Biomarkers, November–December 2006; 11(6): 538–546

informa
healthcare

Plasma-transforming growth factor-alpha expression in residents of an arseniasis area in Taiwan

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Abstract

Epidemiological studies have demonstrated an association between long-term exposure to inorganic arsenic and the related adverse effects such as cancers, skin lesions, and vascular diseases. Although several hypotheses have been proposed for the mechanism of arsenic-induced pathogenesis, it remains imperfectly understood. Recent studies have suggested that alterations in growth signal transduction pathways, particularly involving transforming growth factor-alpha (TGF-alpha), may be important. Immunoassays were used to determine the plasma levels of TGF-alpha and epidermal growth factor receptor (EGFR), which is the receptor for TGF-alpha, in residents of an arseniasis area of Taiwan in relation to their estimated cumulative arsenic exposure from drinking water. No relationship between arsenic exposure and EGFR was found. However, among the high cumulative exposure group (>6 ppm-years), levels of plasma TGF-alpha (25.5 ± 38.2 pg ml^{-1}) and the proportion of individuals with TGF-alpha over-expression (29.4%) were significantly higher ($p < 0.05$) than normal, healthy unexposed controls (8.1 ± 5.6 pg ml^{-1}, 8.6%, respectively). There was a significant linear trend between cumulative arsenic exposure and the prevalence of plasma TGF-alpha over-expression after adjusting for age and sex ($p = 0.019$). The results suggest that plasma TGF-alpha expression may be a useful biomarker when detecting adverse effects on arsenic exposed population.

(Received November 2004; accepted May 2006)

Introduction

Arsenic is present in the environment in a variety of chemical forms, and exposures can occur from natural sources as well as herbicides, insecticides, rodenticides, cotton defoliators, and by-products of a number of industrial processes (IARC 1980).

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500600881488

Arsenic enters the human body mainly through ingestion and inhalation, and absorption via the gastrointestinal tract and lungs into the bloodstream is rapid. Absorbed arsenic distributes to a number of sites, including the lungs, liver, kidneys and skin (Vahter & Norin 1980). Long-term exposure may affect many organ systems, including the respiratory, gastrointestinal, cardiovascular, nervous and haematopoietic systems (World Health Organization (WHO) 1981). Arsenic has been associated with human cancers (Hutchinson 1987, 1988), including the skin, lungs, liver, kidney and urinary bladder (Chen et al. 1992). In the area of the south-west coasts of Taiwan, arsenic has also been associated with a peripheral vascular disorder termed Blackfoot Disease (Wu et al. 1961), as well as cancers of the skin, lung, liver, kidneys, prostate and urinary bladder, with a significant dose–response relationship to inorganic arsenic in drinking water and its metabolism capacity (Tseng et al. 1968, Chen et al. 1988, 1992, 1995, 1996, Yu et al. 2000). Recent studies in the area of the north-east coast of Taiwan have also demonstrated that toxicities were related with chronic arsenic exposure as well as the methylation capacity (Hsu et al. 1997, Chiou et al. 2003).

Although several hypotheses have been proposed for the mechanism of arsenic-induced carcinogenesis, it remains unknown, with limited evidence for either genetic or epigenetic mechanisms or both. In particular, over-expression of several growth factors, including transforming growth factor-alpha (TGF-alpha) and granulocyte/macrophage colony-stimulating factor (GM-CSF) has been associated with arsenic-mediated skin disease and neoplasia (Germolec et al. 1996). In Bangladesh, urinary TGF-alpha levels were shown to be significantly correlated with urinary arsenic levels in individuals drinking arsenic-containing well water, particularly in those individuals with arsenic-associated skin lesions (Ahsan et al. 2000, Rahman et al. 2001). It has been proposed that TGF-alpha expression may thus be a useful biomarker for the epidermal effects of arsenic (Do et al. 2001).

TGF-alpha is a known potent mitogenic polypeptide synthesized as a 160 amino acids, transmembrane precursor that undergoes sequential, external, proteolytic cleavage to a mature, active, stable form of 5.5 kDa (Todare et al. 1985). Since mature TGF-alpha is present in the extracellular environment, it is readily detectable in a variety of biological fluids. Elevated levels have been detected in the urine, serum or plasma of patients with various malignancies, including the colon, liver, ovaries, breast, oesophagus, stomach, pancreas, lung, and skin (Yeh et al. 1987, Ellis et al. 1990, Katoh et al. 1990). In addition, mature TGF-alpha is known to bind to the extracellular domain (ECD) of the epidermal growth factor receptor (EGFR) and mediates tyrosine phosphorylation of the intracellular domain of the receptor which stimulates an intracellular cascade of growth signal transduction, explaining its mitogenic effects (Brandt-Rauf 1995). This growth signal transduction can be accompanied by the proteolytic release of the ECD of EGFR with its accumulation in the extracellular environment, so that it too could be readily detected in biological fluids (Brandt-Rauf 1995). Elevated levels of the ECD of EGFR have been detected in the serum of patients with cancers of the lung, stomach and cervix, as well as asbestos-exposed workers before the development of malignancy (Brandt-Rauf 1995). Thus, it is possible that the EGFR ECD may also be a useful biomarker of the mitogenic effects of arsenic exposure.

The present study has used enzyme-linked immunosorbent assays (ELISAs) to determine the levels of TGF-alpha and EGFR ECD in the plasma of residents in the Ilan arseniasis area of Taiwan and to examine their relationship with cumulative arsenic exposure from drinking water.

Materials and methods

Subjects

From approximately 2000 residents of an arseniasis-endemic area in Ilan county of north-east Taiwan, 150 individuals were randomly selected according to the arsenic levels in their residential well water with 30 individuals in each of the following groups: 0–50, >50–100, >100–300, >300–600 and >600 ppb. Of these, 68 agreed to participate in medical surveillance, and plasma was collected from them during medical evaluation from March to April 1998. In addition, for these individuals information on age, gender, history of arsenic-containing well water consumption, and history of lung, bladder or skin cancer was available. There was no significant difference in terms of arsenic levels in well water between participants and non-participants. Plasma samples from 35 individuals with no known arsenic exposure were also collected.

Plasma TGF-alpha immunoassay

Plasma analysis for TGF-alpha was performed using a sandwich ELISA (Oncogene Research Products, Cambridge, MA, USA), which utilizes affinity-purified goat polyclonal antibodies specific for mammalian TGF-alpha, according to the manufacturer's instructions. Microtiter plate wells were precoated with these antibodies, and 50 µl of plasma or standards (diluted 1:5) and biotinylated TGF-alpha polyclonal antibody were added to each well and incubated for 3 h at room temperature. After washing, 100 µl of horseradish peroxidase-conjugate was added to each well and allowed to incubate for 30 min at room temperature. After washing again, the colour was developed by incubation with the chromogenic substrate *o*-phenylenediamine hydrochloride. Absorbance of each well was read on a spectrophotometric plate reader at 490 nm, and the concentration of TGF-alpha in the samples was determined by comparison with the absorbance of known concentrations of TGF-alpha from the standard curve.

Plasma EGFR immunoassay

Plasma analysis for EGFR was performed using a sandwich ELISA (Oncogene Research Products) in which a mouse monoclonal capture antibody and a rabbit polyclonal detector antibody that specifically recognize the ECD of EGFR were used, according to the manufacturer's instructions. Microtiter plate wells were precoated with the mouse monoclonal antibody, and 100 µl of plasma or standards (diluted 1:10) were added to each well and incubated overnight at room temperature. After washing, 100 µl of the rabbit polyclonal antibody was added to each well and incubated for 1 h at room temperature. After washing, 100 µl of goat anti-rabbit IgG conjugated to horseradish peroxidase was added to each well and incubated for 30 min at room temperature. After washing again, 100 µl of tetramethylbenzidine substrate solution were added to each well and incubated for 30 min at room temperature in the dark. Absorbance of each well was read on a spectrophotometric plate reader at 450/595 nm, and the concentration of EGFR ECD in the samples was determined by comparison with the absorbance of known concentrations of EGFR ECD from the standard curve.

Data analysis

Exposure groups were categorized by cumulative arsenic dose (total drinking water arsenic levels $\times$ years of exposure) into three groups: 34 high exposed (>6 ppm-years), 32 low exposed (>0, ≤ 6 ppm-years), and 35 unexposed healthy controls. The demographic data and test results were encoded, entered and analysed using the Statistical Analysis System (SAS 8.0). A Student's t-test and χ^2-tests were performed to analyse significant differences among groups. Odds ratios and 95% confidence intervals were calculated to test the magnitude and significance of differences in the prevalence of TGF-alpha and EGFR ECD over-expression among exposure groups, where over-expression was defined as values more than 2 standard deviations (SDs) above the mean of the controls. For TGF-alpha and EGFR ECD, the cut-off points of over expression were defined as 19.38 pg ml^{-1} and 237.06 fm ml^{-1}, respectively. Multivariable regression analysis was used to analyse the association between plasma TGF-alpha level and arsenic exposure after adjusting for other factor. Multivariable logistic regression analysis was also used to confirm the dose–response relationship between over-expression and arsenic exposure after adjusting for other factors. Mantel–Haenszel χ^2-tests for monotonic trend were also performed to test the linear trend between exposure and growth factor positivity. A Kruskal–Wallis test was applied to differentiate non-normal numerical variables among three groups, a Wilcoxon rank sum test was used to test the difference of numerical variables with non-normality between cancer group and control group.

Results

The basic characteristics of the study cohort and the results of plasma growth factor expression are summarized in Table I. The arsenic exposure groups had a significantly higher proportion of cancer cases compared with the unexposed controls ($p<0.05$). Plasma TGF-alpha levels were statistically significantly elevated in the high arsenic exposure group (25.5 ± 38.2 pg ml^{-1}) compared with the low arsenic exposure group (7.7 ± 9.2 pg ml^{-1}) or the unexposed controls (8.1 ± 5.6 pg ml^{-1}) ($p<0.05$). There were no significant differences among the groups with regard to EGFR ECD levels.

The prevalence of plasma TGF-alpha over-expression by exposure category is presented in Table II. Over-expression was found in ten of 34 (29.41%) individuals in the high exposure group, four of 32 (12.50%) in the low exposure group, and three of 35 (8.57%) in the unexposed controls. Compared with the unexposed controls, the age- and sex-adjusted odds ratios and 95% confidence intervals for TGF-alpha over-expression were 1.98 (0.37, 10.50) in the low exposure group and 5.24 (1.21, 22.68) in the high exposure group. Compared with the low exposure group, the age- and sex-adjusted odds ratio and 95% confidence interval for TGF-alpha over-expression were 2.61 (0.67, 10.23) in the high exposure group. There was a statistically significant linear trend between cumulative arsenic exposure and the prevalence of plasma TGF-alpha over-expression after adjusting for age and sex ($p=0.019$).

In multivariate regression, high cumulative arsenic exposure was statistically significantly associated with high plasma TGF-alpha level ($p=0.0014$) and positive TGF-alpha over-expression ($p=0.033$), after adjusting for other factors, as shown in Table III. Males were significantly lower with plasma TGF-alpha level as opposed to females ($p=0.012$).

Table I. Results of the expression of plasma growth factors by categories of cumulated arsenic exposure level, and the characteristics of study cohorts.

	High exposure (>6 ppm-years) ($n=34$)	Low exposure (>0, ≤6 ppm-years) ($n=32$)	Controls ($n=35$)
Arsenic level in well water (ppm)*	0.47** ±0.61 (293.17; 138.94–614.30)[a]	0.037 ±0.293 (34.10; 9.72–59.00)	0 ±0
Cumulated water arsenic level (ppm-years)*	29.30** ±35.92 (1.74; 8.47–37.63)	2.46 ±1.92 (2.20; 0.64–3.85)	0 ±0
Plasma TGF (pg ml^{-1})	25.5** ±38.2 (8.30; 2.75–31.05)	7.7 ±9.2 (2.15; 0.00–16.28)	8.1 ±5.6 (6.30; 5.30–9.30)
Plasma EGFR (fm ml^{-1})	134.3 ±38.5 (138.0; 102.7–158.4)	142.0 ±67.2 (128.7; 97.1–174.2)	134.6 ±51.24 (124.9; 98.7–159.8)
Age (years)	64.2 ±7.9 (64.0; 59.0–69.0)	66.47 ±8.1 (65.0; 61.0–72.0)	62.7 ±11.6 (62.0; 53.0–72.0)
Sex (male)	21(61.8%)[b]	23(67.7%)	21 (63.6%)
Cancers	5(14.7%)	4 (12.5%)	1 (2.86%)

*p <0.05, performed with a non-parametric method: Kruskal–Wallis test.
**p <0.05, while comparing with the control group performed with a Duncan procedure after analysis of variance (ANOVA).
[a]Values in parenthesis are (median; inter-quartile range).
[b]Value in parenthesis is a percentage.

Table IV shows the characteristics of the cohort members with cancer. Among the 68 exposed cohort members, ten (14.7%) were found to have cancer, including four lung cancers, three stomach cancers, one liver cancer, one lymphoma, and one ovarian cancer. One of these, a 65-year-old female ovarian cancer patient in the high cumulative arsenic exposure group (exposure of 6.133 ppm-years) was positive for TGF-alpha over-expression (98.65 pg ml^{-1}), and another 64-year-old male lymphoma patient in the low cumulative arsenic exposure group (exposure of 1.080 ppm-years) was also positive for TGF-alpha over-expression (22.45 pg ml^{-1}). Table V summarizes the data for the cohort members with and without cancer. There were no

Table II. Prevalence of plasma TGF-alpha over-expression by categories of cumulated arsenic level in well water.

	High exposure (>6 ppm-years) ($n=34$)	Low exposure (>0, ≤6 ppm-years) ($n=32$)	Controls ($n=35$)	Total ($n=101$)
TGF over-expression[a] frequency (%)	10 (29.41%)	4 (12.50%)	3 (8.57%)	17 (16.83%)
OR[b] (95% CI)	4.44* (1.1, 17.92)	1.52 (0.31, 7.4)	1.00	Linear trend $\chi^2=4.93$, $p=0.026$
AOR[c] (95% CI)	5.24* (1.21, 22.68)	1.98 (0.37, 10.5)	1.00	$\chi^2=5.48$, $p=0.019$
OR[a] (95% CI)	2.92** (0.81, 10.5)	1.00		
AOR[b] (95% CI)	2.61** (0.67, 10.23)	1.00		

[a]TGF over-expression is defined as plasma TGF >19.38 pg ml^{-1}.
[b]Crude odds ratio.
[c]Odds ratio is adjusted for age and sex.
*p <0.05; **p <0.1.

Table III. Multivariable regression analysis between plasma TGF-alpha expression and related cofactors.

	Plasma TGF over-expression[a]	Plasma TGF
Intercept:		
Parameter estimate ± standard error (p-value)	0.95 ± 2.07 (0.65)	35.35 ± 15.41 (0.024)
Cumulated well water As level (high exposure versus control): Parameter estimate ± standard error (p-value) (low exposure versus control)	1.59 ± 0.75 (0.033)	17.86 ± 5.43 (0.0014)
Parameter estimate ± standard error (p-value)	0.54 ± 0.84 (0.52)	1.78 ± 5.48 (0.75)
Age:		
Parameter estimate ± standard error (p-value)	−0.05 ± 0.034 (0.15)	−0.32 ± 0.24 (0.19)
Sex (males versus females):		
Parameter estimate ± standard error (p-value)	−0.45 ± 0.57 (0.43)	−11.95 ± 4.65 (0.012)

[a]TGF over-expression is defined as plasma TGF > 19.38 pg ml^{-1}.

significant differences in growth factor expression between non-cancer and cancer cases. Cancer cases were found to have statistically significantly lower cumulative arsenic exposure and to be significantly older than non-cancer cases.

Discussion

The results suggest that high cumulative exposure to arsenic in drinking water (> 6.00 ppm-years or the equivalent of levels >0.10 ppm over a 60-year lifetime) may significantly increase the expression of plasma TGF-alpha. Such levels are well above the WHO water arsenic permissible exposure level of 0.050 ppm (or 3.00 ppm-years over a 60-year lifetime). There was a significant linear trend between cumulative arsenic exposure and the prevalence of plasma TGF-alpha over-expression after adjusting for age and sex. These findings are consistent with the previous observation of a significant relationship between urinary total arsenic levels and urinary levels of TGF-alpha in individuals with drinking water arsenic exposure in Bangladesh (Do et al. 2001), although in that case the relationship was confined to individuals with arsenic-associated skin lesions. In the present study urine samples were not available and individuals had no arsenic-associated skin lesions that might be indicative of a healthy selection effect among the subjects, i.e. since this was voluntary, cross-sectional medical surveillance, individuals with disease may not have come for examination. However, based on past experience, one would expect approximately one-third of this cohort to develop arsenic-associated skin lesions in the future. Therefore, continued follow-up of this cohort will allow a more clear delineation of the relationship between TGF-alpha expression and the development of arsenic-associated skin lesions including skin cancer, as well as the potential predictive value of plasma TGF-alpha as a biomarker for such lesions and perhaps other arsenic-associated diseases such as internal malignancies, if plasma-negative individuals remain healthy and plasma-positive individuals develop disease.

As noted, several hypotheses have been proposed for the mechanism of arsenic-induced carcinogenesis, with limited evidence for either genetic or epigenetic processes. Increased cell proliferation related to ornithine decarboxylase activity (Brown & Kitchin 1996), the DNA binding of AP-1 and AP-2 transcription factors, and growth factor expression appears to play a role (Germolec et al. 1996). Arsenic is

Table IV. Characteristics of arseniasis residents with cancer.

	Type of cancer	Cumulated arsenic level (ppm-years)	As (water) (ppm)	Exposure categories	Plasma TGF (pg ml^{-1})	TGF(+)	Plasma EGFR (fm ml^{-1})	Age (years)	Sex
A	liver	0	0	low	7.7	(−)	117.6	69	male
B	lung	21.528	0.299	high	1.55	(−)	180.7	72	male
C	lung	3.222	0.0565	low	0	(−)	173.3	57	male
D	lung	8.474	0.103	high	6.45	(−)	138	82	male
E	lung	5.707	0.0761	low	6.45	(−)	76.7	75	male
F	stomach	4.630	0.0520	low	1.55	(−)	180.7	89	male
G	stomach	16.918	0.229	high	0	(−)	93.4	74	female
H	stomach	6.885	0.0956	high	8.9	(−)	69.3	72	male
I	lymphoma	1.080	0.0169	low	22.45	(+)	175.1	64	male
J	ovary	6.133	0.0944	high	98.65	(+)	89.7	65	female

also co-mutagenic with ultraviolet light light, X-rays or alkylating agents, inducing SCEs in lymphocytes (Jha et al. 1992) causing gene amplification in 3T3 cells in culture (Lee et al. 1988). Over-expression of several growth factors, including TGF-alpha and GM-CSF, have been suggested to be involved in arsenic-mediated skin diseases including neoplasia (Germolec et al. 1996). Other studies have demonstrated that TGF-alpha can synergize with c-myc oncogene expression to accelerate spontaneous and chemical-induced neoplastic development (Takagi et al. 1993, Amundadottir et al. 1996, Thorgeirsson & Santoni-Rugui 1996) and serve as a tumour enhancer or co-promoter for cancer. Thus, it is plausible that individuals with elevated TGF-alpha expression may be at increased risk of cancer.

In the present study, ten of 68 (14.7%) participants were found to have cancers including four lung, three stomach, one liver, one lymphoma and one ovarian. Of these, one ovarian cancer case in the high cumulative arsenic exposure category (6133 ppb-years) had an elevated TGF-alpha level (98.65 pg ml^{-1}), which is consistent with previous reports of TGF-alpha over-expression in ovarian cancer patients (Katoh et al. 1990, Ridderheim et al. 1994); and another lymphoma patient in the low cumulative arsenic exposure category (1.08 ppm-years) had an elevated TGF-alpha level (22.45 pg ml^{-1}), which is also consistent with other reports of TGF-alpha expression in differentiating human granulocyte precursor cells (Walz et al. 1993) and TGF-alpha involvement in lymphoma progression (Brissenden et al. 1985). Although

Table V. Results of plasma growth factors expression by cancer status.

	Arseniasis resident with cancer ($n=10$)	Arseniasis resident without cancer ($n=58$)
Cumulated water arsenic level (ppm-years)	7.58$^{\star}\pm$6.68 (0, 21.53)	17.23$\pm$30.75 (0, 197.46)
Arsenic level in well water (ppm)	0.10$^{\star}\pm$0.09 (0, 0.30)	0.28$\pm$0.52 (0, 3.46)
Plasma TGF (pg ml^{-1})	15.38$\pm$30 (0, 98.65)	16.96$\pm$28.98 (0, 149)
Plasma EGFR (fm ml^{-1})	120$\pm$43.6 (69.3, 180.7)	142.5$\pm$55.9 (67.4, 442.5)
Age (years)	71.9$^{\star}\pm$9.1 (57, 89)	64.2$\pm$7.4 (45, 81)
Sex (male)	8 (80%)	36 (62%)

$^{\star}p<0.05$, performed with a Wilcoxon rank sum test.

four other cancer cases were in the high cumulative arsenic exposure group, none had elevated TGF-alpha expression, and overall, cancer cases had lower plasma TGF-alpha levels and significantly lower cumulative arsenic exposure levels than non-cancer cases. These results suggest that arsenic exposure and TGF-alpha over-expression could not demonstrate in this study the association with the cancer occurrence due to limitations of study design. However, as noted above, prospective follow-up of the cohort may be able to demonstrate a link between high arsenic exposure, TGF-alpha over-expression and the subsequent development of cancer in the future.

This study is limited by its small sample size, resulting in values of borderline significance, and cross-sectional design, prohibiting causal inferences. Nevertheless, the association between cumulative arsenic exposure and TGF-alpha supports the contention that TGF-alpha expression may be a useful biomarker of effect of arsenic exposure and that further longitudinal studies are warranted.

Acknowledgements

This study was supported by grants from the Chang Gung Medical Centre (CMRP599, CMRP1004, CMRP1210) and the National Science Council in Taiwan (NSC92-2320-B-182-042, NSC91-2320-B-182-039, NSC90-2320-B182-056, NSC90-2320-B-182-054; NSC87-2314-B-182-079, NSC85-2331-B-182-106).

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