International Journal of Occupational Medicine and Environmental Health 2013;26(1):173–182
DOI 10.2478/S13382-013-0079-1

CORRELATION OF CHROMOSOME DAMAGE AND PROMOTER METHYLATION STATUS OF THE DNA REPAIR GENES MGMT AND hMLH1 IN CHINESE VINYL CHLORIDE MONOMER (VCM)-EXPOSED WORKERS

FEN WU[1], JING LIU[1], YU-LAN QIU[2], WEI WANG[3], SHOU-MIN ZHU[1], PIN SUN[1], WEN-BIN MIAO[1], YONG-LIANG LI[4], PAUL W. BRANDT-RAUF[4], and ZHAO-LIN XIA[1]

[1] School of Public Health, Fudan University and Key Laboratory of Public Health and Safety of the Ministry of Education of China, Shanghai, China
Department of Occupational Health and Toxicology
[2] School of Public Health, Shanxi Medical University, Taiyuan, China
Department of Health Toxicology
[3] School of Public Health, Zhengzhou University, Zhengzhou, China
Department of Occupational Health and Occupational Medicine
[4] School of Public Health, University of Illinois in Chicago, Chicago, USA

Abstract
Objective: To explore the association of the methylation status of *MGMT* and *hMLH1* with chromosome damage induced by vinyl chloride monomer (VCM). **Materials and Methods:** Methylation of *MGMT* and *hMLH1* was measured in 101 VCM-exposed workers by methylation-specific PCR. Chromosome damage in peripheral blood lymphocytes was measured by the cytokinesis-block micronucleus assay. The subjects were divided into chromosome damaged and non-damaged groups based on the normal reference value of micronuclei frequencies determined for two control groups. **Results:** *MGMT* promoter methylation was detectable in 5 out of 49 chromosome damaged subjects, but not in the chromosome non-damaged subjects; there was a significant difference in *MGMT* methylation between the two groups ($p < 0.05$). **Conclusions:** We detected aberrant promoter methylation of *MGMT* in a small number of chromosome damaged VCM-exposed workers, but not in the chromosome non-damaged subjects. This preliminary observation warrants further investigation in a larger study.

Key words:
Vinyl chloride monomer, Chromosome damage, *MGMT*, *hMLH1*, DNA methylation

The study is supported by the National Natural Science Foundation of China (NSFC 30070650 and 30671740), Shanghai Bureau of Public Health (08GWD12, 08GWZX0402), the National Institutes of Health (R01-OH04192 and P30-ES09089), and China Postdoctoral Science Foundation (20100471583).
Received: March 29, 2012. Accepted: July 11, 2012.
Corresponding author: Z.-L. Xia, Department of Occupational Health and Toxicology, School of Public Health, Fudan University, Shanghai 200032, China (e-mail: zlxia@shmu.edu.cn).

INTRODUCTION

Vinyl chloride monomer (CH2 = CHCl, VCM), a chemical widely used in the manufacture of polyvinyl chloride (PVC) plastics, causes angiosarcoma and hepatocellular cancer (HCC) [1–3] and is a known human and rodent carcinogen [4]. In the liver, VCM is primarily metabolized by the cytochrome P450 system to chloroethylene oxide (CEO) [5], which can either undergo hydrolysis or rearrange rapidly to the more stable chloroacetaldehyde (CAA). Acting as a bifunctional agent, CAA can react with nucleic acid bases to produce the four exocyclic DNA adducts: 3,N(4)-ethenocytosine; 1,N(6)-ethenoadenine; N(2)-3-ethenoguanine, and 1,N(2)-ethenoguanine [6]. These adducts are pro-mutagenic and genotoxic and, additionally, may give rise to chromosomal aberrations, micronuclei (MN), sister chromatid exchange, and DNA strand breaks which are observed in the lymphocytes of individuals occupationally exposed to VCM [7–9].

VCM-induced DNA damage is subject to repair, which is partly executed by O6-methylguanine-DNA methyltransferase (MGMT) and mismatch repair (MMR). MGMT is a DNA repair enzyme that plays an important role in the defense against the carcinogenic and cytotoxic effects of alkylating agents in cellular DNA [10]. The MGMT expression was frequently lost in a variety of human tumors and was found to be a significant prognostic factor [11]. Since the loss of the MGMT expression was not commonly observed due to a genetic change, it has been suggested that other causes, such as epigenetic changes, might be involved [12]. MMR is an essential system by which cells correct errors in DNA replication during proliferation to maintain the fidelity of the genome [13,14]. One of the MMR genes, *hMLH1*, has been demonstrated to play a pivotal role in DNA MMR [15]. Additionally, it has been shown that $Mgmt^{-/-}$ $Mlh1^{-/-}$ (double knockout) mice treated with alkylating agents exhibited high susceptibility to carcinomas [16].

It has been proposed that aberrant DNA methylation of CpG islands in the promoter region is correlated with the inactivation of tumor suppressor genes in human cancers. Several investigators have reported that CpG islands on the promoters of the *MGMT* and *hMLH1* genes are hypermethylated in several malignancies and aberrant hypermethylation represses the expression of MGMT and hMLH1 [17–20]. A high frequency of *p16* hypermethylationin was observed in vinyl chloride (VC)-associated hepatocellular carcinomas (HCC) [21]. Aberrant methylation of tumor suppressor genes might also act in the early stages of the multistep process of carcinogenesis [22].

Based on the information above, we hypothesized that epigenetic silencing of *MGMT* and *hMLH1*, by promoter hypermethylation, might be involved in VCM-exposure-induced chromosome damage which is the early stage in the process of liver angiosarcoma. In the present study, we attempted to identify the *MGMT* and *hMLH1* promoter methylation status in genomic DNA isolated from peripheral blood lymphocytes from workers exposed to VCM using a methylation-specific polymerase chain reaction (MS-PCR) and to analyze the correlation between *MGMT* and *hMLH1* gene promoter methylation and chromosome damage induced by VCM exposure.

MATERIALS AND METHODS

Study population

Workers employed in a VCM polymerization plant in China were studied. Prior to the study, written informed consent had been obtained from each subject and a standardized questionnaire had been used to obtain personal information, smoking and alcohol habits, medication and occupational history. The subjects exposed to VCM for longer than one year were selected if the following criteria were met: detailed questionnaires had been completed, the CBMN test results and a blood sample had been provided and the MSP analysis for the *MGMT* and *hMLH1* genes was completed successfully. A total of 101 workers met these criteria. Individuals who smoked once a day for

over 6 months were defined as smokers, and individuals who consumed one or more alcohol drinks a week for over 6 months were considered as drinkers.

In addition, two groups were selected as controls: group 1 consisted of 41 male and 56 female workers from the same factory and another VCM polymerization plant who were not exposed to VCM or other known toxicants occupationally; group 2 comprised 23 male and 21 female healthy residents living in the same city. A normal reference value of the MN frequency was determined for the controls on which the grouping of the VCM-exposed subjects was based, such that the MN frequency above 3‰ in the VCM-exposed workers is an indicator of chromosome damage and below or equal to 3‰ is considered normal [23].

Assessment of the VCM exposure

The level of VCM was measured for different work sites of the plant using gas chromatography. Since the VCM plant had kept VCM air concentration data for different work sites from the beginning of its establishment, we were able to estimate the cumulative exposure dose of each worker with a relatively high level of precision. The cumulative exposure dose was calculated according to an equation as described previously [7]. The VCM-exposed subjects were then divided into high-exposure and low-exposure groups according to the median dose (26 642.28 mg).

Cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was performed according to the standard methods as described previously [24]. For each subject, 1000 binucleated (BN) lymphocytes with well-preserved cytoplasm were scored blindly by the same reader.

Collection of blood samples and DNA preparation

Blood clot was immediately frozen at –80°C after collection and sent to the laboratory on dry ice. Genomic DNA was extracted from the blood samples by a routine phenol-chloroform method [25].

DNA modification (bisulfite treatment)

DNA modification with sodium bisulfite causes unmethylated cytosine bases to convert to uracil, while methylated cytosine is resistant and remains unchanged. After treatment, subsequent PCR using primers specific for either methylated or modified unmethylated DNA was performed [26]. Briefly, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, USA), treated again with NaOH, precipitated with ethanol, and resuspended in water.

Methylation-specific PCR (MSP)

The methylation status of *MGMT* and *hMLH1* genes in the samples was determined by MSP [26]. Two sets of primers (Table 1) had been previously described [13,17]. For PCR amplifications, 2 μl of bisulfite-modified DNA was added to PCR mixture containing 1× buffer with 2 mmol/l $MgCl_2$, 500 nmol/l each primer, 0.2 mmol/l dNTPs, 1 U Hot Start Taq polymerase. The samples were amplified for *MGMT* under the following conditions: 95°C for 8 min, followed by 35 cycles of 95°C for 30 s, 59°C (unmethylated reaction) and 65°C (methylated reaction) for 30 s, 72°C for 30 s and the final extension at 72°C for 10 min. The annealing temperature and time for *hMLH1* were 60°C (unmethylated reaction) and 45 s. Normal lymphocytes treated with CpG methyltransferase (M.SssI) (New England BioLabs, USA) before the bisulfite treatment were used as a positive control for the methylated alleles of *MGMT*. Also, control experiments without DNA were performed for each set of PCRs. Each PCR product (5 μl) was loaded directly onto 3% agarose gels stained with ethidium bromide and visualized under UV illumination.

Statistical analysis

Statistical analysis was performed using SPSS (ver. 15.0, SPSS Inc., Chicago, IL) and SAS (ver. 9.1)

Table 1. Primers for MSP

Primers	Sequence (5'→3')	Size (bp)	Annealing temperature (°C)
MGMT Uf	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93bp	59
MGMT Ur	AACTCCACACTCTTCCAAAAACAAAACA		
MGMT Mf	TTTCGACGTTCGTAGGTTTTCGC	81bp	65
MGMT Mr	GCACTCTTCCGAAAACGAAACG		
hMLH1 Uf	TTTTGATGTAGATGTTTTATTAGGGTTGT	124bp	60
hMLH1 Ur	ACCACCTCATCATAACTACCCACA		
hMLH1 Mf	ACGTAGACGTTTTATTAGGGTCGC	115bp	65
hMLH1 Mr	CCTCATCGTAACTACCCGCG		

Uf – unmethylation forward primer; Ur – unmethylation reverse primer; Mf – methylation forward primer; Mr – methylation reverse primer.

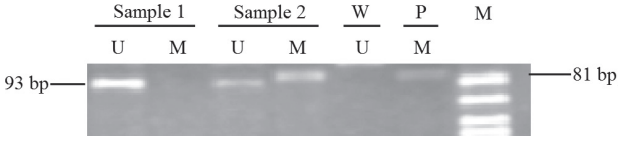
software. The influence of gender, age, cumulative exposure dose, smoking, and alcohol consumption on the frequencies of MN was determined using univariate and multiple Poisson regression analyses. Frequency ratio (FR) and its 95% confidence interval (95% CI) were estimated using $FR = e^{\beta}$ ($e \approx 2.71828$), where β is the regression coefficient for a categorical variable (i.e. binary) in the Poisson model fitted to the MN frequency data. Thus, FR is the ratio of the mean MN frequency in a study group to that in the reference group. The difference of the mean micronuclei frequencies between the two groups was evaluated in the Poisson model and the difference of methylation was compared using Pearson's χ^2 and Fisher's exact test. Odds ratio was estimated to quantify the relative risk of chromosome damage caused by VCM exposure. Statistical significance was defined as $p < 0.05$.

RESULTS

The habitual drinkers had higher mean MN frequencies than the non-habitual drinkers (4.36 vs. 3.38, respectively; $p < 0.05$). No significant difference was observed in the mean MN frequencies grouped by gender, age, cumulative exposure dose and smoking habit ($p > 0.05$; Table 2).

The mean and median MN frequencies of the VCM-exposed workers were 3.70±1.95 (‰) and 3.00 (‰), respectively, with a range of 0–9 (‰). Unadjusted Poisson regression showed a significant difference in MN frequencies between the exposed group and the pooled control group (mean: 1.22±1.24 (‰); median: 1.00 (‰); range: 0–5 (‰); $p < 0.05$).

Based on the normal reference value of MN frequency (3‰), there were 8 and 49 cases of chromosome damage in the controls and exposed workers, respectively. There was a 15.67-fold increase in the risk of chromosome damage in the exposed workers compared to that in the control group (unadjusted OR: 15.67; 95% CI: 6.95–35.33; $p < 0.05$). Among



Two samples are shown in lanes 1–4.
U and M represent PCR products from the unmethylated and methylated alleles, respectively; W, PCR reaction with deionized water; P, PCR product with M.SssI-treated DNA as a positive control for hypermethylated DNA; and M, molecular weight markers.
Sample 1 is unmethylated and sample 2 has a methylated allele.

Fig. 1. Methylation-specific PCR of *MGMT* in DNA from the peripheral lymphocytes

Table 2. Comparison of micronuclei (MN) frequency by demographic and lifestyle factors among vinyl chloride monomer (VCM)-exposed workers

Variables	Exposed workers n (%)	MN (‰), M±SD	FR (95% CI)
Gender			
male	74 (73.3)	3.41±1.915	0.91 (0.69–1.20)
female	27 (26.7)	3.81±2.434	1.00
Age (years)			
younger (≤ 35)	55 (54.5)	3.35±1.898	0.88 (0.69–1.12)
older (> 35)	46 (45.5)	3.72±2.248	1.00
Cumulative exposure dose			
low exposure	55 (54.5)	3.58±1.969	1.12 (0.87–1.43)
high exposure	46 (45.5)	3.43±2.187	1.00
Smoking			
never	53 (52.5)	3.64±2.228	1.12 (0.86–1.47)
ever	48 (47.5)	3.38±1.875	1.00
Drinking			
non-habitual + never	87 (86.1)	3.38±2.070	0.71 (0.52–0.96)*
habitual	14 (13.9)	4.36±1.865	1.00

M – mean; SD – standard deviation; FR – frequency ratio.
* Significant at p = 0.05 from multiple Poisson regression analyses.

the 49 VCM-exposed workers with chromosome damage, the median cumulative dose was 28 015.13 mg, as compared with 25 348.64 mg in the 52 exposed workers without chromosome damage.

Methylation of *MGMT* was detected in 10.2% (5/49) of the 'chromosome damaged' group, but not in the 'chromosome non-damaged' group. There was a significant difference in *MGMT* methylation between the two groups ($p < 0.05$). Representative data for MSP of *MGMT* is shown in Figure 1. The basic information for the five methylated workers is summarized in Table 3. The five methylated subjects never smoked and rarely drank alcohol, and their exposure lasted longer than 9 years. Aberrant methylation was not observed in the promoter region of *hMLH1* gene, in both the 'chromosome damaged' and 'chromosome non-damaged' group.

Table 3. Basic information of *MGMT*-methylated workers

Subject	Sex	Worked years (n)	Smoking	Drinking	Cumulative exposure dose (mg)
1	female	14	no	no	11.070
2	female	15	no	no	21.810
3	male	9	no	occasionally, a little	7.170
4	male	12	no	occasionally, a little	9.580
5	male	16	no	no	42.000

DISCUSSION

Methylation is a major epigenetic modification in humans and changes in methylation patterns play an important role in tumorigenesis [27,28]. Abnormal methylation of CpG islands can efficiently repress transcription of the associated gene in a manner akin to mutations and deletions and act as one of the 'hits' in Knudson's 2-hit hypothesis for tumor generation. Tumor cells exhibit global hypomethylation of the genome accompanied by region-specific hypermethylation events. So far, numerous examples of aberrant CpG island promoter hypermethylation have been observed in tumor-suppressor genes, genes involved in cell-cell adhesion, and genes that play an important role in DNA repair [28].

Aberrant methylation of tumor-suppressor genes has also been observed in normal tissues adjacent to tumors in cases of OSCC, HCC, HNSCC, and gastric cancers [22, 29–32]. In all studies, the incidence of hypermethylation was higher in tumor tissues than in the corresponding adjacent normal tissues, suggesting that aberrant DNA methylation is an early event of carcinogenesis, including hepatocarcinogenesis.

As the majority of VCM-exposed workers do not develop hepatoma and angiosarcoma of the liver, our study focused on the chromosome damage stage in the process of carcinogenesis following VCM exposure. The induction of MN represents sensitive cytogenetic end points for the detection of genotoxic activity of environmental mutagens and carcinogens and the increased frequency of MN has been observed in lymphocytes of individuals occupationally exposed to VCM [8]. Our data also showed that workers exposed to VCM had higher frequency of MN than the unexposed controls. This implies that the induction of MN is a sensitive cytogenetic endpoint for detecting genotoxicity caused by VCM exposure. The CBMN assay is the preferred method for measuring MN in cultured human cells, so it was employed in the present study. The frequency of MN in lymphocytes was used to assess the genetic effect of occupational exposure to VCM. Furthermore, the 95-percentile of the controls' MN frequencies was used to define the 'chromosome damaged' and 'chromosome non-damaged' groups in workers exposed to VCM. We found the risk of chromosome damage in the VCM-exposed workers to be significantly elevated (48.5%, 49 out of 101) over that of the controls (5.67%, 8 out of 141).

Although VCM is known to cause cancer and other diseases via genotoxicity, we hypothesize that long-term exposure to VCM may contribute to disease development through epigenetic reprogramming. Indeed, *p16* methylation is a frequent event in VC-associated HCCs [21]. In the present study, *MGMT* promoter methylation was only detected in the chromosome damaged subjects. However, the percentage of methylation (10%) was lower than those reported in other tumors, including liver carcinoma (12.4% to 58%) [33–44]. One explanation might be that workers were in the chromosome damage stage, which is an early effect of VCM exposure. In addition, the difference may be accounted for by the use of blood-derived DNA methylation as a surrogate for tissue methylation. Given the difficulty in obtaining DNA from some tissues of interest in studies in humans, the use of blood-derived DNA as a surrogate is commonplace [45,46]. Thirdly, unspecific amplifications may occur especially in patient samples with potentially suboptimal quality and limited quantity of DNA. Therefore, we would like to stress the importance of DNA sequencing to confirm the obtained MSP results. Although MSP following bisulfite treatment is generally an extremely sensitive method to analyze the promoter methylation status [26], its results have to be confirmed by a more specific method such as bisulfide direct sequencing to definitely exclude unspecific amplifications and incomplete bisulfite treatment [47].

As the consumption of tobacco and alcohol has been implicated in the methylation of tumor suppressor gene p15 [48], it indicates the need to exclude the effect of smoking and drinking on methylation. We found that the five

methylated subjects never smoked and rarely drank alcohol. Their exposure duration ranged from 9 to 16 years, with cumulative exposure dose from 7170 mg to 42000 mg. Although the five subjects were not exposed to a significantly higher level of VCM, we could still surmise that methylation of *MGMT* might be due to VCM exposure, given the fact that the cumulative VCM exposure employed in this study might not completely reflect personal exposure, and the use of more accurate methods such as personal air sampling may improve the assessment of the actual exposure. Further studies comparing the methylation level between VCM-exposed and non-exposed subjects will be required to elucidate the effect of VCM exposure on *MGMT* methylation.

We also investigated the promoter methylation status of *hMLH1*. Silencing *hMLH1* has been detected in different tumors [17,32,39,41–43,49–51]. However, *hMLH1* promoter methylation was not detected in sporadic renal cell carcinoma (RCC) and multiple myeloma (MM) [52,53]. In this analysis, only unmethylated promoter sequences of *hMLH1* were detectable in all the subjects.

Although VCM is a known hepatic carcinogen, which results from its metabolic activation to the reactive intermediate that interacts with the sensitive tissue in the liver, we used peripheral lymphocytes to evaluate VCM-induced chromosome damage. In our previous attempts to find out alternative biological samples for VCM-induced adverse effects to the liver, we compared VCM-induced DNA damage of liver cells and peripheral lymphocytes in rats. We found that VCM induced increased DNA damage both in liver cells and lymphocytes compared to the controls and the effect was dose- and time-dependent. DNA damage detected in lymphocytes was significantly correlated with that in liver cells, demonstrating that peripheral lymphocytes could serve as an ideal alternative for liver cells to investigate VCM-related damage [54]. On the other hand, alterations in blood-derived DNA methylation patterns, both at the global level and specific loci, by environmental exposures (benzene, persistent organic pollutants, lead, arsenic, and air pollution) have been observed in epidemiologic studies, though the patterns are far from consistent [55].

In summary, aberrant promoter methylation of the DNA repair gene *MGMT* was detectable in a small number of the chromosome damaged workers, but not in the non-damaged subjects. Although there was a significant difference in *MGMT* methylation between the two groups, these preliminary findings require confirmation in larger studies.

ACKNOWLEDGMENTS

We would like to thank physicians Shang-jian Chai and Jun Li for their help with physical examination of the workers and data collection of VCM exposure, and Dr Jie Liu (NIEHS, USA) for his critical review and scientific editing.

Fen Wu, Jing Liu, and Yu-lan Qiu contributed equally to this work.

REFERENCES

1. Mundt KA, Dell LD, Austin RP, Luippold RS, Noess R, Bigelow C. *Historical cohort study of 10 109 men in the North American vinyl chloride industry, 1942–72: update of cancer mortality to 31 December 1995*. Occup Environ Med 2000;57:774–81.
2. Ward E, Boffetta P, Andersen A, Colin D, Comba P, Deddens JA, et al. *Update of the follow-up of mortality and cancer incidence among European workers employed in the vinyl chloride industry*. Epidemiology 2001;12:710–8.
3. Wong RH, Chen PC, Du CL, Wang JD, Cheng TJ. *An increased standardised mortality ratio for liver cancer among polyvinyl chloride workers in Taiwan*. Occup Environ Med 2002;59:405–9.
4. Creech JL, Jr, Makk L. *Liver disease among polyvinyl chloride production workers*. Ann NY Acad Sci 1975;246:88–94.
5. 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.

6. Dosanjh MK, Chenna A, Kim E, Fraenkel-Conrat H, Samson L, Singer B. *All four known cyclic adducts formed in DNA by the vinyl chloride metabolite chloroacetaldehyde are released by a human DNA glycosylase*. Proc Natl Acad Sci USA 1994;91:1024–8.
7. Zhu S, Wang A, Xia Z. *Polymorphisms of DNA repair gene XPD and DNA damage of workers exposed to vinylchloride monomer*. Int J Hyg Environ Health 2005;208:383–90.
8. Fucic A, Horvat D, Dimitrovic B. *Mutagenicity of vinyl chloride in man: comparison of chromosome aberrations with micronucleus and sister-chromatid exchange frequencies*. Mutat Res 1990;242:265–70.
9. Fucic A, Barkovic D, Garaj-Vrhovac V, Kubelka D, Ivanic B, Dabo T, et al. *A nine-year follow up study of a population occupationally exposed to vinyl chloride monomer*. Mutat Res 1996;361:49–53.
10. Pegg AE. *Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents*. Cancer Res 1990;50:6119–29.
11. Matsukura S, Miyazaki K, Yakushiji H, Ogawa A, Harimaya K, Nakabeppu Y, et al. *Expression and prognostic significance of O6-methylguanine-DNA methyltransferase in hepatocellular, gastric, and breast cancers*. Ann Surg Oncol 2001;8:807–16.
12. Bhakat KK, Mitra S. *Regulation of the human O(6)-methylguanine-DNA methyltransferase gene by transcriptional coactivators cAMP response element-binding protein-binding protein and p300*. J Biol Chem 2000;275:34197–204.
13. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. *Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia*. Cancer Res 1999;59:793–7.
14. Lahue RS, Au KG, Modrich P. DNA *mismatch correction in a defined system*. Science 1989;245:160–4.
15. Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, et al. *Mutation of a mutL homolog in hereditary colon cancer*. Science 1994;263:1625–9.
16. Kawate H, Sakumi K, Tsuzuki T, Nakatsuru Y, Ishikawa T, Takahashi S, et al. *Separation of killing and tumorigenic effects of an alkylating agent in mice defective in two of the DNA repair genes*. Proc Natl Acad Sci USA 1998;95:5116–20.
17. Fleisher AS, Esteller M, Tamura G, Rashid A, Stine OC, Yin J, et al. *Hypermethylation of the hMLH1 gene promoter is associated with microsatellite instability in early human gastric neoplasia*. Oncogene 2001;20:329–35.
18. Kang GH, Shim YH, Jung HY, Kim WH, Ro JY, Rhyu MG. *CpG island methylation in premalignant stages of gastric carcinoma*. Cancer Res 2001;61:2847–51.
19. Qian XC, Brent TP. *Methylation hot spots in the 5' flanking region denote silencing of the O6-methylguanine-DNA methyltransferase gene*. Cancer Res 1997;57:3672–7.
20. Watts GS, Pieper RO, Costello JF, Peng YM, Dalton WS, Futscher BW. *Methylation of discrete regions of the O6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene*. Mol Cell Biol 1997;17:5612–9.
21. Weihrauch M, Benicke M, Lehnert G, Wittekind C, Wrbitzky R, Tannapfel A. *Frequent k- ras -2 mutations and p16(INK4A)methylation in hepatocellular carcinomas in workers exposed to vinyl chloride*. Br J Cancer 2001;84:982–9.
22. Zhang YJ, Ahsan H, Chen Y, Lunn RM, Wang LY, Chen SY, et al. *High frequency of promoter hypermethylation of RASSF1A and p16 and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma*. Mol Carcinog 2002;35:85–92.
23. Ji F, Wang W, Xia ZL, Zheng YJ, Qiu YL, Wu F, et al. *Prevalence and persistence of chromosomal damage and susceptible genotypes of metabolic and DNA repair genes in Chinese vinyl chloride-exposed workers*. Carcinogenesis 2010;31:648–53.
24. Fenech M. *The cytokinesis-block micronucleus technique: a detailed description of the method and its application to genotoxicity studies in human populations*. Mutat Res 1993;285:35–44.

25. Miller SA, Dykes DD, Polesky HF. *A simple salting out procedure for extracting DNA from human nucleated cells*. Nucleic Acids Res 1988;16:1215.
26. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. *Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands*. Proc Natl Acad Sci USA 1996;93: 9821–6.
27. Feinberg AP. *Cancer epigenetics takes center stage*. Proc Natl Acad Sci USA 2001;98:392–4.
28. Robertson KD. *DNA methylation, methyltransferases, and cancer*. Oncogene 2001;20:3139–55.
29. Shen L, Ahuja N, Shen Y, Habib NA, Toyota M, Rashid A, et al. *DNA methylation and environmental exposures in human hepatocellular carcinoma*. J Natl Cancer Inst 2002;94:755–61.
30. Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. *Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis – A comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma*. Hepatology 2000;32:970–9.
31. Wong TS, Man MW, Lam AK, Wei WI, Kwong YL, Yuen AP. *The study of p16 and p15 gene methylation in head and neck squamous cell carcinoma and their quantitative evaluation in plasma by real-time PCR*. Eur J Cancer 2003;39:1881–7.
32. Sakata K, Tamura G, Endoh Y, Ohmura K, Ogata S, Motoyama T. *Hypermethylation of the hMLH1 gene promoter in solitary and multiple gastric cancers with microsatellite instability*. Br J Cancer 2002;86:564–7.
33. Kitajima Y, Miyazaki K, Matsukura S, Tanaka M, Sekiguchi M. *Loss of expression of DNA repair enzymes MGMT, hMLH1, and hMSH2 during tumor progression in gastric cancer*. Gastric Cancer 2003;6:86–95.
34. Kulkarni V, Saranath D. *Concurrent hypermethylation of multiple regulatory genes in chewing tobacco associated oral squamous cell carcinomas and adjacent normal tissues*. Oral Oncol 2004;40:145–53.
35. Yang HJ, Liu VW, Wang Y, Chan KY, Tsang PC, Khoo US, et al. *Detection of hypermethylated genes in tumor and plasma of cervical cancer patients*. Gynecol Oncol 2004;93:435–40.
36. Bello MJ, Alonso ME, Aminoso C, Anselmo NP, Arjona D, Gonzalez-Gomez P, et al. *Hypermethylation of the DNA repair gene MGMT: association with TP53 G:C to A:T transitions in a series of 469 nervous system tumors*. Mutat Res 2004;554: 23–32.
37. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. *Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia*. Cancer Res 1999;59:793–7.
38. Hasselblatt M, Muhlisch J, Wrede B, Kallinger B, Jeibmann A, Peters O, et al. *Aberrant MGMT (O6-methylguanine-DNA methyltransferase) promoter methylation in choroid plexus tumors*. J Neurooncol 2009;91(2):151–5.
39. Matsukura S, Soejima H, Nakagawachi T, Yakushiji H, Ogawa A, Fukuhara M, et al. *CpG methylation of MGMT and hMLH1 promoter in hepatocellular carcinoma associated with hepatitis viral infection*. Br J Cancer 2003;88:521–9.
40. Martin P, Santon A, Garcia-Cosio M, Bellas C. *hMLH1 and MGMT inactivation as a mechanism of tumorigenesis in monoclonal gammopathies*. Mod Pathol 2006;19:914–21.
41. Lenz G, Hutter G, Hiddemann W, Dreyling M. *Promoter methylation and expression of DNA repair genes hMLH1 and MGMT in acute myeloid leukemia*. Ann Hematol 2004;83:628–33.
42. Oue N, Sentani K, Yokozaki H, Kitadai Y, Ito R, Yasui W. *Promoter methylation status of the DNA repair genes hMLH1 and MGMT in gastric carcinoma and metaplastic mucosa*. Pathobiology 2001;69:143–9.
43. Ye C, Shrubsole MJ, Cai Q, Ness R, Grady WM, Smalley W, et al. *Promoter methylation status of the MGMT, hMLH1, and CDKN2A/p16 genes in non-neoplastic mucosa of patients with and without colorectal adenomas*. Oncol Rep 2006;16:429–35.
44. Carvalho B, Pinto M, Cirnes L, Oliveira C, Machado JC, Suriano G, et al. *Concurrent hypermethylation of gene promoters*

is associated with a MSI-H phenotype and diploidy in gastric carcinomas. Eur J Cancer 2003;39:1222–7.

45. Bjornsson HT, Sigurdsson MI, Fallin MD, Irizarry RA, Aspelund T, Cui H, et al. *Intra-individual change over time in DNA methylation with familial clustering*. JAMA 2008;299:2877–83.
46. Heijmans BT, Kremer D, Tobi EW, Boomsma DI, Slagboom PE. *Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human IGF2/H19 locus*. Hum Mol Genet 2007;16:547–54.
47. Rein T, DePamphilis ML, Zorbas H. *Identifying 5-methylcytosine and related modifications in DNA genomes*. Nucleic Acids Res 1998;26:2255–64.
48. Chang HW, Ling GS, Wei WI, Yuen AP-W. *Smoking and drinking can induce p15 methylation in the upper aerodigestive tract of healthy individuals and patients with head and neck squamous cell carcinoma*. Cancer 2004;101:125–32.
49. Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki H, Yin J, et al. *Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability*. Cancer Res 1999;59:1090–5.
50. Guan H, Ji M, Hou P, Liu Z, Wang C, Shan Z, et al. *Hypermethylation of the DNA mismatch repair gene hMLH1 and its association with lymph node metastasis and T1799A BRAF mutation in patients with papillary thyroid cancer*. Cancer 2008;113:247–55.
51. Fang DC, Wang RQ, Yang SM, Yang JM, Liu HF, Peng GY, et al. *Mutation and methylation of hMLH1 in gastric carcinomas with microsatellite instability*. World J Gastroenterol 2003;9:655–9.
52. Rubio-Del-Campo A, Salinas-Sanchez AS, Sanchez-Sanchez F, Gimenez-Bachs JM, Donate-Moreno MJ, Pastor-Navarro H, et al. *Implications of mismatch repair genes hMLH1 and hMSH2 in patients with sporadic renal cell carcinoma*. BJU Int 2008;102:504–9.
53. Galm O, Wilop S, Reichelt J, Jost E, Gehbauer G, Herman JG, et al. *DNA methylation changes in multiple myeloma*. Leukemia 2004;18:1687–92.
54. Zhu S WA, Liu G, Sun Z, Jin F, Zhou Y and Xia Z. *DNA damages and changes of certain hepatic biochemical indexes in rats exposed to vinyl chloride monomer*. J Hyg Res 2004;33:273–5.
55. Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. *DNA methylation in white blood cells: Association with risk factors in epidemiologic studies*. Epigenetics 2011;6: 828–37.