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Mutant c-Ki-*ras* p21 protein in chemical carcinogenesis in humans exposed to vinyl chloride

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Mutations in *ras* oncogenes and expression of their encoded p21 protein products are believed to play an important role in carcinogenesis in humans. Detection of mutant p21 proteins in serum may be a useful molecular epidemiologic biomarker with which to study this process, and workers with heavy exposure to vinyl chloride (VC) represent a model population for such study. We studied the occurrence of a specific *ras* mutation (Asp 13 c-Ki-*ras*) by oligonucleotide hybridization and the expression of the corresponding p21 protein in tumor tissue and serum by immunohistochemistry and immunoblotting with monoclonal antibodies in five individuals with heavy exposure to VC and resultant angiosarcomas of the liver (ASL). Four of five (80 percent) of the cases of ASL were found to contain the mutation and to express the corresponding mutant protein in their tumor tissue and serum. Serum expression of the mutant protein also was examined in nine VC-exposed workers with liver angiomas and 45 VC-exposed workers with no evidence of liver neoplasia; eight of nine (89 percent) of the former and 22 of 45 (49 percent) of the latter were also positive for the mutant p21 in their serum. However, serum immunoblotting results for 28 age-gender-race matched, unexposed controls were all negative. Stratification by years of VC exposure showed a significant linear trend ($P < 10^{-5}$) for the occurrence of the serum mutant p21 protein with increasing duration of exposure. These results suggest that detection of serum mutant p21 protein can be a valid surrogate for *ras* gene expression at the tissue level. Further, serum mutant p21 may be a useful molecular epidemiologic biomarker for the study of chemical carcinogenesis in humans exposed to VC and possibly for the study of other mutant *ras*-related human cancers. *Cancer Causes and Control* 1994, 5, 273 - 278

Key words: Angiosarcoma of the liver; chemical carcinogenesis; p21 protein; *ras* gene; serum biomarker; vinyl chloride.

Introduction

The production of specific point mutations in the *ras* oncogenes, as can be induced by certain chemical carcinogens, is believed to be related to the development of a portion of human cancers.[1,2] A model system for studying this process *in vivo* in humans is provided by workers who have been exposed occupationally to high levels of vinyl chloride (VC), since these individuals are at risk for the development of a rare sentinel

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neoplasm, angiosarcoma of the liver (ASL),[3] which has been associated in a high proportion of cases (83 percent) with G·C→A·T transitions in the second nucleotide of codon 13 of the c-Ki-*ras*-2 oncogene probably due to the formation of VC-DNA adducts.[4] The encoded p21 protein product of such a mutated Ki-*ras* gene would contain an aspartic acid in place of the normal glycine at position 13. This Asp 13 mutant p21 protein can be identified with monoclonal antibodies specific for the protein in lysate and supernatant from cells in culture known to contain the mutation.[5] Also, in analogous situations *in vivo* in animals and humans with cancer, total p21 protein can be detected immunologically in serum.[6] Thus, the ability to detect this Asp 13 p21 in the serum of VC-exposed individuals may be a potential molecular epidemiologic marker for the study of this chemical carcinogenic process in humans.

Here, we use immunohistochemistry to demonstrate the Asp 13 p21 protein in ASLs from VC-exposed workers with corresponding mutations in their tumor DNA. We use immunoblotting to demonstrate the protein in the serum of these cancer patients and in a high proportion of VC-exposed workers without tumors in comparison with matched, unexposed controls.

Materials and methods

Subjects and samples

From a cohort of more than 400 workers employed in VC-polymerization plants in France since 1950 and followed at INSERM, a sample of 60 of the most heavily exposed individuals (defined as at least one year of exposure before 1974 or five years of total exposure) was selected for study. For these individuals, information was available on age, gender, race, smoking status, alcohol consumption, occupational exposure history, and current medical status, including the presence of hepatic or other neoplasia. Estimates of VC exposure were based on years worked with VC (both total years worked and years worked prior to 1974 when strict exposure limits and controls were instituted[3]). As shown in Table 1, the 60 individuals selected for study were White males with an average age of 57.2 years (range = 34-74) and with an average of 19.5 years of exposure to VC, including an average of 12.2 years of very high estimated exposure to VC prior to 1974. These 60 individuals included five cases of ASL; one case of hepatocellular carcinoma (HCC); nine cases of benign angiomatous lesions of the liver; and 45 individuals with no evidence of neoplastic lesions of the liver.

Table 1. Frequency distribution of 28 unexposed controls, by age, and 60 vinyl chloride (VC) -exposed workers, by age and by years of VC exposure, White males

Age (yrs)	Control	Exposed	Exposure (yrs)	Pre-1974	Post-1974
<46	0	8	<5	11	13
46-55	2	10	5-14	31	42
56-65	13	34	15-24	15	5
66+	13	8	25+	3	0

For all 60 exposed 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. For eight of the exposed workers, two separate serum samples that had been collected one to two years apart were available for analysis. In addition, fresh-frozen tumor-tissue samples from autopsy or liver biopsy specimens were available for the six cancer cases (five ASL and one HCC).

A sample of 28 control patients—group-matched for age, gender, and race—was selected from a previously described cohort of 190 patients admitted to the surgical services of Columbia-Presbyterian Medical Center with non-cancer diagnoses.[7] As shown in Table 1, these controls were White males with an average age of 66.1 years (range = 54-81) and with no known exposure to VC; the controls were also comparable to the exposed cohort in terms of cigarette smoking and alcohol consumption. For all 28 controls, serum samples had been collected between 1987 and 1989 by routine venipuncture techniques and stored frozen at −80°C until the time of analysis. In addition, six fresh-frozen tissue samples of normal liver were obtained from autopsy specimens at Columbia-Presbyterian Medical Center for use as normal tissue controls. These six autopsy cases (White; average age = 68.0 years; five males, one female) had no known exposure to VC and died of cardiopulmonary failure with no evidence of hepatic or other neoplasia or other hepatic disease.

Sample analysis

DNA was extracted from liver-tumor tissue samples, amplified by PCR, and analyzed by allele-specific oligonucleotide hybridization for point mutations at codons 12, 13, and 61 of c-Ha-*ras*, ci-Ki-*ras*, and N-*ras* genes, as described previously.[4] DNA extracted from cell lines Calu I (G→T transversion at first base of codon 12 of Ki-*ras* gene)[8] and SW 480 (G ≥ T transversion at second base of codon 12 of Ki-*ras* gene)[9] was used as negative controls, and DNA extracted from cell line MDA-MB 231 (G→A transition at second base of codon 13 of Ki-*ras* gene)[10] was used as a positive control.

Tissue immunohistochemistry for Asp 13 p21 was performed on the liver cancer and the normal-liver autopsy sections as described previously[11] using a

primary mouse monoclonal antibody, D146, raised against a synthetic peptide corresponding to amino acid residues 5-16 of the p21 protein with aspartic acid at position 13[5] and with a secondary antibody (horse anti-mouse)-avidin-biotin-alkaline phosphatase detection system (Vectastain Elite; Vector Labs, Burlingame, CA, USA). Tumor tissue was scored for immunohistochemical staining in comparison with the six normal liver sections and a human colon-cancer cell line, HT 29, with the wild-type c-Ki-*ras* gene[12] (negative controls) and a human colon-cancer cell line, HCT 116, with the c-Ki-*ras* codon 13 aspartic acid mutation[12] (positive control). Staining was confirmed as c-Ki-*ras* p21 with another primary mouse monoclonal antibody, 147-67C6, raised against a synthetic peptide corresponding to amino acid residues 157-180 of the c-Ki-*ras* p21 protein[13] in a similar manner. Immunohistochemistry specificity was confirmed by failure of reactions with a nonspecific mouse myeloma antibody, MOPC-141, and competitive inhibition of positive reactions with cell lysate from positive control cell-lines.

Serum immunoblotting was performed as described previously[14] using the primary mouse monoclonal antibody, D146,[5] as above with a secondary antibody (horse anti-mouse)-avidin-biotin-peroxidase detection system (Vectastain Elite; Vector Labs, Burlingame, CA). Serum 21 kDa bands were scored in comparison with cell lysate or cell culture supernatant from the above control cell-lines. Based on the level of expression of the mutant *ras* in control cell lines, the limit of detection of this assay for Asp 13 p21 is estimated to be 0.25 ng. Bands were confirmed as c-Ki-*ras* p21 with the primary mouse monoclonal antibody, 147-67C6,[13] as above. Immunoblotting specificity was confirmed by failure of reactions with a nonspecific mouse myeloma antibody, MOPC-141, and competitive inhibition of positive reactions with cell lysate from positive control cell-lines.

All assays were performed in triplicate. Mutational analysis, immunohistochemistry, and immunoblotting results were recorded blinded to the results of the other assays and blinded to diagnosis.

Data analysis

For the six cancer cases, results of serum immunoblotting were compared with the results of tissue immunohistochemistry and tissue mutational analysis to establish the validity of the serum marker as a surrogate for mutational activation and gene expression at the tissue level. Serum immunoblotting results for the paired samples from eight exposed workers were compared in order to establish the reproducibility of the assay over time. Percentages of positive serum-immunoblotting results were compared for the cancer cases (five ASLs and one HCC), the benign liver-angioma cases, the exposed workers without liver neoplasia, and the unexposed controls. Serum immunoblotting results were stratified by degree of VC exposure in terms of total years exposed by decades. Using the unexposed controls as reference, odds ratios were calculated for each strata, and a χ^2 for linear trend was determined with a P value less than 0.05 considered to be significant.

Results

Representative examples of mutational analysis, tissue immunohistochemistry, and serum immunoblotting are presented in Figure 1. Results are summarized in Tables 2 and 3. Four of the five ASLs (80 percent) were found to contain G→A transitions at the second base of

Table 2. Results for serum Asp 13 c-Ki-*ras* p21 in vinyl chloride-exposed workers and unexposed controls

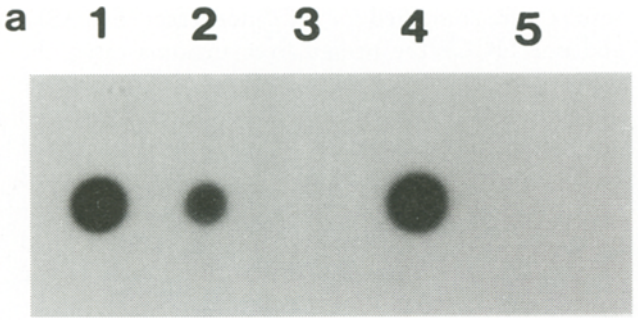
	Age (yrs)	Vinyl chloride exposure (yrs)			Serum Asp 13 c-Ki-*ras* p21
		Pre-1974	Post-1974	Total	
ASL[a] ($n=5$)	59.6	16.8	8.6	25.4	80% +
HCC[a] ($n=1$)	51	2	0	2	0% +
Angioma ($n=9$)	55.3	10.0	9.8	19.8	89% +
Exposed ($n=45$)	57.3	12.3	7.1	19.4	49% +
Control ($n=28$)	66.1	0	0	0	0% +

[a] ASL = angiosarcoma of the liver and HCC = hepatocellular carcinoma; among ASL and HCC patients, individuals who were + for serum Asp 13 c-Ki-*ras* p21 had corresponding tumor tissue samples that stained positively for Asp 13 c-Ki-*ras* p21 by immunohistochemistry and that contained DNA with the mutant GAC codon 13 in the c-Ki-*ras* gene, whereas individuals who were—for serum Asp 13 c-Ki-*ras* p21 had negative results by immunohistochemistry and DNA analysis.

Table 3. Distribution of serum Asp 13 p21 biomarker in exposed workers and controls by years of VC exposure

Years of total VC-exposure	Serum Asp 13 p21		Odds ratio[a]
	+	−	
0	0	28	1
< 10	4	6	37
10-19	11	11	56
20-29	13	7	104
30 +	6	2	168

[a] Odds ratio of 1 assigned to lowest strata; χ^2 for linear trend = 24.986, $P < 10^{-5}$.



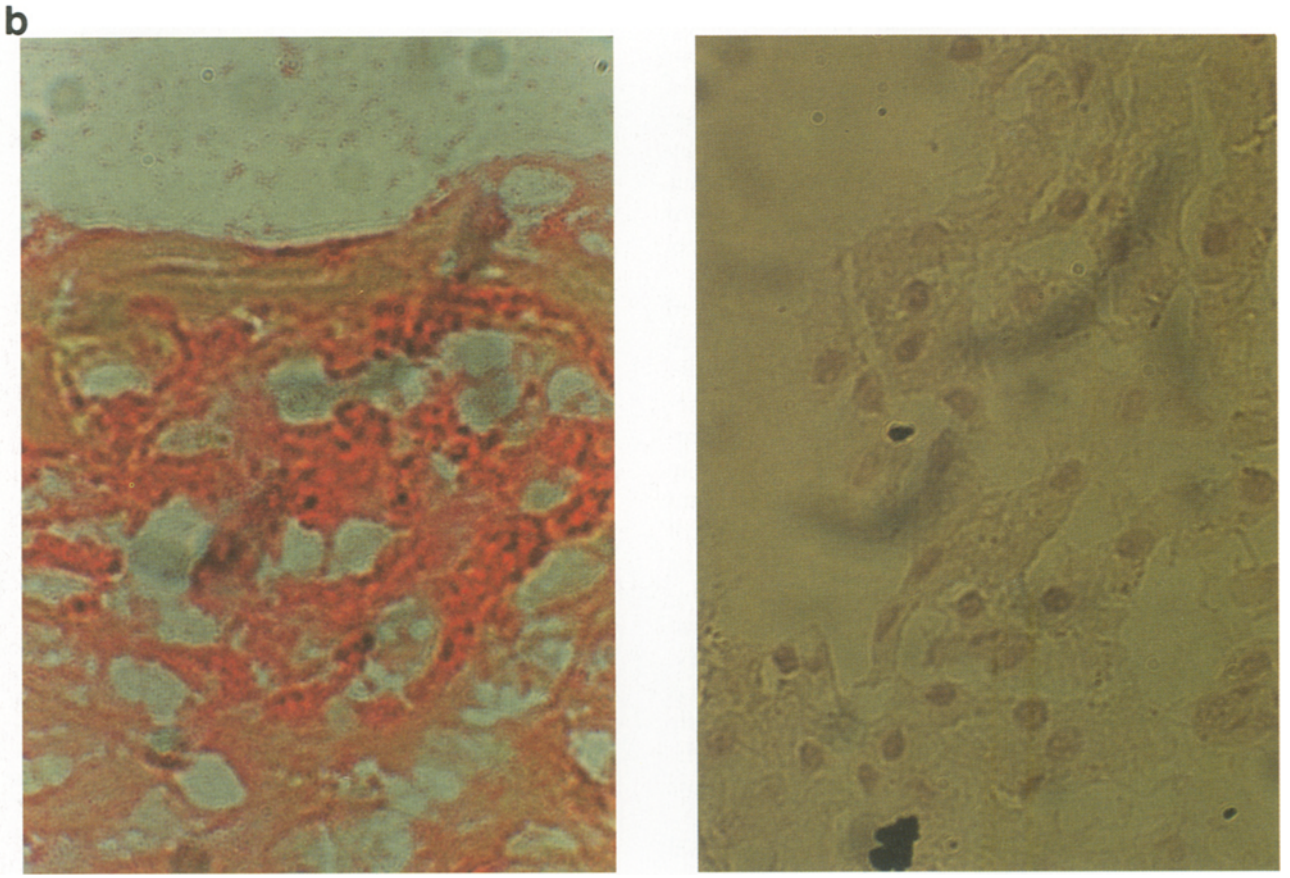


Figure 1. a. Representative hybridization of PCR-amplified DNA from two samples of ASL (Lanes 1 and 2) and control cell lines, Calu I (G→T transversion at 1st base of codon 12 of Ki-*ras*-2 gene) (Lane 3), MDA-MB 231 (G→A transition at 2nd base of codon 13 of Ki-*ras*-2 gene) (Lane 4), and SW 480 (G→T transversion at 2nd base of codon 12 of Ki-*ras*-2 gene) (Lane 5), with radioactive oligonucleotides specific for the mutated (5′-CCTACGTCACCAGCTCCAAC-3′) (Asp 13) sequence (non-coding strand) of the Ki-*ras*-2 gene. **b.** Representative immunohistochemistry of ASL containing Asp 13 mutation (left) and normal liver (right) probed with mouse monoclonal antibody D146 raised against a synthetic peptide corresponding to amino acid residues 5-16 of the p21 protein with aspartic acid at position 13 and with a secondary antibody (horse anti-mouse) -avidin-biotin alkaline phosphatase detection system (600 ×; hematoxylin counterstain).

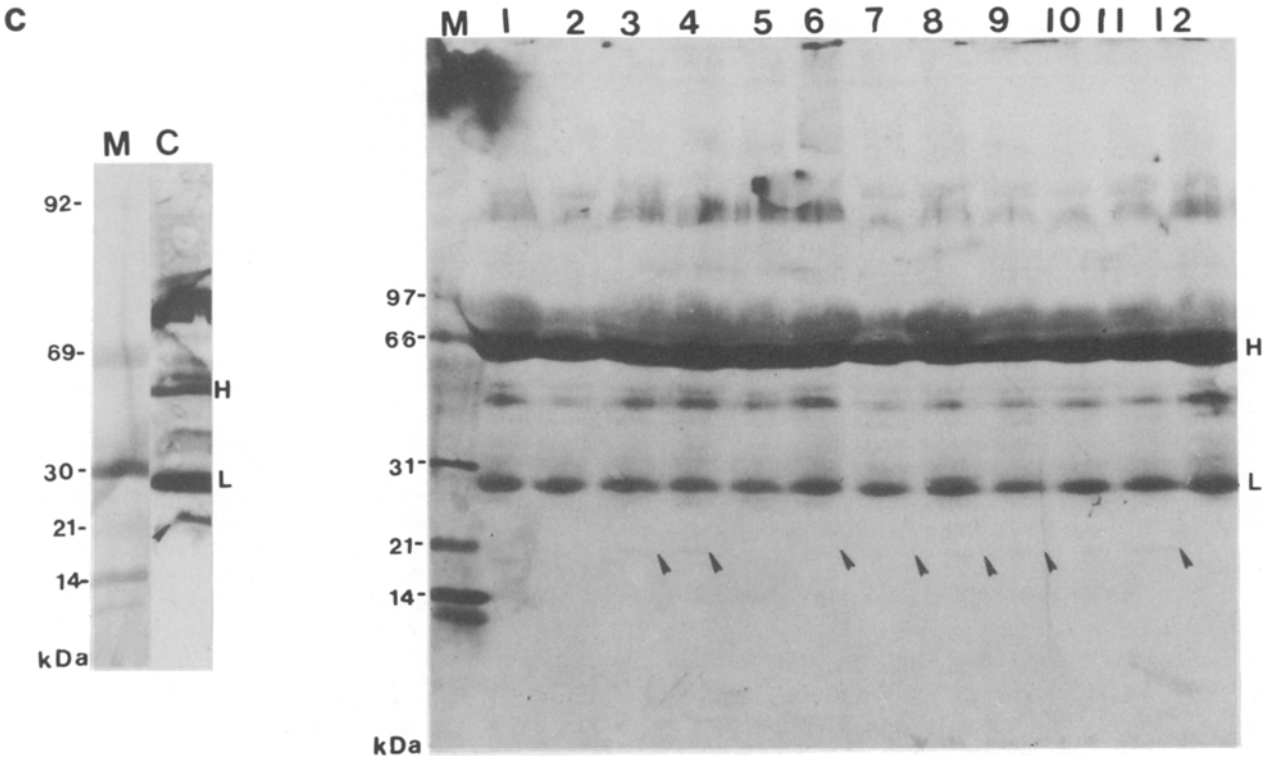


Figure 1. c. Representative serum immunoblots of normal controls (Lanes 1, 2, 5, and 12), patients with ASLs containing the Asp 13 mutation (Lanes 3, 4, 7, and 11), patient with ASL not containing the Asp 13 mutation (Lane 10), and VC-exposed workers without cancer (Lanes 6, 8, and 9) probed with D146 and with a secondary antibody (horse anti-mouse) -avidin-biotin-peroxidase detection system. C is positive control HCT 116 cell lysate; M is biotinylated molecular weight markers; arrowheads indicate positive 21 kDa bands; higher molecular weight bands represent nonspecific cross-reaction of secondary antibody with immunoglobulin components of serum.

codon 13 of the c-Ki-*ras* gene. The four tumors with mutations stained positively for Asp 13 Ki-*ras* p21, and serum from those patients had identifiable Asp 13 Ki-*ras* p21 bands by immunoblotting. One case of ASL and the case of HCC were negative by mutational analysis, immunohistochemistry, and immunoblotting. Immunohistochemistry for all normal liver sections was negative. Eight of nine (89 percent) VC-exposed individuals who had benign (but potentially pre-malignant) angiomatous-liver lesions also were found to have identifiable Asp 13 Ki-*ras* p21 in their sera. Serum immunoblotting results for mutant p21 in all unexposed controls were negative. For the eight pairs of serum samples, six were positive for Asp 13 Ki-*ras* p21 in both samples, one was negative in both samples, and one was negative in the initial sample and positive in the later sample. Results of stratification by decades of VC exposure in terms of the odds ratio for occurrence of the serum biomarker yielded a χ^2 for linear trend of 25 with a P value less than 10^{-5} (Table 2); the results were similarly significant if confined to pre-1974 years of exposure.

Discussion

The results of mutational analysis confirm prior findings that codon 13 Ki-*ras* aspartic acid mutations are a common feature of VC-associated ASLs. This is consistent with the metabolism of VC in terms of formation of DNA adducts and the resultant spectrum of mutations.[4] Further, the results indicate that, in individuals with tumors containing this mutation, the expressed protein product of the mutant gene can be detected reliably by immunologic methods in the tumor tissue and the serum. This suggests that the immunologic detection of the mutant protein in serum may be used as a surrogate biomarker for mutational activation and gene expression at the tissue level. This serum biomarker also appears to be reproducible over time since seven of eight paired samples were concord-

ant for the immunoblotting results; the one discordant pair of serum samples possibly may be attributable to the occurrence of the mutation happening in the intervening one to two years between the time of the first and second samples.

Liver angiomas also have been associated with VC exposure in animal models and may represent a premalignant lesion,[15] so it is also significant that 89 percent of VC-exposed workers with liver angiomas were found to be positive for the serum biomarker. This is also consistent with the previous finding of the characteristic codon 13 Ki-*ras* mutation in a non-dysplastic pre-angiosarcomatous lesion of a VC-exposed individual.[4] Further, since 49 percent of heavily VC-exposed individuals who had no evidence of liver tumors also were found to have Asp 13 Ki-*ras* p21 in their sera, it is possible that this mutational activation of Ki-*ras* may be a very early event in VC carcinogenesis and that identification of the serum protein may be a useful biomarker of cancer risk in these individuals. Confirmation of this awaits further follow-up of this cohort, but, already, one of the serum-positive individuals has been found to have developed a liver lesion suspicious for ASL, whereas all of the serum-negative individuals remain healthy. Of course, it is also noteworthy that serum positivity was correlated significantly with length of VC exposure, since increased exposure is thought to be associated with increased cancer risk.[16,17]

Although this represents a unique model population for study, these results may have significance beyond VC-associated ASLs, since Asp 13 *ras* mutations have been identified in human colonic carcinomas and premalignant adenomas[18] and in human leukemias and premalignant syndromes.[19] In light of the present findings, it is of note that in the latter study, patients with a preleukemic syndrome that progressed to leukemia contained the Asp 13 *ras* mutation whereas patients without the mutation remained in the preleukemic state.[19]

An important goal of molecular epidemiologic studies such as this one is disease prevention by using biological markers of presumed critical events in the disease pathway to better identify individuals at risk before clinical onset in order to allow more effective intervention. Through a combination of early identification of cancer risk via serum oncoprotein biomarkers and oncoprotein specific chemoprophylaxis, it may be feasible in the future to prevent a significant proportion of VC-associated ASLs and other mutant *ras* related cancers.

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