Biomarker		Crude odds ratio (95 % CI)[a]	Adjusted odds ratio (95 % CI)[b]
	Negative	Positive		
< 500 ($N = 54$)	41	13	1	1
501–2500 ($N = 62$)	43	19	1·39 (0·61–3·19)	1·22 (0·52–2·88)
2501–5000 ($N = 51$)	33	18	1·72 (0·74–4·02)	1·27 (0·51–3·16)
> 5000 ($N = 58$)	32	26	2·56 (1·15–5·73)	1·70 (0·68–4·30)

[a] For trend, $p = 0{\cdot}018$
[b] For trend, $p = 0{\cdot}266$; adjusted for age, smoking status and alcohol consumption

(31·6% sero-positive), although this difference was not statistically significant ($\chi^2 = 1{\cdot}48$, $p = 0{\cdot}23$). Also, among the VC-exposed workers, the presence of the biomarker did occur in a higher proportion of smokers (35·8% sero-positive) compared with non-smokers (32·3% sero-positive), although once again this difference was not statistically significant ($\chi^2 = 0{\cdot}30$, $p = 0{\cdot}59$).

In order to account for any interaction between these potential confounders and the exposure–biomarker relationship, the odds ratios and 95 % confidence intervals for the exposure quartiles were adjusted for age, drinking and smoking (table 1), but the effect of adjustment was minimal. The adjusted odds ratio for the presence of biomarker still increased from 10·18 (95 % CI 2·94–35·25) in the lowest exposure group (< 500 ppm-years) to 13·61 (95 % CI 4·26–43·46) in the next highest exposure group (501–2500 ppm-years) to 15·43 (95 % CI 4·83–49·28) in the next highest exposure group (2501–5000 ppm-years) to 21·55 (95 % CI 6·99–66·44) in the highest exposure group (> 5000 ppm-years). The trend for increasing biomarker positivity with increasing exposure remained highly significant ($p < 0{\cdot}001$).

Finally, because of potential problems in terms of comparability between the unexposed controls and the exposed workers for other uncontrollable variables (see

Discussion below), the exposure–biomarker trend analysis was repeated excluding the control group and using the lowest exposure group (<500 ppm-years) as the reference group with an assigned odds ratio of one (table 2). In this case, the adjusted odds ratio for the presence of biomarker increased to 1·22 (95 % CI 0·52–2·88) in the next highest exposure group (501–2500 ppm-years) to 1·27 (95 % CI 0·51–3·16) in the next highest exposure group (2501–5000 ppm-years) to 1·70 (95 % CI 0·68–4·30) in the highest exposure group (> 5000 ppm-years). Thus, the trend for increasing biomarker positivity with increasing exposure remained, although it failed to achieve statistical significance ($p = 0·27$) primarily due to the adjustment for age.

Discussion

The aim of this study was to determine whether the expression of serum mutant p21ras is related to VC exposure, as suggested by prior studies. The finding of a highly significant dose–response relationship strongly supports this hypothesis, providing corroboration for the proposed carcinogenic pathway of VC via *ras* mutation in humans produced by a G –> A transition at the second base of codon 13 of the Ki-*ras* gene due to N^2, 3-ethenoguanine VC–DNA adducts (Brandt-Rauf *et al*. 1995b). These results parallel previous findings of a strong dose–response relationship between VC exposure and the serum expression of mutant p53 tumour suppressor gene protein, presumably produced by A –> T transversions in codons 179, 249 and 255 of the p53 gene due to 1, N^6-ethenoadenine VC–DNA adducts (Smith *et al*. 1997). These results, together with the findings of these specific *ras* and p53 mutations in a high proportion of VC-associated ASLs but not in non-VC-associated ASLs (Soini *et al*. 1995, Przygodzki *et al*. 1997), suggest that the production of mutant p21ras and mutant p53 are important steps in the development of ASL in VC-exposed individuals.

Four positives for mutant p21ras were also identified among the controls who presumably had no VC exposure. There are several possible explanations for this. These could represent false-positives due to antibody cross-reactivity with other proteins of similar sequence and molecular weight, which is possible but not highly likely. These individuals could represent true-positives from unknown VC exposure, also seemingly unlikely. Alternatively, it is possible that these individuals are sero-positive due to mutant p21ras expression from another source in the body. For example, Asp 13 K-*ras* mutations have also been noted in a small proportion of colonic adenomas and carcinomas (Vogelstein *et al*. 1988), and, since these neoplasms are relatively common in the general population, they could potentially provide a source for mutant p21ras protein. We have previously been able to identify an increase in total p21ras protein in the serum of individuals who have colonic adenomas and carcinomas that over-express the protein in their tumour tissue (Luo *et al*. 1996). The finding of mutant p21ras in the unexposed individuals in this study could thus have significance for the detection of colonic tumours, as well as for the development of ASLs in exposed individuals.

The results of this study could also have relevance for the risk assessment of VC. For example, the presence or absence of sero-positivity 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 VC has been 1 ppm since 1974. However, if some individuals

with such low exposure levels (e.g. below 40 ppm-years of 1 ppm for 40 working years) tested positive for these biomarkers, this could suggest that current permissible exposure limits may not be adequately protective. 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%) were seropositive. The adjusted odds ratio for seropositivity for this subgroup compared with the unexposed controls was statistically significant (OR = 17·89, 95 % CI 3·34–95·67). Within this subgroup, the sero-positive individuals tended to have higher estimated exposures compared with the sero-negative individuals, suggesting a possible safe level of exposure somewhat below the current permissible exposure limit. All three individuals with less than 8 ppm-years were sero-negative. In addition, within this subgroup, sero-positive individuals were much more likely to be alcohol drinkers (80%) compared with sero-negative individuals (22%), again supporting a possible positive interaction between VC exposure and alcohol consumption as suggested from animal studies (Radicke *et al*. 1981).

Several limitations of this study should be emphasized. First, no direct VC exposure data for this cohort is available. As noted, exposure is based on job category, years worked and exposures extrapolated from historical evaluations of similar work situations. The present cohort tends to be a stable worker population spending long periods of time in job categories generally recognized as having a high likelihood of VC exposure, but the exact VC exposure remains unknown. The amount of error that this uncertainty in exposure classification introduces into the 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 quantification of potential confounders is also somewhat crude based on the data available. Lastly, the controls were drawn from a population of hospital patients which may not be the same as healthy controls drawn from the general working population in terms of risk for this biomarker, although analysis of the data within the exposed group omitting the control group still demonstrated a trend with exposure, suggesting any potential effect from this would not be large. Future studies will be addressing these issues further. Despite these limitations, the results of this study strongly support the existence of a dose–response relationship between VC exposure and serum biomarker status.

Acknowledgements

This work was supported in part by grants from the US Environmental Protection Agency (R-825361), the National Cancer Institute (R01-CA69243; T32-CA09529), the Association pour la Recherche sur le Cancer, and la Fondation de France.

References

Bishop, J. M. 1991, Molecular themes in oncogenesis. *Cell*, **64**, 235–248.

Brandt-Rauf, P. W., DeVivo, I., Marion, M.-J. and Hemminki, K. 1995a, The molecular epidemiology of growth signal transduction proteins. *Journal of Occupational and Environmental Medicine*, **37**, 77–83.

Brandt-Rauf, P. W., Marion, M.-J. and DeVivo, I. 1995b, Mutant p21 protein as a biomarker of chemical carcinogenesis in humans. In *Biomarkers and Occupational Health: Progress and Perspectives*, M. L. Mendelsohn, J. P. Peeters and M. J. Normandy, eds (Washington, DC: John Henry Press), pp. 163–173.

DeVivo, I., Marion, M.-J., Smith, S. J., Carney, W. P. and Brandt-Rauf, P. W. 1994, Mutant c-Ki-ras p21 protein in chemical carcinogenesis in humans exposed to vinyl chloride. *Cancer Causes and Control,* **5**, 273–278.

Heldaas, S. S., Langard, S. L. and Anderson, A. 1984, Incidence of cancer among vinyl chloride workers. *British Journal of Industrial Medicine*, **41**, 25–30.

LaVecchio, J. A., Hamer, P. J., Ng, S. C., Trimpe, K. L. and Carney, W. P. 1990, Characterization of monoclonal antibodies specific to the activated ras p21 with aspartic acid at position 13. *Oncogene*, **5**, 1173–1178.

Luo, J.-C., Neugut, A. I., Garbowski, G., Forde, K. A., Treat, M., Smith, S., Niman, H. and Brandt-Rauf, P. W. 1996, Expression of p21ras-related protein in the plasma and tissue of patients with adenomas and carcinomas of the colon. *Biomarkers*, **1**, 29–33.

Przygodzki, R. M., Finkelstein, S. D., Keohavong, P., Zhu, D., Bakker, A., Swalsky, P. A., Soini, Y., Ishak, K. G. and Bennett, W. P. 1997, Sporadic and thorotrast-induced angiosarcomas of the liver manifest frequent and multiple point mutations in K-ras-2. *Laboratory Investigation*, **76**, 153–159.

Radicke, M. J., Stemmer, K. and Bingham, E. 1981, Effect of ethanol on vinyl chloride carcinogenesis. *Environmental Health Perspectives*, **41**, 59–62.

Smith, S. J., Li, Y., Whitley, R., Marion, M.-J., Partilo, S., Carney, W. P. and Brandt-Rauf, P. W. 1997, The molecular epidemiology of mutant p53 in vinyl chloride exposed workers. *American Journal of Epidemiology,* **147**, 302–308.

Soini, Y., Welsh, J. A., Ishak, K. G. and Bennett, W. P. 1995, p53 mutations in primary hepatic angiosarcomas not associated with vinyl chloride exposure. *Carcinogenesis*, **16**, 2879–2881.

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M. M. and Bos, J. L. 1988, Genetic alterations during colorectal tumor development. *New England Journal of Medicine*, **319**, 525–532.