ELSEVIER

Cancer Detection and Prevention 27 (2003) 94–101

Cancer Detection and Prevention

www.elsevier.com/locate/cdp

Molecular epidemiology of plasma oncoproteins in vinyl chloride monomer workers in Taiwan

Jiin-Chyuan John Luo, MD [a,b,*], Tsun-Jen Cheng, MD [c], Chung-Li Du, MD [c], Jung-Der Wang, MD [c]

[a] *Department of Public Health, Chang Gung Medical College, 259 Wen-Hua 1st Road, Kwei-Shan, Tao-Yuan, Taiwan, ROC*
[b] *Department of Family Medicine, Chang Gung Medical Center, Tao-Yuan, Taiwan, ROC*
[c] *Institution of Occupational Medicine and Industrial Hygiene, National Taiwan University, Taipei, Taiwan, ROC*

Accepted 8 January 2003

Abstract

Aims: To determine the presence of Asp13-p21-ki-ras oncoprotein and p53 oncoprotein in the plasma of vinyl chloride monomer (VCM)-workers in Taiwan. *Methods*: We used enhanced chemiluminescence (ECL) western blotting to detect Asp13-p21-ki-ras and ELISA to detect mutant p53 protein (p53-Ag) and anti-p53 antibodies (p53-Ab) in the plasma of VCM-exposed workers. *Results*: Twenty-five out of 251 (10%) VCM-workers were positive for Asp13-p21-ki-ras in plasma, but 0 out of 36 controls were positive. There were 15 out of 95 (15.8%) plasma-positives among the more highly exposed (>480 ppm-month) workers and 10 out of 156 (6.4%) plasma-positives among the lesser exposed (≤480 ppm-month). Compared to the unexposed controls, age and drinking adjusted odds ratios (95% CI) were 1.2 (0.1, 9.8) in the lower exposed workers, and 4.8 (0.8, 28) in the higher exposed workers, and there was a significant linear trend between exposure and plasma positivity ($P = 0.001$). Thirty-three out of 251 (13.2%) VCM-workers were positive for the p53 over-expression (10% with positive p53-Ag and 2.8% with positive p53-Ab). There was a significant association between cumulative VCM exposure concentration and positive p53 expression ($P = 0.032$) among VCM-workers after adjusting for age, hepatitis, drinking and smoking status. *Conclusions*: Asp13-p21-ki-ras oncoprotein and p53 over-expression (p53-Ag or p53-Ab) can be found in the plasma of VCM-workers in Taiwan, and a significant dose–response relationship exists between plasma oncoproteins expression and VCM exposure.

Keywords: Vinyl chloride monomer; Asp13-p21-ki-ras oncoprotein; p53 oncoprotein

1. Introduction

Molecular epidemiology attempts to use bio-markers to study various stages of pathogenesis in vivo, from exposure to an etiologic agent to the clinical expression of disease [1]. Studies in model systems suggest that subsequent stages in the biological response to these carcinogenic insults are likely to be mediated by alterations produced in the oncogenes and tumor suppressor genes [2]. Thus, the study of these genes offers a new and potentially useful avenue of exploration for the molecular epidemiology of bio-markers of cancer.

The mutant *ras* oncogenes encode a 21 kDa protein whose expression is responsible for the cellular changes that ultimately manifest as malignant transformation [3]. The p53 tumor suppressor gene is mutated in a wide variety of human cancers, including cancer of the liver [4,5]. The predominant genetic alterations are missense mutations in four common regions of the most conserved domains of the protein, exons 5–8, resulting in altered protein configuration, loss of suppressor function, and immunochemically detectable accumulation of p53 in the nucleus of tumor cells [6–8]. It is possible to use monoclonal antibodies to detect mutant ras-p21 and mutant p53 in the extra-cellular supernatant, including plasma [9,10].

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 (CEO) and chloroacetaldehyde (CAA). CEO and CAA react with DNA bases to form adducts that are mutagenic in bacterial systems and mammalian cells, including: 7-(2′-oxoethyl) guanine; 1, N^6-ethenoadenine; 3, N^4-ethenocytosine; and N^2-3-etheno-quanine [11]. The

* Corresponding author. Tel.: +886-3-3283016-5485; fax: +886-3-3283031.
E-mail address: luo5485@ms29.hinet.net (J.-C. John Luo).

0361-090X/03/$30.00
doi:10.1016/S0361-090X(03)00021-7

etheno adducts are known to be capable of causing miscoding [12], and could account for the occurrence of specific point mutations (including G.C → A.T transitions and A.T → T.A transversions) in vitro assays [13].

One site where such G → A transitions are apparently produced in vivo is at the second base of codon 13 of the ras oncogenes [14–16]. In a study of *ras* gene mutations in tumors of vinyl chloride monomer (VCM)-exposed workers, 15 of 18 (83%) angiosarcomas of the liver (ASLs) have been found to contain a G → A transition at the second base of codon 13 of the c-ki-ras-2 gene [14,15]. Serum Asp13-p21-ki-ras expression has been shown to be a useful marker for the study of this VC-induced carcinogenesis [17]. Four out of five ASLs in VCM-exposed workers were found to have an Asp13-ki-ras mutation, and Asp13-p21-ki-ras oncoprotein was also found in the tumor tissue and serum and tissues [17]. Expression of serum Asp13-p21-ki-ras oncoprotein was also found in 50% of VCM-exposed workers without evidence of liver angiosarcoma and in eight out of nine (89%) VC-exposed angiomas patients [17]. In a study of *p53* gene mutations in tumors of VCM-exposed workers, two of four (50%) ASLs examined contained an A → T transversion at the first base of codon 249 or 255 of p53 [18]. The mutations result in the substitution of a tryptophan for the normal arginine at amino acid residue 249, or of a phenylalanine for the normal leucine at amino acid residue 255 in the encoded p53 protein product.

One previous study [10] showed that two of four cases of ASLs (50%) known to contain p53 mutations in their tumor DNA were found to have increased amounts of mutant p53 protein in their tumor tissue and serum, whereas the two cases of ASL and one case of hepatic cell carcinoma (HCC) known not to contain p53 mutations in their tumor DNA were found not to have increased amounts of mutant p53 protein in their tumor or serum. This study also examined the serum p53 in 18 VCM-exposed workers without ASLs and five controls. Three of 18 exposed workers (16%) had elevated serum mutant p53, compared with none of the control subjects. All of the five p53 serum-positive individuals in this group had histories of potential high exposure to VCM (>10 years exposure, >1500 ppm-year), whereas the VCM-exposed workers with lesser exposure were all serum-negative. An enlarged follow-up study of this cohort [19,20] demonstrated a statistically significant trend for plasma p53-Ag and p53-Ab with increasing VCM exposure. Another study [21] showed that 5 of 15 individuals with VCM-induced ASL were positive for p53-Ab, 4 of 77 VCM-exposed without ASL were positive for p53-Ab, and none of 15 control subjects without cancer had p53-Ab. The above results suggest that the detection of serum p53-Ag and p53-Ab may be a useful bio-marker for the study of VCM-induced carcinogenesis in exposed human populations.

PVC manufacture is a major industry in Taiwan, with annual production totaling several hundreds thousand tons. Production increased from 12,000 tonnes in 1971 to nearly 70,000 tonnes in 1986, and the figure will increase to 2 millions tonnes in the near future. Thousands of workers could be exposed to VCM. Currently in Taiwan, there is an increased odds ratio of primary liver cancer among vinyl chloride monomer workers [22]. More tumors in VCM-exposed workers can be expected in the future. Previously, we had detected the presence of plasma Asp13-p21-ki-ras or p53 protein in Taiwan VCM-exposed workers in two preliminary studies [23,24]. In this paper, we used immunoassays to determine the presence of Asp13-p21-ki-ras oncoprotein and p53 oncoproteins (p53-Ag and p53-Ab) in the plasma of entire Taiwan VCM study cohorts, and explored the VCM-induced carcinogenesis.

2. Study design and method

The study cohort included 251 male workers from five vinyl chloride factories in Taiwan. Plasma had been collected from March to April, 1995. For these individuals, the information available included age, gender, total months of employment, exposure concentration, results of abdominal ultrasonogram, hepatitis status (HBsAg or anti-HCV), smoking and alcohol drinking status, and history of tumors. All seven liver tumors were assumed to be angiomas on the basis of abdominal echography. Estimates of VCM exposure were based on estimated accumulated ppm-months of VC exposure [25].

We also selected 36 healthy males without any tumors or vinyl chloride exposure history from the Health Examination Ward of Chang Gung Hospital as a control group. Exposure and control groups were group-matched by age, and sex. Each VCM-worker and control provided a 20 ml sample of blood via routine venipuncture techniques, and the plasma was stored at −80 °C.

2.1. Enhanced chemiluminescence (ECL) immunoblotting assay-(Asp13-p21 monoclonal antibody)

Western immunoblotting for oncoproteins was performed as described previously [15]. We used 5–17% gradient polyacrylamide (Sigma Chemical Company, St. Louis, MO) resolving gels with 15 combed wells of 5% polyacrylamide stacking gels which were prepared using a gel gradient-maker (Hoefer Scientific Instrument, San Francisco, CA). An amount of 2 ul of plasma was mixed with 8 ul of 1X treatment buffer (0.0625 M trizma, pH 6.8) and 10 ul of sample buffer (0.125 M trizma base, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol) (Sigma Chemical Company, St. Louis, MO). A 10 ul of Rainbow molecular weight markers (Amersham Medi-Physics, Arlington Heights, IL), which range from M_W 14.3 to 200 kD, were also mixed with 10 ul of sample buffer. All the above preparations (except the molecular weight markers) were placed in a bath of boiling water for 5 min (the molecu-

lar weight markers were boiled for 1 min). An amount of 2 ul of 0.05% bromophenol blue (plus a few crystals of methyl green) was then added to each sample except the molecular weight markers. Then 13 prepared unknown samples, one known positive serum and one molecular weight marker solution were loaded into each of the 15 combed gel wells.

Samples were electrophoretically separated using a Hoefer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA) for 5 h at 12–15 mA constant current per gel, then transferred electrophoretically with a Hoefer Transphor unit to nitrocellulose membrane (Hoefer Scientific Instruments, San Francisco, CA) overnight at 20 mA constant current per gel. After blocking with phosphate buffered saline (PBS), pH 7.4, containing 3% bovine serum albumin and 0.1% Triton X-100 (Sigma Chemical Company, St. Louis, MO), the nitrocellulose was incubated overnight at 4 °C with a mouse monoclonal antibody, D146 (1:250), raised against a synthetic peptide corresponding to amino acid residues 5–16 of the p21 protein with aspartic acid at position 13. After washing with PBS, biotinylated goat antimouse immunoglobulin (Vectastain) was applied for 30 min. The blots were developed with 20 ml of ECL substrate solution, and ECL films supplied by the manufacturer. The presence of the Asp13-*ras* oncoprotein-antibody band on the films was determined using the molecular weight marker 21 kD band as a reference as well as the band of known positive serum.

2.2. Enhanced chemiluminescence (ECL) immunoblotting assay-(ki-ras monoclonal antibody)

If samples were found to be positive for expression of Asp13-p21 oncoprotein, we repeated the ECL assay to confirm it to be specifically ki-*ras* p21 oncoprotein. The procedure and agents were all the same as the above, except that the monoclonal antibody used was 147-67C6 (National Cancer Institute Repository, Microbiological Associate Inc., Bethesda, MD), which was raised against a synthetic peptide incorporating residues 157–180 of the human ki-*ras* sequence (ascites fluid diluted 1:2000).

2.3. Plasma mutant p53 ELISA

Plasma analysis for mutant p53 was performed using an ELISA (Oncogene Science, Cambridge, MA) based on the mouse monoclonal antibody PAb 240 which identifies an epitope between amino acids 210 and 214 that is normally concealed in the wild-type p53 protein, but which is revealed in many mutant p53 proteins due to the aforementioned conformational changes produced by the mutations.

Microtiter wells were precoated with PAb 240, and then 50 ul of plasma and 50 ul sample dilutent were added to each well and incubated overnight at 4 °C. After washing, 100 ul of a rabbit polyclonal reporter antibody for p53 was added to each well and allowed to incubate at room temperature for 2 h. After washing again, the remaining reporter antibody was bound to a horseradish peroxidase-conjugated goat anti-rabbit IgG, and color was developed by incubation with the chromogenic substrate 2,2′-azino-di-[3-ethylbenzthiazoline sulfate]. Absorbance of each well was read on a spectrophotometric plate reader at 405 nm, and the concentration of mutant p53 was determined by comparison to the absorbance of standard solutions of purified, recombinant, human mutant p53. Positive plasma mutant p53 protein was defined by any level greater than 0.878 ng/ml which was calculated by mean plus two standard deviations of mutant p53 levels from 36 unexposed controls.

2.4. Plasma anti-p53 antibody ELISA

Diluted (1:100) samples and prediluted controls (negative control, low-control, medium-control, and high-control) were loaded at 100 ul on p53 plate which was precoated with recombinant human p53 protein and incubated at 37 °C for 1 h according to the instructions of the manufacturer (Oncogene Science, Cambridge, MA). After washing, the remaining anti-p53 antibody was bound to peroxidase-conjugated goat anti-human IgG polyclonal antibody, and color was developed by incubation with TM blue (3,3,5,5-tetramethylbenzidine) substrate and HCl stop solution. Absorbance of each well was read on a spectrophotometric plate reader at 450/595 nm to determine the p53 OD. The OD of patients plasma above the 90% OD of low-control was treated as positive for anti-p53 antibody expression.

2.5. Data analysis

The data were encoded, entered, and analyzed with the assistance of the statistical analysis system (SAS) PC software package [26] and Database III plus [27]. Logistic regression analyses was performed to calculate the association between positive total p53 or Asp13-p21-ki-ras expression rate and cumulative VCM-exposure concentration after adjusting for age, hepatitis, smoking, and drinking status. The degree of VCM exposure was also further stratified with exposure of more than 480 ppm-months considered to be the high exposure group and exposure less than or equal to 480 ppm-months considered to be the low exposure group, with the controls as unexposed. *T*-test, ANOVA and chi-square were performed to test significant difference among groups. Odds ratios with 95% confidence intervals were calculated to test the magnitude and the significance of difference in prevalence of p53 or Asp13-p21-ki-ras positives among exposure and control groups. Mantel–Haenszel chi-square tests were applied to calculate drinking, smoking and age adjusted odds ratios. Chi-square was also performed to test the linear trend between exposure concentration and positivity.

Table 1
Characteristics of study cohorts

Variables	High exposure[a] ($n = 95$)	Low exposure ($n = 156$)	Total VCM-workers ($n = 251$)	Controls ($n = 36$)
Age (years-old)[b]	42.5 ± 8.9 (24, 62)	39.2 ± 10.4 (23, 65)	40.5 ± 10 (23, 65)	40.8 ± 10.1 (23, 58)
Duration of employment (years)[b]	9.21 ± 7.1 (0.5, 28.8)	9.26 ± 8.8 (0.5, 33.9)	9.24 ± 8.2 (0.5, 33.9)	0 ± 0
Exposure concentration (ppm)[b]	11.6 ± 21.2 (0.4, 73.8)	3.5 ± 10.5 (0.4, 63.7)	6.6 ± 15.9 (0.4, 73.8)	0 ± 0
Cumulative concentration (ppm-months)[b]	3305 ± 4478 (482, 34521)	145 ± 125 (0, 467)	1341 ± 3148 (0, 34521)	0 ± 0
Smoker*, *n* (%)	42 (44.2)	58 (37.2)	100 (40)	23 (63.9)
Drinker*, *n* (%)	18 (19)	14 (9)	32 (12.8)	21 (58.3)
Hepatitis status[c], *n* (%)	25 (26.3)	29 (18.6)	54 (21.5)	NA[e]
HBsAg(+)	23 (24.2)	27 (17.3)	50 (19.9)	
Anti-HCV(+)	2 (2.1)	4 (2.6)	6 (2.4)	
Liver tumors, *n* (%)	3 (3.2)	4 (2.6)	7 (2.8)	0 (0)
Asp13-p21-ki-ras(+), *n* (%)	15 (15.8)	10 (6.4)	25 (10)	0 (0)
Total p53(+)[d], *n* (%)	14 (14.7)	19 (12.2)	33 (13.2)	2 (5.6)
p53-Ag(+)	11 (11.6)	14 (9)	25 (10)	1 (2.8)
p53-Ab(+)	4 (4.2)	5 (3.2)	9 (3.6)	1 (2.8)

[a] High exposure (>480 ppm-months), low exposure (≤480 ppm-months).
[b] Values shown as mean ± S.D. (range).
[c] HBsAg, hepatitis B surface antigen; anti-HCV, anti-hepatitis C virus.
[d] p53-Ag, p53 protein; p53-Ab, p53 antibody.
[e] NA, not available.
* $P < 0.05$, difference of the prevalence of drinkers or smokers among the high, low exposure and control groups.

3. Results

The basic characteristics of the study cohort are summarized in Table 1. There were 25 (10%) VCM-workers found to have detectable amounts of Asp13-ki-ras oncoprotein in their plasma, whereas none of 36 controls were positive. There were 33 (13.2%) VCM-workers found to have positive p53 expression (positive p53-Ag or p53-Ab) in their plasma including 25 (10%) positive for p53-Ag and 9 (3.6%) positive for p53-Ab, whereas 2 (5.6%) controls were positive including 1 (2.8%) with positive p53-Ag and 1 (2.8%) with positive p53-Ab. Control workers were significantly more likely to be alcohol drinkers and smokers than high exposure (>480 ppm-months) or low exposure (≤480 ppm-months) workers. There were no significant differences among exposure groups for age, duration of employment, exposure concentration, smoking status, hepatitis status (HBsAg or anti-HCV), and liver tumors.

Table 2 showed dose–response relationship between positive plasma Asp13-ki-ras expression and vinyl chloride monomer exposure. There were 15 (15.8%) high exposure workers found to have Asp13-p21-ki-ras in their plasma, and 10 (6.4%) low exposure workers found to have Asp13-p21-ki-ras in their plasma. Compared to the unexposed controls, the crude odds ratios (and 95% confidence intervals) of positive plasma Asp13-p21-ki-ras expression for the other exposure groups were: high exposure, 14.1 (0.8, 241); low exposure, 5.2 (0.3, 91.4), respectively; and there was a significant linear trend between exposure concentration and positive rate ($P = 0.001$). After adjusting for age and drinking status, the odds ratios were 4.8 (0.8, 28), and 1.2 (0.1, 9.8), respectively, and there was a significant linear trend between exposure concentration and positive rate ($P = 0.001$).

Table 3 showed dose–response relationship between positive plasma p53 expression and vinyl chloride monomer exposure. There were 14 (14.7%) high exposure workers found to have positive p53 expression in their plasma, 19 (12.2%) low exposure controls found to have positive p53 expression in their plasma, and 2 (5.6%) unexposed workers

Table 2
Dose–response relationship between positive plasma Asp13-p21-ki-ras expression and vinyl chloride monomer exposure in ppm-months

	Variables		
	Asp13-p21-ki-ras(+), *n* (%)	OR[b] (95% CI)	Adjusted[c] OR (95% CI)
High exposure[a] ($n = 95$)	15 (15.8)	14.1 (0.8, 241)	4.8 (0.8, 28)
Low exposure ($n = 156$)	10 (6.4)	5.2 (0.3, 91.4)	1.2 (0.1, 9.8)
Unexposed ($n = 36$)	0 (0)	1	1
Linear trend	–	Chi-square = 10.25, $P = 0.001$	Chi-square = 11.2, $P = 0.001$

[a] High exposure (>480 ppm-months), low exposure (≤480 ppm-months).
[b] OR, odds ratio; 95% CI, 95% confidence interval.
[c] Adjusted for age, drinking status.

Table 3
Dose–response relationship between positive plasma p53 expression and vinyl chloride monomer exposure in ppm-months

	Variables		
	p53(+)[a], *n* (%)	OR[b] (95% CI)	Adjusted[c] OR (95% CI)
High exposure[d] ($n = 95$)	14 (14.7)	2.94 (0.67, 12.9)	2.5 (0.5, 12.4)
Low exposure ($n = 156$)	19 (12.2)	2.36 (0.54, 10.2)	1.49 (0.24, 9.2)
Unexposed ($n = 36$)	2 (5.6)	1	1
Linear trend	–	Chi-square = 1.81, $P = 0.18$	Chi-square = 0.95, $P = 0.33$

[a] p53(+), if p53-Ag(+) or p53-Ab(+).
[b] OR, odds ratio; 95% CI, 95% confidence interval.
[c] Adjusted for age, drinking and smoking status.
[d] High exposure (>480 ppm-months), low exposure (≤480 ppm-months).

found to have positive p53 expression in their plasma. Compared to the unexposed controls, the crude odds ratios (and 95% confidence intervals) of positive plasma p53 expression for the other exposure groups were: high exposure, 2.94 (0.67, 12.9); low exposure, 2.36 (0.54, 10.2); and there was a non-significant linear trend between exposure concentration and positive p53 over-expression ($P = 0.18$). After adjusting for age, smoking and drinking status, the odds ratios were 2.5 (0.53, 12.4), and 1.49 (0.24, 9.2), respectively, and there remained a non-significant linear trend between exposure concentration and positive p53 expression ($P = 0.33$).

In multivariate logistic regression analysis (Table 4), there was a significantly higher Asp13-ki-ras expression in the high exposure group than in the low exposure group, with odds ratio (and 95% CI) of 3.35 (1.34, 8.41) after adjusting for other factors. There was also a significant association between cumulative VCM exposure concentration and total positive p53 expression ($P = 0.032$) in VC-workers after adjusting for age, hepatitis, drinking, and smoking status. There was no association between oncoproteins expression and age, hepatitis, drinking, or smoking status.

Tumor status was not associated with cumulative exposure concentration, hepatitis status, drinking status, smoking status, positivity of Asp13-p21-ki-ras expression and p53 expression (Table 5).

As shown in Table 6, 29 of 95 (>40 ppm-years) exposed workers (30.5%) were found to have one oncoprotein expression in their plasma, 13 of 77 (10–40 ppm-years) exposed workers (16.9%) had one positive, 12 of 71 (0–10 ppm-years) exposed workers (16.9%) had one positive, whereas 2 of 44 (0 ppm-years) exposed workers (4.5%) had one positive. Only one worker in the (0 to10 ppm-years) exposed or (10–40 ppm-years) exposed groups had two positives. For one positivity, using the (0 ppm-years) group as the comparison group with assigned odds ratio of 1, the odds ratios (and 95% confidence intervals) for the other

Table 4
Logistic regression analyses between plasma Asp13-p21-ki-ras, p53 positivity and related indicators among vinyl chloride monomer workers

	Asp13-p21-ki-ras(+)	p53(+)
Intercept	−1.22 ± 0.98[a] (0.21)*	−2.08 ± 0.86 (0.015)
Cumulative concentration (ppm-months)	–	0.0001 ± 0.00005 (0.032)
Cumulative concentration[b]		
1 (high)	1.21 ± 0.46 (0.008)	–
0 (low)	3.35 (1.34, 8.41)[c]	–
Age (years)	−0.035 ± 0.024 (0.15)	0.0096 ± 0.02 (0.62)
Hepatitis status[d]		
1 (+)	0.29 ± 0.49 (0.56)	−0.77 ± 0.56 (0.17)
0 (−)	1.34 (0.5, 3.56)	0.46 (0.15, 1.42)
Drinking		
1 (yes)	−0.62 ± 0.8 (0.44)	−0.89 ± 0.79 (0.26)
0 (no)	0.54 (0.11, 2.66)	0.41 (0.08, 1.99)
Smoking		
1 (yes)	−0.47 ± 0.48 (0.32)	−0.44 ± 0.43 (0.31)
0 (no)	0.63 (0.24, 1.63)	0.64 (0.27, 1.52)

[a] Parameter estimate ± S.E.
[b] High exposure (>480 ppm-months), low exposure (≤480 ppm-months).
[c] Adjusted odds ratio.
[d] HbsAg(+) or anti-HCV(+).
* *P*-value.

Table 5
Characteristics of vinyl chloride monomer workers according to liver tumor status

	Tumor[a] ($n = 7$)	Non-tumor ($n = 244$)
Age (years)[b]	37.6 ± 13.1 (24, 58)	40.5 ± 9.9 (23, 65)
Duration of employment (years)[b]	10.4 ± 11.3 (0.5, 28.8)	9.2 ± 8.1 (0.5, 33.9)
Exposure concentration (ppm)[b]	9.3 ± 14.9 (0.4, 38.2)	6.5 ± 15.9 (0.4, 73.8)
Cumulative exposure (ppm-months)[b]	572 ± 582 (60, 1413)	1363 ± 3189 (5.4, 34521)
Smoker, *n* (%)	2 (28.6)	98 (40.2)
Drinker, *n* (%)	2 (28.6)	30 (12.3)
Hepatitis status[c], *n* (%)	3 (42.9)	51 (20.9)
Asp13-p21-ki-ras(+), *n* (%)	1 (14.3)	24 (9.8)
Total p53(+), *n* (%)	1 (14.3)	32 (13.1)
p53-Ag(+)	0 (0)	25 (10.3)
p53-Ab(+)	1 (14.3)	8 (3.3)

[a] Benign liver tumors by abdominal ultrasonogram.
[b] Values as shown as mean ± S.D. (range).
[c] HbsAg(+) or anti-HCV(+).

Table 6
Prevalence of positive oncoproteins expression according to vinyl chloride monomer exposure categories in ppm-years

Exposure category (ppm-years)	None positive, *n* (%)	One[a] positive, *n* (%)	Two[b] positive, *n* (%)	OR[c] (95% CI)	OR[d] (95% CI)
0 ($n = 44$)	42 (95.5)	2 (4.5)	0	1	1
0–10 ($n = 71$)	58 (81.7)	12 (16.9)	1 (1.4)	4.35 (1.02, 18.5)	2.18 (0.09, 54.8)
10–40 ($n = 77$)	63 (81.8)	13 (16.9)	1 (1.3)	4.33 (1.03, 18.3)	2.01 (0.08, 50.5)
>40 ($n = 95$)	66 (69.5)	29 (30.5)	0	9.23 (2.57, 33.1)	–
Linear trend	–	–	–	Chi-square = 12.43, $P = 0.0004$	Chi-square = 0.048, $P = 0.83$

[a] Positive expression of Asp13-p21-ki-ras or p53.
[b] Positive expression of Asp13-p21-ki-ras and p53.
[c] Odds ratio comparing one to none positive.
[d] Odds ratio comparing two to none positive.

exposure groups were: (0–10 ppm-years) group, 4.35 (1.02, 18.5); (10–40 ppm-years) group, 4.33 (1.03, 18.3); (>40 ppm-years) group, 9.23 (2.57, 33.1). There was a significant linear trend between exposure concentration and one positive over-expression ($P = 0.0004$).

4. Discussion

The results from our study are consistent with those from previous reports [19,20,28] and indicate that Asp13-p21-ki-ras and p53 proteins (p53-Ag and p53-Ab) can be detected in the plasma of Taiwan VCM-workers. There was a significant dose–response relationship between plasma Asp13-p21-ki-ras expression and VCM exposure ($P = 0.001$) and a significant association between cumulative VCM exposure concentration and positive total p53 expression ($P = 0.032$) among VCM-workers after adjusting for age, hepatitis, drinking, and smoking status. There was also a significant dose–response relationship between one oncoprotein (p21 or p53) positivity and VCM exposure ($P = 0.0004$). Because the physical examination performed on these workers were a voluntary and cross-sectional one, there might be a healthy selection among the subjects. Namely, workers with disease could not come for the physical. Thus, the above finding supports the hypothesis that the production of Asp13-p21-ki-ras, and mutant p53 protein may be important steps in VCM-induced carcinogenesis, and that p21 and p53 proteins may be potential bio-markers of cancer risk in these workers. Continued follow-up of this cohort should help confirm the predictive value of these bio-markers, if plasma-negative workers remain healthy and some of the plasma-positive workers develop liver angiosarcoma.

It is interesting to note that 12 out of 58 (16.9%) (0–10 ppm-years) exposure workers, whose exposures are below the current permissible exposure limit of estimated 480 ppm-months (40 ppm-years) in most Western countries (1 ppm for 40 working years), tested positive for one oncoprotein (p21 or p53) expression. The odds ratio for positivity in this subgroup in comparison with the unexposed controls was 4.35 (1.02, 18.5). One p21-positive worker had an estimated cumulative exposure of only 0.56 ppm-years (6.7 ppm-months). Another two (p21 and p53) positive worker had an estimated cumulative exposure of 1.07 ppm-years (12.9 ppm-months). This may indicate that current permissible exposure limits are not adequate to protect VCM-workers, as previously suggested [23,24,28].

Among 25 p53-Ag-positive workers and nine p53-Ab-positive workers, only one worker was positive for both.

This may support our previous notion [29] that the presence of p53-Ab could accelerate clearance of the p53-Ag from the circulation or diminish the ability to detect the protein in blood, causing a false-negative result. In this study, we treated either presence as positive to avoid underestimation based on this effect.

VCM is metabolized to chloroethylene oxide (CEO), which rearranges to chloracetaldehyde (CAA), and subsequently metabolized by the alcohol dehydrogenase (ALDH) pathway [30,31]. Alcohol can induce ALDH to cause CAA accumulation and potentiate the carcinogenicity of VC monomers [32]. Smoking is a known carcinogenic exposure that can produce p53 mutation [33], and causes serum p53 expression in unexposed controls [34,35]. The only p53 antibody-positive unexposed control in our study cohort is a heavy smoker with history of smoking 1.5 packs per day for 25 years, and the only mutant p53 protein-positive unexposed control is also a current smoker. This findings are consistent with those from other studies [34,35]. By considering smoking effect on p53 protein expression, we include smoking status, besides age and drinking as indicators to examine the relationship between VCM exposure and positive p53 protein. Smoking is unlikely to cause serum ras oncoprotein in unexposed controls [36,37]. In this study, we only included age, hepatitis, drinking as confouders to study Asp13-p21-ki-ras expression among VCM-workers. Our results did not show significant association between alcohol consumption, smoking, hepatitis status and oncoproteins expression. The crude estimation of the above factors may cause the insignificance. Further precise delineation may clarify the effect.

Prior studies have found increased Asp13-p21-ki-ras expression in VCM-workers with pre-malignant angioma [17]. In this study, the presence of liver angiomas did not associate with VC exposure, and also did not increase the risk of positive expression of oncoproteins. Further pathological classification of these tumors according to the mutational profiles may provide more information to resolve these differences.

Currently, the most common VCM-induced liver cancer in Taiwan is hepatic cell carcinoma (HCC), not ASL [22]. Most HCC patients are hepatitis B virus (HBV) carriers. The mechanisms of VCM carcinogenesis are still unclear. The combination of HBV and VCM may lead to primary liver cancer before occurrence of the ASL. One recent study identified p53 mutations in VC-induced HCC [38]. The point mutations detected were comprised of five transversions and five transitions. Five of 11 mutations (codons 175, 245, 248, 273, and 282) occurred at CpG sites which were different from the mutation patterns of VC-induced ASL of A → T transversion at the first base of codon 249 or 255 of p53 [18]. Further DNA assays of hepatocytes or vascular endothelium cells from HCC of Taiwan VC workers to identify different mutation patterns of p53 or *ras* will test the above hypotheses and explore the VCM carcinogenesis.

Several limitations of the study need to be addressed. One of the disadvantages of p53-Abs assay is its lack of sensitivity inasmuch as only 20–40% of patients with p53 mutations will develop p53-Abs, even with 95% specificity [39]. The major drawbacks of the serum p53 protein assay is its high background and false-positives [39]. Some false-positive sera were caused either by the presence of human antibodies with broad antispecies specificities that can cross-react with some antibodies used in the assay or by other nonspecific reagents that interfere with the assay [40]. No complete vinyl chloride exposure data were available; estimates of accumulated VC exposure were based on the job category, years worked, and exposures extrapolated from recent exposure data [25]. We may have underestimated the cumulative VC exposure, so the real exposure concentration could be higher. Previous reports have demonstrated that VC workers have genetic polymorphisms in the CYP2E1 and ALDH-2, this could cause an increased formation of DNA adducts and lead to increasing sister chromatid exchanges [41]. The genetic polymorphisms in the repair enzymes for the DNA adducts could have a similar effect. The inclusion of the above two genetic polymorphisms as susceptible bio-markers will provide more information to clarify VC-induced carcinogenesis in the Taiwan cohort.

In conclusion, Asp13-p21-ki-ras, and p53 over-expression (p53-Ag or p53-Ab) can be found in the plasma of VCM-workers in Taiwan, and a significant dose–response relationship exists between plasma one oncoprotein (Asp13-p21-ki-ras or p53) and VCM cumulative exposure concentration.

Acknowledgements

We thank Dr. Paul Brandt-Rauf for his assistance with the ECL immunoblotting techniques and kindly advice. This work was supported by grants from the Taiwan National Science Council (NSC87-2314-B-182-063), Taiwan Bureau of Health (DOH86-TD-077), and the Chang Gung Medical College (NSC85-2331-B182-106).

References

[1] Schulte PA, Perera FP. Molecular epidemiology: principles and practice. San Diego: Academic Press; 1993.
[2] Balmain A, Brown K. Oncogene activation in chemical carcinogenesis. Adv Cancer Res 1988;51:147–82.
[3] Barbacid M. *Ras* genes. Annu Rev Biochem 1987;56:779–827.
[4] Hollstein M, Sidransky D, Vogelstein B, et al. P53 mutations in human cancers. Science 1991;253:49–53.
[5] Hollstein M, Rice K, Greenblatt MS, et al. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res 1994;22:3551–5.
[6] Volgelstein B, Kinzler KW. P53 function and dysfunction. Cell 1992;70:523–6.
[7] Prives C, Manfredi JJ. The p53 tumor suppressor protein: meeting review. Genes Dev 1993;7:529–34.
[8] Lane DP. On the expression of the p53 protein in human cancer. Mol Biol Rep 1994;19:23–9.

[9] Brandt-Rauf PW, Smith S, Hemminki K, et al. Serum oncoproteins and growth factors in asbestosis and silicosis patients. Int J Cancer 1992;50:881–5.
[10] Smith S, Luo JCJ, Brandt-Rauf P. Mutant p53 protein as a biomarker of chemical carcinogensis in human. JOEM 1996;38:743.
[11] Agency for Toxic Substances and Disease Registry. Toxicology Profile for Vinyl Chloride. TP-92/20. Atlanta, USDHHS/PHS, 1993.
[12] Barbin A, Bartsch M. Mutagenic and promutagenic properties of DNA adducts formed by vinyl chloride metabolites. In: Singer B, Bartsch H, editors. The role of Cyclic Nucleic Acid Adducts in Carcinogensis and Mutagenesis. Lyon: IARC; 1986. p. 345–58.
[13] Cheng KC, Preston BD, Cahill DS, et al. The vinyl chloride DNA derivative N^2-3-ethenoquanine produces G → A transitions in *Escherichia coli*. Proc Natl Acad Sci USA 1991;88:9974–8.
[14] Marion MJ, Froment O, Trepo C. Activation of ki-*ras* gene by point mutations in human liver angiosarcoma associated with vinyl chloride exposure. Mol Carcinog 1991;4:450–4.
[15] Marion MJ, Froment O, Contassot JC, et al. *Ras* gene as molecular epidemiologic marker in human cancers induced by chemicals. Proc Int Cong Occup Health 1993;24:175.
[16] Boivin S, Marion MJ, Froment O, et al. *Ras* genes in liver cancers induced by vinyl chloride in rats. Proc Int Cong Occup Health 1993;24:258.
[17] DeVivo I, Marion MJ, Smith SJ, et al. Mutant c-ki-*ras* p21 protein in chemical carcinogenesis in humans exposed to vinyl chloride. Cancer Causes Control 1994;5:273–8.
[18] Hollstein M. P53 mutations at A:T base pairs in angiosarcomas of vinyl chloride-exposed factory workers. Carcinogenesis 1994;15:1–3.
[19] Smith SJ, Li Y, Whitley R, Brandt-Rauf PW. Molecular epidemiology of p53 protein mutations in workers exposed to vinyl chloride. Am J Epidemiol 1998;147:302–8.
[20] Li Y, Asheron M, Marion MJ, et al. Mutant oncoprotein biomarkers in chemical carcinogenesis. In: Meadelsohn ML, Mohr LC, Peters JP, editors. Biomarkers: Medical and Workplace Applications. Washington, DC: Joseph Henry Press; 1998. p. 345–53.
[21] Trivers GE, Cawley HL, Hollstein M, et al. Anti-p53 antibodies in sera of workers occupationally exposed to vinyl chloride. J Natl Cancer Inst 1995;87:1400–7.
[22] Du CL, Wang JD. Increased morbidity odds ratio of primary liver cancer and cirrhosis of the liver among vinyl chloride monomer workers. Occup Environ Med 1998;55:528–32.
[23] Luo J-C, Liu H-T, Cheng T-J, et al. Plasma p53 protein and anti-p53 antibody expression in vinyl chloride monomer workers in Taiwan. JOEM 1999;41:521–6.
[24] Luo J-C, Liu H-T, Cheng T-J, et al. Plasma Asp13-ki-ras oncoprotein expression in vinyl chloride monomer workers in Taiwan. JOEM 1998;40:1053–8.
[25] Du CL, Chan CC, Wang JD. Comparison of personal and area sampling strategies in assessing workers' exposure to vinyl chloride monomer. Bull Environ Contam Toxicol 1996;56:534–42.
[26] SAS Institute. SAS Statistical Package, Version 6. Cary, NC: SAS Institute Inc.; 1990.
[27] Database III plus. Torrance, CA: Ashton-Tate; 1986.
[28] Marion MJ, DeVivo I, Smith S, et al. The molecular epidemiology of occupational carcinogenesis in vinyl chloride workers. Int Arch Occup Environ Health 1996;68:394–8.
[29] Luo JC, Zehab R, Anttila S, et al. Detection of serum p53 protein in lung cancer patients. J Occup Med 1994;36:155–60.
[30] Hefner RE, Watanabe PG, Gehring PJ. Preliminary studies of the fate of inhaled vinyl chloride monomer in rats. Ann NY Acad Sci 1975;246:135–48.
[31] Guengerich FP, Kim DH, Iwasaki M. Role of human cytochrome p-450 IIE1 in the oxidation of many low molecular weight cancer suspects. Chem Res Toxicol 1991;4:168–79.
[32] Radike MJ, Stemmer KL, Binghem E. Effect of ethanol on vinyl chloride carcinogenesis. Environ Health Perspect 1981;41:59–62.
[33] Kondo K, Tsuzuki H, Sasa M. A dose–response relationship between the frequency of p53 mutations and tobacco consumption in lung cancer patients. J Surg Oncol 1996;41:59–62.
[34] Partanen R, Koskinen H, Oksa P. Serum oncoproteins in asbestosis patients. Clin Chem 1995;41:1844–7.
[35] Hemminki K, Partnanen R, Koskinen H. The molecular epidemiology of oncoproteins: serum p53 protein in patients with asbestosis. Chest 1996;109(Suppl):22S–6S.
[36] Brinkworth MH, Yardley-Jones A, Edwards AJ, et al. A comparison of smokers and nonsmokers with respect to oncogene products and cytogenetic parameters. J Occup Med 1992;34:1181–8.
[37] Luo JCJ. Blood oncoprotein expression in colonic neoplasia. Doctorial Thesis, Columbia: Columbia University; 1994.
[38] Weihrauch M, Lehnert G, Kockerling F, et al. P53 mutation pattern in hepatocellular carcinoma in workers exposed to vinyl chloride. Cancer 2000;88:1030–6.
[39] Soussi T. P53 antibodies in the sera of patients with various types of cancer: a review. Cancer Res 2000;60:1777–88.
[40] Levesque MA, Dcosta M, Diamandis EP. P53 protein is absent from the serum of patients with lung cancer. Br J Cancer 1996;74:1434–40.
[41] Wong RH, Wang JD, Hsieh LL, et al. Effects on sister chromatid exchange frequency of aldehyde dehydrogenase 2 genotype and smoking in vinyl chloride workers. Mut Res 1998;420:99–107.