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Oxidative DNA damage and global DNA hypomethylation are related to folate deficiency in chromate manufacturing workers

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ABSTRACT

Exposure to hexavalent chromium [Cr (VI)] can cause DNA damage, genetic instability and increase the risk of cancer development. Folate deficiency affects DNA methylation and reduces the stability of the genetic material. However, the correlation between folate deficiency and DNA damage has never been clearly elucidated in chromate workers. In this study, we recruited one hundred and fifteen workers from chromate producing facilities as testing subjects and sixty local residents without chromium exposure history served as controls. The results showed an evident accumulation of Cr in peripheral red blood cells accompanied by a significantly decreased serum folate in chromate exposed workers. The decreased serum folate was associated with an increased urinary 8-hydroxy-2′-deoxyguanosine, DNA strand breaks and global DNA hypomethylation. These findings suggest that chronic occupational chromate exposure could induce folate depletion, which may further promote DNA damages and global DNA hypomethylation. Adequate folate supplement may provide benefit to chromate sufferers in stabilization of genetic material and reduce the risk of cancer development.

1. Introduction

Chromium (Cr) compounds, broadly used in industry, have shown serious toxic effects on humans and animals [1–3]. There are two major forms of Cr, hexavalent Cr [Cr (VI)] and trivalent Cr [Cr (III)] present in the environment. Whereas Cr (III) shows certain beneficial effects on human; Cr (VI) has been identified as a carcinogenic agent [4–6]. Epidemiological studies have indicated that exposure to Cr (VI) significantly increases the risk of respiratory cancer [7,8]. Substantial evidences have also shown that Cr (VI) exposure can cause chromosome aberration, sister chromatid exchanges, gene mutation, apoptosis, DNA strand breaks and oxidative DNA damage [9–12].

Folate, one kind of water soluble B vitamin enriched in green leafy vegetables, is involved in the synthesis of a wide variety of essential biological substances including nucleic acids, phospholipids and proteins through donating carbon units in cellular metabolism [13]. Folate plays a key role in maintaining the genomic stability, and its deficiency could increase risk of carcinogenesis and other metabolic disorders such as oxidative stress, DNA strand break, micronucleus formation and aberrant DNA methylation, and these changes can further result in chromosomal instability and increase the frequency of mutation events [14–17].

Therefore, both environmental exposure of Cr (VI) and folate deficiency have been associated with increased oxidative DNA damage, DNA strand breaks and aberrant DNA methylation *in vitro* and *in vivo* [18–28]. However, the correlation between the severity of folate deficiency and the extent of DNA abnormality in Cr (VI) exposed subjects has not been thoroughly studied. The purpose of this study was to investigate the detriment effects of folate deficiency on cellular genetic material in chronic chromate exposed workers.

Abbreviations: Cr, Chromium; Cre, Creatinine; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; tHcy, total homocysteine; RBC–Cr, Cr in red blood cells; U-Cr, Urinary Cr; Air–Cr, Cr in Air; ICIA, ion capture immunoassay; SPE, solid-phase extraction; HPLC, high performance liquid chromatography; ECD, electrochemical detection.

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2. Materials and methods

2.1. Subjects

The study was conducted in a chromate production plant located in the suburb of Jinan city, Shandong province of China. One hundred and fifteen subjects (female/male is 29/86) with a minimum exposure time to sodium dichromate ($Na_2Cr_2O_7$) for 6 months were enrolled. The average weekly working time of the chromate exposed workers was 40 h. Sixty healthy volunteers (female/male is 15/45) living in the same city without chromate exposure history were served as controls. Any subjects with a medical history of liver or renal diseases, hypertension, diabetes, cardiovascular disorders or pregnancy were excluded from the study. This research was approved by the ethical committee of Health Science Center of Peking University and local government authorities. A written informed consent was obtained from all participants prior to the enrollment.

2.2. Air–Cr concentration in the working environment

The Air–Cr concentration in the working environment was determined by FCS-30 point dust sampler (Jinqi Elect. Tech. Co. Shanghai, China). The air samples were collected at multiple posts in the workshop and the Cr levels were measured with electrothermal atomic absorption spectrometry (ET-AAS). All the samples were below 50 $\mu g/m^3$, which is the threshold limit as recommended by both the American Conference of Governmental Industrial Hygienists (ACGIH) and Chinese National Standard.

2.3. Urine and blood samples collection

The post-shift urine and blood samples were collected after 5 consecutive working days from Cr exposed workers. For each sample, 30 mL urine was collected into a metal free bottle. Red blood cells (RBC) were separated as reported previously [29]. Serum and plasma samples were obtained by centrifugation at 3500 rpm for 10 min to precipitate the cellular components from the blood specimen. Subsequently, the urine, serum, plasma and RBC samples were stored at −80 °C for later analysis.

2.4. Chemicals and equipments

Reagents and solvents were of at least analytical or chromatographic grade. 8-hydroxy-2′-deoxyguanosine (8-OHdG, $C_{10}H_{13}N_5O_5$, $M_W = 283.2$) was obtained from Sigma (St Louis, MO, USA). Ultra-pure water (>15 MΩ) was used in all analysis to eliminate background interference. Bond Elut Certify SPE columns with a capacity of 10 mL were obtained from Varian Corp. (Palo Alto, CA. USA). High performance liquid chromatography (HPLC) system was purchased from ESA Inc. (Sunnyvale CA. USA), which including the Model 582 pump, Model 542 autosamplers, Model 5600A Coularray electrochemical detection and data station.

2.5. Assessments of Cr personal exposure and cumulative dose

The Aircheck 2000 air samples pump (SKC Inc., USA) with a flow rate of 2.0 L/min was used for personal air sample collection. The Air–Cr was assessed with a full-shift (8 h) personal exposure sampler. The Cr in the air was absorbed onto a 0.8 μm mixed cellulose ester (MCE) filter and analyzed by ET-AAS method. The cumulative Cr exposure dose was calculated according to an equation as described previously [30].

2.6. RBC–Cr analysis

The RBC–Cr concentration was measured by Inductively Coupled Plasma Mass Spectrometer (ICP-MS) with ELAN DRC II ICP-MS (PerkinElmer Corp. USA) as described previously [29].

2.7. Serum folate and plasma total homocysteine (tHcy) concentration

The serum folate and plasma tHcy were measured by ion capture (ICIA) and fluorescence polarization (FPIA) method, respectively. The reagent kits were purchased from Abbott Laboratories Diagnostics Division and measured with the Abbott AxSYM system (Abbott Laboratories Diagnostics Division, USA).

2.8. MDA concentration in RBC, serum GSH-Px and SOD activity measurements

Lipid peroxide malondialdehyde (MDA) in hemolyzed RBC was assayed by using a colorimetric method after incubation with thiobarbituric acid reactive substances (TBARS). Briefly, 0.1 mL of hemolyzed RBC was mixed with 0.2 mL thiobarbituric acid (TBA) reagent containing 15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25 M hydrochloric acid. The mixture was placed in a boiling water bath for 15 min. The samples were cooled down to room temperature and centrifuged at 1000 g for 10 min. The absorbance of the supernatant was measured at 532 nm. The total protein in hemolyzed RBC was measured by using the Bradford assay. The results were expressed as nmol/gram-protein (g-pr). Serum glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity were measured as described by Gokirmak et al. [31]. Briefly, the GSH-Px activity was initiated by introducing H_2O_2 and the absorbance was read at 340 nm with a spectrophotometer. The SOD activity was assayed after inhibition of nitroblue tetrazolium reduction with xantine–xantine oxidase system which was used as a superoxide generator. Superoxide radicals can reduce nitro blue tetrazolium chloride monohydrate (NBT) to formazone. SOD prevented this reaction and its activity was inversely proportional to the absorbance value of formazone at 560 nm. All colorimetric assays above were measured with UV-2401PC UV–vis spectrophotometer (Shimadzu Corp., Japan)

2.9. Urinary 8-OHdG assay

The urinary 8-OHdG concentrations were determined by solid phase extraction (SPE) and high performance liquid chromatography and electrochemical detection (HPLC–ECD) as described previously [32]. In order to minimize the influence of urinary volume difference among subjects, the 8-OHdG level was normalized with urinary creatinine (Cre). The urinary Cre was determined by alkaline picric acid assay with a commercial kit from Ausbio Laboratories Co., Ltd. (China) and measured with a Hitachi 7170A automatic analyzer (Hitachi Corp., Japan).

2.10. DNA strand break and damage

The DNA strand breaks in peripheral blood lymphocytes were detected by single cell gel electrophoresis assay (Comet assay) as described previously [33]. Briefly, cells from 10 μL of blood were suspended in 30 μL of 20 mM EDTA/Ca^{2+} and Mg^{2+} free PBS and embedded in low-melting point agarose. After incubation with a lysis solution, the samples were subjected to electrophoresis followed by ethidium bromide staining. The intensity of the staining signal was measured under a fluorescence microscopy (Olympus optical Co. Tokyo, Japan). Quantitative analysis was performed by randomly analyzing one hundred cells on three slides for each

sample. Classification was made into four groups according to the length and fluorescence intensities of comet tails. Comet scores (degree of DNA strand breaks) were calculated as: (0 × *n* of type 1) + (1 × *n* of type 2) + (2 × *n* of type 3) + (3 × *n* of type 4), where "*n*" represents the number of a specific comet type per 100 counted cells. Types 1–4 were weighted by special factors of 0–3, respectively.

2.11. Genomic DNA isolation and global DNA methylation assay

Genomic DNA was extracted from whole blood by the QIAamp DNA Blood Midi Kit (Model 51185, Qiagen Inc., Germany) based on the protocol provided by the manufacturer. Briefly, 100 μL Qiagen protease was mixed thoroughly with 1 mL blood sample and 1.2 mL buffer AL in a 15 mL falcon tube, followed by incubation at 70 °C for 10 min. Subsequently, 1 mL ethanol was added to the solution, and the mixed solution was transferred onto the QIAamp Midi column, and centrifuged at 1850 g for 3 min. The column was further washed by using 2 mL buffer AW1 and AW2, respectively. The membrane was incubated at room temperature for 5 min after 200 μL buffer AE was added, and centrifuged at 4500 g for 2 min. Quality and quantity of DNA in the filtrate was measured by a DNA/RNA/Protein analyzer (BioSpec-mini, Shimadzu Corp., Kyoto, Japan) by measuring the absorbance at 260 nm and 280 nm. DNA with A260/280 nm ratio ranged from 1.8 to 2.0 was considered as purified DNA and used for global DNA methylation assay.

The global DNA methylation level was measured by using ELISA kit (MethylFlash Methylated DNA Quantification Kit, Colorimetric assay, Epigenetic Group Inc., NY, USA). Briefly, 1–8 μL of DNA at a concentration of 12.5–100 ng/μL was added to the strip wells that are specifically treated to have a high DNA affinity. The samples were incubated at 37 °C for 90 min followed by introduction of capture and detection antibodies. The absorbance was read at 450 nm. A standard curve generated from the plot of OD values versus the amount of positive controls (methylated polynucleotide containing 50% of 5-methylcytosine (5-mC)) at each concentration point was used to quantify the percentage of methylated DNA (5-mC) in the total DNA sample.

2.12. Statistical analysis

The Statistical Package of Social Science (SPSS) 17.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data normality was tested with one sample Kolmogorov–Smirnov method. For the normal data, the Student's *t*-test was used for analysis. The non-normal data was analyzed with Mann–Whitney U nonparametric test. The χ^2 test was used to compare the differences of frequency distribution on gender, smoking and drinking populations in the two groups. Pearson or Spearman correlation was performed in bivariate analyses. Multiple linear stepwise regression analysis was used to analyze the main factors that affected serum folate concentration. For all tests, *p* values reported, were two-tailed and results were considered statistically significant when $p < 0.05$.

3. Results

3.1. General information, RBC–Cr concentration and cumulative exposure level of Cr

Information about the subjects was summarized in Table 1. The mean Cr exposure time of the workers was 12.86 years. There was no significant difference in age and gender distribution, alcohol intake, smoking and diet habit between the chromate exposed workers and the controls. The RBC–Cr in chromate exposed workers was 12.45 μg/L, which was significantly higher than that of controls

Table 1
Subject characteristics of occupational chromate exposed workers and controls.

		Chromate exposure workers			Control subjects			*p* value
		Mean (SD)	Range	95% CI	Mean (SD)	Range	95% CI	
Age	year	37.96 (5.93)	25–54	36.65–38.94	38.77 (10.34)	24–62	36.10–41.44	0.577
Gender	M/F (%)		86/29 (74.8/25.2)			45/15(75.0/25.0)		0.975
Smokers	Y/N (%)		49/66 (42.6/57.4)			17/43(28.3/71.7)		0.064
Drinkers	Y/N (%)		52/63 (45.2/54.8)			24/36 (40.0/60.0)		0.509
Exposed time	year	12.86(6.02)	1–33	11.75–13.97		–		–
Cumulative exposed dose	g	240.30(410.23)	0.23–3206.17	153.96–322.93		–		–
RBC–Cr	μg/L	12.45 (20.28)	0.96–115.01	8.71–16.19	2.81 (0.48)	1.92–4.02	2.68–2.93	<0.001
Plasma tHcy	μmol/L	17.82 (7.82)	8.80–43.35	16.29–19.38	12.11 (2.47)	5.58–19.80	11.48–12.76	<0.001
Serum folate	ng/mL	6.86 (2.45)	2.40–12.80	6.46–7.36	8.14 (3.82)	3.0–16.30	7.15–9.13	0.020
Global DNA methylation	%	1.48 (0.67)	0.33–3.94	1.35–1.60	2.10 (1.04)	0.46–5.74	1.35–1.60	<0.001
MDA in RBC	nmol/g-pr	8.05 (4.21)	2.76–26.61	7.27–8.83	6.59 (3.27)	1.77–16.85	5.74–7.43	0.031
Serum SOD	U/mL	1.96 (0.44)	0.10–3.80	1.87–2.05	2.24 (0.68)	0.98–3.55	2.07–2.41	0.040
Serum GSH-Px	U/mL	1.19 (0.21)	0.56–2.00	1.15–1.24	1.46 (0.40)	0.83–2.08	1.36–1.52	<0.001
Urinary 8-OHdG	μmol/Mol-Cre	3.18 (3.08)	0.05–12.47	2.53–3.84	1.85 (1.35)	0.02–4.29	1.50–2.20	0.028
Score of DNA damaged	–	54.87 (23.62)	10–114	49.98–59.76	24.41 (11.70)	7–58	21.38–27.42	<0.001

χ^2 test, nonparametric test (Mann–Whitney test) or Student's *t*-test.

(2.81 μg/L, $p < 0.001$). The average cumulative chromate exposure dose in the testing workers was 340.3 g.

3.2. Serum folate and plasma tHcy concentration

In the chromate exposed workers, the serum folate concentration was 6.86 ng/mL, as compared to 8.14 ng/mL in control group ($p = 0.020$, Table 1). The plasma tHcy in chromate exposed workers was 17.82 μmol/L as compared to 12.11 μmol/L in control group ($p < 0.001$). The detailed data information was summarized in Table 1.

3.3. MDA in hemolyzed RBC, serum GSH-Px and SOD activity

MDA in hemolyzed RBC, activity of GSH-Px and SOD in serum were listed in Table 1. The MDA value in RBC from the chromate exposed workers and control groups were 8.05 and 6.59 nmol/g-pr, respectively. There was a significant increase of MDA level in chromate exposed workers comparing to controls ($p = 0.031$). The GSH-Px and SOD activity in chromate exposed workers was 1.19 and 1.96 U/mL, respectively, which were significantly lower than the values of 1.46 ($p < 0.001$) and 2.24 U/mL ($p = 0.040$) in the control group.

3.4. Urinary 8-OHdG concentration

The status of oxidative DNA damage was assessed with the urinary 8-OHdG level as listed in Table 1. The urinary 8-OHdG concentration in chromate exposed workers was 3.18 μmol/mol-Cre comparing to 1.85 μmol/mol-Cre in the control group ($p = 0.028$).

3.5. DNA strand breaks (Comet Assay)

The occurrence of type 1 and type 2 in the chromate exposed workers were lower, but the levels of type 3 and type 4 were higher than the controls. The mean comet score of DNA strand breaks in occupational chromate exposed workers was 54.87, which was significantly higher than the score of 24.41 in the controls ($p < 0.001$, Table 1).

3.6. Global DNA methylation level

The global DNA methylation level in the Cr exposed workers was 1.48%, as compared to 2.10% observed in the control group, ($p < 0.001$, Table 1). The reduced global DNA methylation level in the Cr exposed workers is presumed to be related to folate depletion.

3.7. Relationship among RBC–Cr, serum folate, urinary 8-OHdG concentrations and percent of global DNA methylation

In the chromate exposed workers, RBC–Cr concentration revealed a significantly negative correlation with the level of folate in the serum ($r = -0.228$, $p = 0.014$, Fig. 1). The cumulative exposure dose of Cr was also negatively related to serum folate concentration ($r = -0.334$, $p = 0.001$, Fig. 2). In contrast, the RBC–Cr concentration demonstrated a positive correlation with urinary 8-OHdG ($r = 0.211$, $p = 0.047$, Fig. 3). The serum folate was negatively correlated with urinary 8-OHdG concentration ($r = -0.249$, $p = 0.019$, Fig. 4). Furthermore, the serum folate concentration was significantly inversely correlated with the plasma tHcy ($r = -0.341$, $p < 0.001$). A significant positive association was observed between the serum folate concentration and the percentage of global DNA methylation in both Cr exposed workers and controls ($r = 0.163$, $p = 0.032$). The percentage of global DNA methylation was negatively associated with RBC–Cr level ($r = -0.241$, $p = 0.001$). Multiple line regression analysis showed that the serum folate concentration in chromate exposed workers was closely related to cumulative exposure dose and urinary 8-OHdG level, but was not significantly affected by the age, gender, smoking, drinking, serum SOD and GSH-Px activity and RBC–Cr level (Table 2).

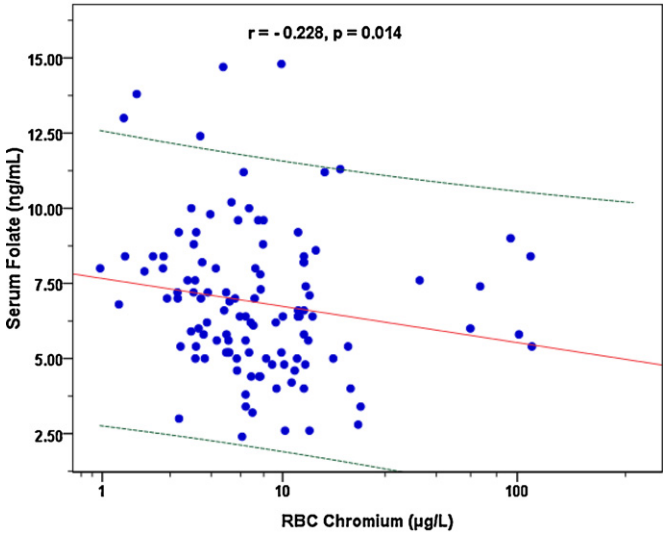

Fig. 1. Correlations between the levels of RBC–Cr and serum folate in occupational chromate exposed workers. Regression prediction line and 95% individual confidence intervals (dotted line) are provided.

4. Discussion

Available data suggest that Cr exposure may induce ROSs generation and undermine the antioxidant defenses [34], this could result in lipid peroxidation and cell damage. ROSs induced by Cr exposure can also cause DNA damage [8–10,35]. Several studies have indicated that increased chromosomal aberrations and/or sister chromatid exchange frequency occurred in lymphocytes from the chromate exposed workers [10–12]. Additionally, studies have concluded that Cr exposure increases the risk of lung cancer development [8,22].

SOD and GSH-Px are widely distributed in tissues with major function of being antioxidants. The antioxidative function can be

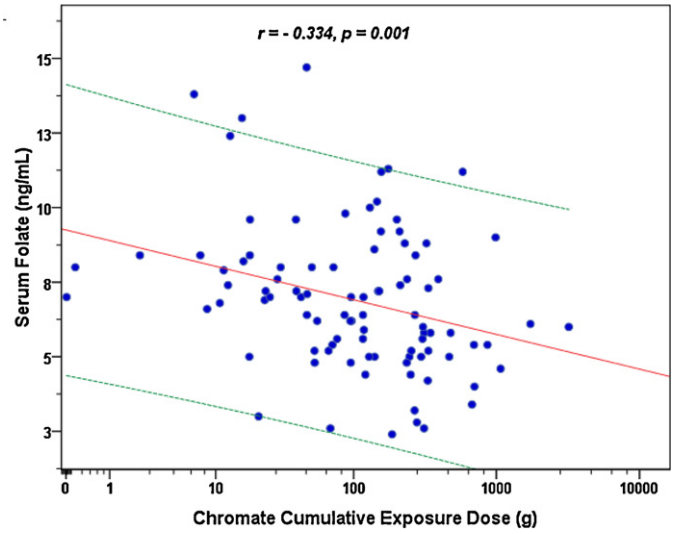

Fig. 2. Correlations between the levels of serum folate and Cr cumulative exposure dose in chronic occupational chromate exposed workers. Regression prediction line and 95% individual confidence intervals (dotted line) are provided.

Table 2
Multiple regression analysis of serum folate in occupational chromate exposed workers.

	Unstandardized coefficients		Standardized coefficients	*p*-value
	β value	SE		
Constant	12.128	1.518	7.989	0.000
Ln cumulative exposure dose	−0.560	0.209	−0.305	0.009
Ln urinary 8-OHdG	−0.483	0.220	−0.250	0.032

The variables of age, gender, smoking, drinking, Ln RBC–Cr, Ln cumulative exposure dose, Ln serum SOD, serum GSH-Px, Ln urinary 8OHdG concentration were including in the regression model (stepwise method).

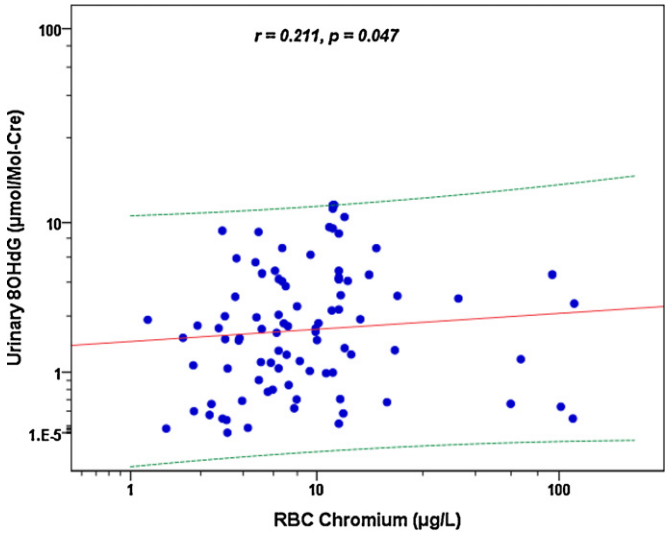

Fig. 3. Correlations between the levels of RBC–Cr and urinary 8-OHdG in chronic occupational chromate exposed workers. Regression prediction line and 95% individual confidence intervals (dotted line) are provided.

damaged during and after Cr exposure [10,34,35]. In this study, we observed a significant decrease of serum GSH-Px and SOD activity in the chromate exposed workers. The MDA is an indirect index of lipid peroxidation. A significant increase of MDA in RBC, from the chromate exposed workers further indicates that chronic chromate exposure results in depletion of the antioxidant defense elements.

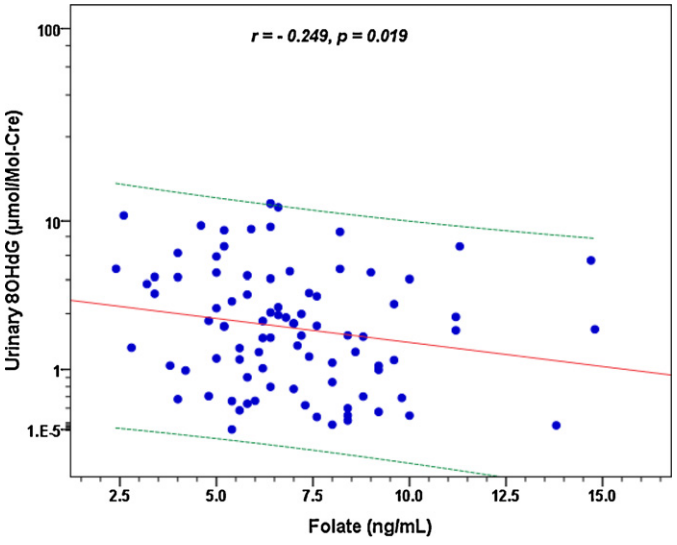

Fig. 4. Correlations between the levels of serum folate and urinary 8-OHdG in chronic occupational chromate exposed workers. Regression prediction line and 95% individual confidence intervals (dotted line) are provided.

Previous studies indicate that folate also has direct antioxidant effects, declining of folate in the circulation could increase oxidative stresses in both animal and human [36–39]. The protective role of folate has been confirmed by animal experiments, in which rabbits were pretreated with Cr followed by folate depletion resulting in significant DNA damage [40]. The urinary 8-OHdG has been used as a biomarker of oxidative DNA damage during the process of Cr exposure [10]. In present study we noticed lowered serum folate, elevated plasma tHcy and urinary 8-OHdG concentration in the chronic occupational chromate exposed workers. Multivariate analysis revealed that the serum folate concentration was significantly related to cumulative Cr exposure dose and urinary 8-OHdG level. Our results imply that oxidative DNA damage in the Cr exposed workers is related to folate depletion.

The importance of folate to genome stability has been acknowledged [13]. Folate can play a crucial role against genomic instability [15]. Previous studies showed that folate deficiency can reduce thymidylate synthesis and then bring about nucleotide imbalance and DNA disintegrity, finally accelerate double-strand DNA breaks, genetic instability and chromosome fragility [41,42]. Excessive DNA uracil content as well as increased numbers of chromosomal breaks is observed in subjects with folate deficiency [42,43]. The function of folate in modifying and repairing the genotoxicity effects caused by exposure to mutagenic and carcinogenic agents has also been confirmed [13–16]. In this study, the chromate exposed workers had an increased score of DNA strand breaks and an obvious decrease of serum folate compared to those of controls, which further supported that the deficiency of folate induced by long-term Cr (VI) exposure could exacerbate DNA strand breaks and damages.

The aberrant DNA methylation is a critical step in mutagenesis and can further induce carcinogenesis and neoplastic transformation by impeding DNA repair [20–22]. Previous studies have shown that folate depletion can alter the structure of DNA methylation and moderate folate depletion can induce a significant DNA hypomethylation without clinical manifestation [17,44]. On the other hand, oxidative DNA damages can also induce DNA hypomethylation by blocking DNA from being a substrate of DNA methyltransferases [45,46]. The abnormal DNA methylation has been observed under Cr (VI) exposure. Klein et al. found that potassium chromate salts can induce aberrant DNA methylation in mammalian cells [18]. Sun et al. reported that Cr (VI) may target histone methyltransferases and demethylases, which in turn affect both global gene and gene promoter specific histone methylation, leading to the silence of specific tumor suppressor genes of human mutL homolog 1 (hMLH1) in human lung A549 cells [20]. Ali et al. examined the frequency of methylation on the genes including p16, hMLH1, methylguanine-DNA methyltransferase (MGMT) and adenomatous polyposis coli (APC) in lung cancer from workers with chromate exposure and found that multiple DNA aberrant methylation of tumor suppressor genes contribute to chromate carcinogenesis [22]. However, the loss of global DNA methylation could be reversed when the folate was supplemented [47], which suggests that an optimal folate concentration may bring

DNA methylation back to normal and thus prevent carcinogenesis at early stage.

In this study, the chromate exposed workers exhibited a global DNA hypomethylation patterns which might resulted from folate deficiency or oxidative DNA damage. Our findings provide further evidence to support the existence of DNA hypomethylation with low level of folate in the chromate exposed workers. The reasons underlying folate reduction in Cr exposed workers might be due to its over consumption for oxidative DNA repairing or metabolic disturbance after a long-term Cr exposure. The exact mechanism warrants further investigation.

To our knowledge, this is the first study to demonstrate a relationship between folate deficiency and global DNA hypomethylation as well as oxidative DNA damage in chronic occupational chromate exposed workers. This study suggested that the folate deficiency was an important impact factor on global DNA methylation and oxidative DNA damage in chromate exposed workers. Our finding also proposed that folate supplementation for the chromate exposed workers may help to reduce the long term detrimental effects from this toxic hazard, which needs to be further validated in future studies.

5. Conclusions

Long-term chromate exposure can cause folate deficiency which is related to the genotoxicity of chromate as evidenced by the global DNA hypomethylation and oxidative DNA damage. Sufficient folate supply should be considered in order to prevent the chromate exposed workers from the risk of genetic abnormalities and cancer development.

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The authors declare they have no competing financial interests.

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