Cancer Epidemiology 41 (2016) 122–128

Contents lists available at ScienceDirect

Cancer Epidemiology

The International Journal of Cancer Epidemiology, Detection, and Prevention

journal homepage: www.cancerepidemiology.net

Hypomethylation of repetitive elements in blood leukocyte DNA and risk of gastric lesions in a Chinese population

Duo Chen, Xin-ran Zhang, Yang Zhang, Lian Zhang, Jun-ling Ma, Wei-cheng You*, Kai-feng Pan*

Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Cancer Epidemiology, Peking University Cancer Hospital & Institute, Beijing 100142, China

ARTICLE INFO

Article history:
Received 22 December 2015
Received in revised form 6 February 2016
Accepted 9 February 2016
Available online xxx

Keywords:
Methylation
Repetitive elements
LINE-1
ALU
Sat2
Gastric lesions

ABSTRACT

Background: To explore the association between hypomethylation of repetitive elements (*LINE-1*,*Sat2,* and *ALU*) in blood leukocyte DNA and risks of gastric lesions, and development of gastric cancer (GC), a population-based study was conducted in a high-risk area of GC in China.
Materials: Methylation levels were determined by MethyLight in 902 subjects with various gastric lesions from two cohort studies at baseline and 276 subjects with long-term follow-up data.
Results: The frequency of *LINE-1* or *Sat2* hypomethylation was significantly increased in subjects with dysplasia (DYS) compared with superficial gastritis/chronic atrophic gastritis. The odds ratios (ORs) were 2.22 [95% confidence interval (CI): 1.45–3.40] for *LINE-1* and 1.58 (95% CI: 1.14–2.21) for *Sat2*. A dose–response pattern was found for the risk of DYS and *LINE-1* hypomethylation (P-trend < 0.001). Further stratified analysis indicated that the frequency of *LINE-1* or *Sat2* hypomethylation was higher in subjects with *Helicobacter pylori* infection. The ORs were 1.83 (95% CI: 1.12–2.99) for *LINE-1* and 1.44 (95% CI: 1.01–2.05) for *Sat2*. The follow-up data indicated that the risk of progression to GC was increased in intestinal metaplasia (IM) subjects with *LINE-1* hypomethylation (OR = 2.82; 95% CI: 1.17–6.77) or *Sat2* hypomethylation (OR = 2.78; 95% CI: 1.15–6.74). The risk of progression to GC was also increased in DYS subjects with *Sat2* hypomethylation (OR = 5.24; 95% CI: 2.00–13.74).
Conclusions: These findings suggest that hypomethylation of repetitive elements in blood leukocytes is associated with the risks of advanced gastric lesions and development of GC.

1. Introduction

Gastric cancer (GC) is the second most frequent cause of cancer death worldwide, including China [1,2]. GC is thought to develop from chronic gastritis, intestinal metaplasia (IM), dysplasia (DYS), and subsequently to GC. Our cohort study in a high-risk Chinese population revealed that the risk of GC was significantly increased by baseline histopathologic severity [3,4], indicating a multistep process of gastric carcinogenesis [5]. However, the roles of genetic and epigenetic changes from primary to advanced gastric lesions or GC need further investigation.

Genomic hypomethylation contributes to genomic instability [6–8], which is an early event during carcinogenesis, thus potentially leading to an increased risk of cancer. Genomic hypomethylation mainly results from hypomethylation of repetitive DNA elements which comprise 45% of the human genome [9]. Long interspersed nucleotide element-1 (*LINE-1*) and *ALU* are major constituents of repetitive DNA elements, constituting 17% and 11% of the human genome, respectively [9]. Satellite 2 (*Sat2*) DNA sequences are located as tandem repeats in the pericentromeric and juxtacentromeric heterochromatin of several chromosomes [10]. Demethylation in these repetitive elements can be used as surrogate markers of genome wide hypomethylation [11].

Several studies have demonstrated an association between *LINE-1* or *ALU* hypomethylation in cancer tissues and poor clinical outcomes [12,13], suggesting the potential predictive value of repetitive elements. However, hypomethylation of repetitive elements in blood leukocytes associated with cancer risk remained

Abbreviations: CAG, chronic atrophic gastritis; CI, confidence interval; DYS, dysplasia; GC, gastric cancer; *H. pylori*, *Helicobacter pylori*; IM, intestinal metaplasia; Ind DYS, indefinite dysplasia; OR, odds ratio; SG, superficial gastritis.

* Corresponding authors at: Key Laboratory of Carcinogenesis and Translational Research, Department of Cancer Epidemiology, Peking University Cancer Hospital & Institute, 52 Fu-cheng Road, Hai-dian District, Beijing 100142, China.
Fax: +86 1088 12243.
E-mail addresses: weichengyou@yahoo.com (W.-c. You), pankaifeng2002@yahoo.com (K.-f. Pan).

http://dx.doi.org/10.1016/j.canep.2016.02.004
1877-7821/

heterogeneous. Two recent meta-analyses indicated the inconsistency results in association of cancer risk and repetitive elements hypomethylation in blood leukocytes [14,15], while only a few studies examined the association between blood leukocyte genomic hypomethylation and risks of GC and its precursors.

In the present population-based study, we investigated hypomethylation of repetitive elements (*LINE-1, Sat2* and *ALU*) in blood leukocytes in association with risks of various precancerous gastric lesions and its progression to GC in a long-term follow-up cohorts in Linqu County, a high-risk area of GC in Shandong Province, China.

2. Materials and methods

2.1. Study population

Two cohort studies were conducted in Linqu County in 1994 (Cohort I, registered as NCI-OH-95-C-N029 in the U.S. National Cancer Institute PDQ database) and 2002 (Cohort II, registered as HARECCTR0500053 in accordance with WHO ICTRP requirements) [16,17]. Endoscopic screening was performed at baseline and followed the repeated endoscopic examinations at 1999, 2003 and 2009 for Cohort I, and 2006 for Cohort II, respectively. Briefly, for each subject, the biopsy specimens were taken from 5–7 standard sites of the stomach, and given the corresponding histopathological diagnosis by three senior pathologists independently from Peking University Cancer Hospital according to the Updated Sydney System [17] and Padova International Classification [18]. Each biopsy was given a diagnosis based on the most severe histology, and each subject was assigned a 'global' diagnosis based upon the most severe diagnosis among any of the biopsies. To standardize both cohorts, precancerous lesions were classified into superficial gastritis (SG), chronic atrophy gastritis (CAG), IM, and DYS. Information on gender, date of birth, cigarette smoking and alcohol drinking were obtained from the questionnaires of the two cohorts, respectively. The study was approved by the Institutional Review Board of Peking University Cancer Hospital, and all subjects gave written informed consent.

For the present study, a total of 902 subjects with a spectrum of SG, CAG, IM, and DYS were selected randomly from each pathology strata at baseline. Furthermore, 69 GC cases accrued from IM or DYS in two cohorts with blood samples collected at 1 to 10 years before diagnosis during the follow-up period were selected and 207 subjects who remained IM and DYS were further matched (1:3) with same gender- and *Helicobacter pylori* status as the references.

2.2. Assessment of H. pylori infection status

H. pylori status was determined by enzyme-linked immunosorbent assay (ELISA) in Cohort I and by ^{13}C-urea breath test (^{13}C-UBT) in Cohort II. Details of *H. pylori* antibody assay were described previously [19]. In brief, serum anti-*H. pylori* IgG and IgA levels were measured separately in duplicate with ELISA procedures. Quality control samples were assayed at Vanderbilt University, Nashville, TN. An individual was considered to be positive for *H. pylori* infection if the mean optical density for either the IgG or IgA > 1.0, a cut-off value based on *H. pylori* negative persons and the reference sera.

For ^{13}C-UBT, briefly, all participants were requested to drink 20 ml of water with a pill of 80 mg ^{13}C-urea (>99%). Breath samples were collected at baseline and 30 min after ingesting the pill. $^{13}CO_2$ values were determined using a gas isotopic ratio mass spectrometer. The ^{13}C-UBT was identified positive if any concentration of $^{13}CO_2$ at 30 min was over the baseline concentration more than 4 parts per 1000 (>0.4%) [20].

2.3. DNA preparation and sodium bisulfite conversion

All of the blood samples were collected at baseline and followed up period before the endoscopic process. High molecular weight genomic DNA was isolated by standard proteinase K digestion and phenol-chloroform extraction from 5 ml whole blood samples. DNA bisulfite treatment was carried out using the EZ DNA Methylation-Gold Kit™ (Zymo Research, CA, USA) according to the manufacturer's instruction. Briefly, blood DNAs (1 μg) denatured at 98 °C for 10 min were incubated with modification reagents for 2.5 h at 64 °C and cleaned and desulphonated. The DNA was stored immediately at −20 °C.

2.4. Methylation analysis

After sodium bisulfite conversion, genomic DNA was analyzed by the MethyLight technique as described previously [21,22]. In brief, DNA was PCR-amplified in a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA) using TaqManR Universal PCR Master Mix (Applied Biosystems) at the following conditions; 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1.5 min. Primers and probes for repetitive elements including *LINE*-1-M1, *Sat2*-M1, *ALU*-M2 and *ALU*-C4 were previously described [21,22]. Universal methylated DNA served as a methylated reference and *ALU*-C4 was used as a reference gene in control reaction to normalize the levels of input DNA for each methylation reaction.

Relative quantification was determined based on the threshold cycles of the target gene and the internal reference gene. Each MethyLight reaction was performed in duplicate. The percentage of methylation of repetitive elements was expressed as percent of methylated reference (PMR) values. PMR = $100\% \times 2^{-[\Delta Ct \text{ (target gene in sample} - \text{control gene in sample)} - \Delta Ct \text{ (100\% methylated target in reference sample} - \text{control gene in reference sample)}]}$. The amplification efficiencies of the target genes and reference gene must be approximately equal. This was examined using standard curves of the reference gene with 100-fold serial dilutions of CpGenome universal methylated and unmethylated DNAs. The MethyLight assay was further validated by using mixtures of fully methylated and unmethylated DNA to give 0, 1, 5, 10, 25, 50 or 100% methylation. The range of intra-assay coefficients of variation (CVs) was 1.5%.

2.5. Statistical analysis

We used the χ^2 test for categorical variables and Wilcoxon rank-sum test for continuous variables to assess the difference in selected characteristics between different groups. We utilized unconditional logistic regression model to calculate the ORs and 95% confidence intervals (CIs) for association of methylation with the risk of advanced gastric lesions (IM and DYS), adjusting for age, gender, *H. pylori* infection, smoking and drinking status. DNA methylation levels were assessed both as continuous measures and as quartiles based on the distribution among controls to accommodate possible non-linear associations. The highest quartile was used as the reference for risk estimation. Linear trend test was applied to evaluate the changing trend in risk for advanced gastric lesions with consecutive scores 1, 2, 3 and 4 assigned to the respective quartiles. All statistical analyses were carried out using Statistical Analysis System software (version 9.0; SAS Institute, Cary, NC). All statistical tests were two tailed, and the significance level was set at $P < 0.05$.

3. Results

A total of 902 subjects (449 males and 453 females) from the baseline of Cohort II was enrolled in our study including 63 subjects