Joint effects of WT1, CA10 methylation in peripheral blood leukocyte and dietary zinc on breast cancer risk: a case-control study

Anqi Ge  
Harbin Medical University School of Public Health

Song Gao  
Tumor Hospital of Harbin Medical University

Yupeng Liu  
Harbin Medical University School of Public Health

Hui Zhang  
Harbin Medical University School of Public Health

Xuan Wang  
Harbin Medical University School of Public Health

Lei Zhang  
Harbin Medical University School of Public Health

Da Pang  
Tumor Hospital of Harbin Medical University

Yashuang Zhao (zhao_yashuang@263.net)  
Harbin Medical University  https://orcid.org/0000-0002-7425-5773

Research article

Keywords: breast cancer, CA10, WT1, DNA methylation, leukocytes, zinc

DOI: https://doi.org/10.21203/rs.3.rs-27678/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License
Abstract

Background

Studies have shown that abnormal changes of specific-gene DNA methylation in leukocytes may be associated with elevated risk of cancer. However, associations between the methylation of zinc-related genes, *WT1* and *CA10*, and breast cancer risk, and interactions between *WT1*, *CA10* methylation and dietary zinc intake on breast cancer risk remain unknown.

Methods

The methylation of *WT1*, *CA10* was analyzed by methylation-sensitive high-resolution-melting (MS-HRM) in a case-control study with female subjects (N = 959). Logistic regression was used to analyze the associations and propensity score (PS) method was used to adjust confounders.

Results

The results showed that *WT1* hypermethylation was associated with an increased risk of breast cancer with an odds ratio (OR) of 3.069 [95% confidence interval (CI): 1.669–5.643, *P* < 0.001]. Subgroup analyses showed that *WT1* hypermethylation was specifically associated with an elevated risk of Luminal A subtype (OR = 2.620, 95%CI: 1.107–6.200, *P* = 0.029) and Luminal B subtype (OR = 3.231, 95%CI: 1.339–7.796, *P* = 0.009). *CA10* hypermethylation was associated with an increased risk of Luminal B subtype (OR = 1.798, 95%CI: 1.085–2.982, *P* = 0.023). Furthermore, the joint effects of methylation of *WT1*, *CA10* and lower dietary zinc intake were associated a strongly elevated risk of breast cancer with ORs of 11.220 (95%CI: 4.057–31.032, *P* < 0.001) and 4.145 (95%CI: 2.717–6.324, *P* < 0.001), respectively.

Conclusion

The study suggested the hypermethylation of *WT1* methylation in leukocytes was significantly associated with an increased risk of breast cancer. Notably, the joint effects of *WT1*, *CA10* methylation and lower dietary zinc intake might significantly associate with breast cancer risk.

Introduction

Breast cancer is one of the most common malignancies in women worldwide [1] and presents with different molecular subtypes, including luminal A, luminal B, HER2-enriched, and basal-like that also called triple negative [2]. As a major type of epigenetic modification, DNA methylation involves in regulating cellular processes, including chromosomal instability [3] and gene expression. Hypermethylation of CpG regions in specific genes contribute to neoplastic formation through the
transcriptional silencing of tumor suppressor genes. Aberrant patterns of specific gene methylation can help identifying differences in breast cancer subtypes [2], and showing promise for utilizing in large-scale epidemiological studies. It is suggested that leukocyte DNA methylation, as simple non-invasive blood markers [4, 5], could serve as a surrogate of systematic methylation activity, which offered great potential for predicting the increasing breast cancer risk [6].

Wilm’s Tumor gene (WT1) is tumor suppressor gene which involved in human cell growth and differentiation. WT1 have been reported significantly different methylated in the tissues of hepatocellular carcinoma [7], lung cancer [8] and breast cancer [9]. WT1 aberrant methylation may lead to the reduction or absence of expression of WT1, which results in the overexpression of the insulin-like growth factor I receptor (IGF 1R) and insulin-like growth factor II (IGF II), therefore promotes breast cancer process [10–12]. CA10 is a member of the carbonic anhydrase family, which is a large family of zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide and the dehydration of carbonic acid [13]. Ivanov et al. suggested that induction or enhancement of carbonic anhydrase expression may contribute to the tumor microenvironment by maintaining extracellular acidic pH and helping cancer cells grow and metastasize [14]. Studies have found the abnormal expression of carbonic anhydrase family by aberrant methylation was related with gastric cancer and metastasis of ovary tumors [15, 16]. Furthermore, Wojdacz et al. reported that both WT1 and CA10 hypermethylation were significantly different between breast cancer tumor tissues and non-malignant tissues [17]. However, the methylation of these two genes in leukocyte DNA affects breast cancer susceptibility remains unclear.

In recent years, nutri-epigenetics, the influence of dietary components on the epigenome, has emerged as an exciting new field [18]. While earlier studies have shown that high dietary zinc intake could decrease cancer risk [19, 20], the association of zinc intake and breast cancer risk remained controversial [21, 22]. Zinc deficiency may influence the function of zinc related genes, including changes the expression of WT1 in zinc deficiency animal model [23], and decreases blood carbonic anhydrase activity in males in a double-blind, randomized crossover study [24]. Hu et al. discovered that zinc deficiency increased BDNF methylation level in in vitro experiment [25]. However, studies have yet examined the associations between dietary intake of zinc and zinc related genes, WT1 and CA10, genes methylation in white blood cells with breast cancer risk.

In this study, we explored the associations between the methylation of WT1, CA10 in peripheral blood leukocyte DNA and breast cancer risk. We further used a dataset of nested case-control cohort within the EPIC-Italy cohort study as external data to validate the association between gene methylation and breast cancer risk. We also investigated the associations between the methylation of two genes and the risk of different molecular types of breast cancer. Furthermore, we examined the interaction and combination effects between methylation of the genes with dietary zinc intake on the risk of breast cancer.

**Materials And Methods**

**Study subjects**
We investigated the relationship between WT1, CA10 methylation and breast cancer risk using a case-control study. All included breast cancer patients were newly diagnosed female and were recruited from the Tumor Hospital of Harbin Medical University from 2010 to 2014. Controls were recruited from patients admitted to the Orthopedic and Ophthalmology Department of the Second Affiliated Hospital of Harbin Medical University and volunteers from the Xiangfang community of Harbin within the same period. All controls were also female. In addition, all control participants were asked about their disease history in the questionnaire and the ones with any cancer history were excluded from our final subjects. Finally, 402 female breast cancer cases and 557 female controls were included in our study. Informed written consent was provided by all the subjects, and 5 mL blood samples were collected and then stored at −80 °C. This study was approved by the Human Research and Ethics Committee of Harbin Medical University.

Data collection

All subjects were interviewed face-to-face by trained investigators with normalized questioning methods. The questionnaire was adopted from the study of Shu et al. [26], which included demographic information (age, ethnicity, and others); daily dietary intake (vegetables, fruits, beverages, and snacks); behaviors (smoking, drinking, physical activity and work activity); female-specific questions involving menstruation status, breast disease history (lobular hyperplasia, cyst, and others); gynecologic surgery history (hysterectomy, ovariotomy) and family history of cancer and breast cancer. The questions involved in dietary and behavioral were about the participants’ daily routine of one year prior to the interview. The daily dietary intake information were collected with Food Frequency Questionnaire contained 9 major food groups, which represented approximately 77 food items including Chinese staple foods, vegetables, fruits, dairy products, soybean products, meat products, egg, sea-food and fungus. The zinc intake derived from nutrient supplement was also included. The content of zinc in different foods were derived and calculated based on country specific food composition, Chinese Food Composition Table [27].

The study was validated with GEO-GSE51032 (IPEC-Italy cohort) dataset with nested case control study design to analyze the association between the methylation of CA10, WT1 and breast cancer risk in female. The blood samples were also collected and other anthropometric measurements were taken. The detailed criteria for sample selection and the methods were reported by Riboli et al. [28]. We extracted all 232 female breast cancer cases and all 340 female controls from this nested case-control study and located the methylation probes from Illumina 450 K array. The annotated CG sites covered by our MS-HRM sequence were illustrated in Fig. 1.

DNA extraction and bisulfite conversion

DNA was extracted from peripheral blood samples using a commercial DNA extraction kit (QIAamp DNA Blood Mini Kit, Hilden, Germany). The concentration and the purity of DNA were detected by Nanodrop 2000 Spectrophotometer (Thermo Scientific). Genomic DNA was bisulfite-modified with an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Bisulfite DNA was normalized at a concentration of 20 ng/mL and
was stored at −20 °C for the following experiment. DNA extraction and DNA sodium bisulfite modification were performed according to the manufacturer’s instructions.

**Gene methylation status analysis**

We adopted methylation-sensitive high-resolution melting analysis (MS-HRM) to evaluate the methylation of *WT1* and *CA10* with the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) equipped with Gene Scanning software (version 2.0). The primers were adopted from a published article [17]. We used universal methylated and unmethylated DNA standards (ZYMO, USA) and mixed them at different ratios to create standards with a 0.5%, 1%, 2%, and 5% methylation levels of *WT1* and *CA10*, respectively (Fig. 2). PCR amplification and MS-HRM were optimized and performed. The conditions, reaction mixture and primer sequences used in the MS-HRM experiments are listed in Table S1-2. Each standard reaction was performed in duplicate in each run. Each plate included duplicate water blanks as negative controls. We also repeated some samples in different runs to exam the consistency of the experiment. There was a significant agreement of these samples in different runs in detection of the methylation and unmethylation status of *WT1* and *CA10*, with kappa value of 1.000 (\(P = 0.001\)) and 0.936 (\(P = 0.001\)), respectively (Table S3).

**Definition of different molecular subtypes of breast cancer**

Four subtypes of breast cancer were defined as luminal A, luminal B, HER-2 enriched and triple negative breast cancer (TNBC) by immunohistochemical analysis based on previously validated clinicopathological criteria [29].

**Statistical analysis**

For the distribution of basic demographic characteristics, continuous variables such as age were analyzed by two-sample t-tests, and categorical variables were analyzed by chi-square (\(\chi^2\)) tests. For missing values in the environmental factors, we applied multiple imputation to generate possible values. To measure the association between methylation of *WT1*, *CA10* and breast cancer risk and different molecular types breast cancer, we used univariate and multivariate unconditional logistic regression analyses to estimate the crude and adjusted odds ratios (ORs) and 95% confidence intervals (95%CIs). For our case-control study, we used 0% methylation as cutoff for both *WT1* and *CA10*. We used ROC curves to calculate the cut-off value of \(\beta\) for validation dataset. Interactions or combination effects between zinc intake and methylation of genes on breast cancer risk were analyzed by multivariate logistic regression and crossover analysis. We also applied propensity score (PS) method to adjust covariates (involving all environmental factors in the questionnaire), in which the study outcome served as the dependent variable and PS served as the confounding variable. Kappa values were calculated to analyze the consistency between same samples in different runs. All two-sided \(P\) values < 0.05 were considered statistically significant. Data were analyzed by using the software SPSS v.24.0 (SPSS Inc., Chicago, IL, USA).
Results

Characteristics of the cases and controls

This study included 402 female cases with a mean age of 51.75 ± 9.39 and 557 female controls with a mean age of 51.85 ± 10.31. Other demographic information of the cases and controls is listed in Table 1.

| Characteristics               | No. of Controls(%) | No. of Cases (%) | P Value |
|-------------------------------|--------------------|------------------|---------|
| **Age**                       |                    |                  |         |
| Mean ± Std                    | 51.85 ± 10.31      | 51.75 ± 9.39     |         |
| < 40                          | 82(14.7)           | 41(10.2)         | 0.024   |
| 40-                           | 333(59.8)          | 274(68.2)        |         |
| ≥ 60                          | 142(25.5)          | 87(21.6)         |         |
| **BMI**                       |                    |                  |         |
| ≤ 18.5                        | 35(6.3)            | 14(3.5)          | 0.115   |
| 18.5-                         | 274(49.2)          | 211(52.5)        |         |
| ≥ 24.0                        | 248(44.5)          | 177(44.0)        |         |
| **Urban and Rural Status**    |                    |                  |         |
| Rural                         | 236(42.4)          | 232(57.7)        | <0.001  |
| Urban                         | 321(57.6)          | 170(42.3)        |         |
| **Education Level**           |                    |                  |         |
| Primary School or Below       | 162(29.1)          | 98(24.4)         | 0.270   |
| Middle School                 | 175(31.4)          | 135(33.6)        |         |
| Senior School and Higher      | 220(39.5)          | 169(42.0)        |         |
| **Occupation Type**           |                    |                  |         |
| White Collar                  | 273(49.0)          | 233(58.2)        | 0.007   |
| Blue Collar                   | 284(51.0)          | 169(41.8)        |         |
| **Ethnicity**                 |                    |                  |         |
| Han                           | 529(95.0)          | 386(96.0)        | 0.273   |
| Other                         | 28(5.0)            | 16(4.0)          |         |

Associations between WT1, CA10 methylation and breast cancer risk
WT1 methylation was associated with breast cancer risk both in multivariate and PS adjusted methods with ORs of 2.420 (95%CI: 1.449–4.040, \( P < 0.001 \)) and 3.069 (95%CI: 1.669–5.643, \( P < 0.001 \)), respectively. CA10 methylation was statistically significant associated with breast cancer in multivariate adjustment with an OR of 1.531 (95%CI: 1.144–2.051, \( P = 0.004 \)), but only marginally associated with breast cancer with an OR of 1.354 (95%CI: 0.967–1.896, \( P = 0.078 \)) after the adjustment of PS (Table 2).
Table 2
The associations between gene methylation and risk of breast cancer and different molecular types of breast cancer.

| Molar types | No. of Unmethylation(%) | No. of Methylation(%) | Crude OR (95% CI) | P Value | OR_{adj} (95% CI) | P Value | OR_{adj} (95% CI) | P Value |
|-------------|-------------------------|-----------------------|-------------------|---------|-------------------|---------|-------------------|---------|
| WT1 Control | 65(11.7) | 492(8.3) | 1.990 (0.937-4.226) | 0.073 | 2.605 (1.183-5.736) | 0.018 | 2.620 (1.107-6.200) | 0.029 |
| Luminal A   | 9(6.4) | 132(9.3) | 2.121 (1.504-2.990) | 0.066 | 2.491 (1.126-5.507) | 0.024 | 3.231 (1.339-7.796) | 0.009 |
| Luminal B   | 8(6.0) | 125(94.0) | 1.341 (0.514-3.499) | 0.549 | 1.910 (0.688-5.303) | 0.214 | 1.913 (0.664-5.512) | 0.230 |
| HER-2 Enriched | 5(8.9) | 51(91.1) | 4.338 (0.582-32.331) | 0.152 | 5.627 (0.726-43.626) | 0.098 | 6.044 (0.763-47.897) | 0.088 |
| TNBC        | 1(2.9) | 33(27.1) | 1.921 (1.178-3.132) | 0.009 | 2.420 (1.449-4.040) | 0.001 | 3.069 (1.669-5.643) | <0.001 |
| All cases   | 26(6.5) | 376(93.5) | 1.921 (1.178-3.132) | 0.009 | 2.420 (1.449-4.040) | 0.001 | 3.069 (1.669-5.643) | <0.001 |

\(a\) The result excluded 38 breast cancer patients with incomplete immunohistochemical records.

\(b\) Adjusted for age, BMI, ethnicity, urban and rural status and family history of breast cancer and cancer.

\(c\) Adjusted by propensity score (potential confounder included age, BMI, urban and rural status, ethnicity, education level, mammography, gynecologic surgery, breast disease history, menstrual cycle, menopause, reproduction, abortion, breast feeding, oral contraceptive, female hormone intake, fruit intake, vegetable intake, tomato intake, broccoli intake, bean products, pungent food, pork, beef and lamb consumption, chicken consumption, sea-fish, egg, diary, fungus, pickles, alcohol consumption, tea consumption, cigarette, physical activity, occupation type, family history of breast cancer and cancer).
| Molecular types | Control | Luminal A | Luminal B | HER-2 Enriched | TNBC | All cases |
|-----------------|---------|-----------|-----------|----------------|-------|-----------|
| CA10            | 209 (37.5) | 101(17.6) | 99(74.4)  | 38(67.9)       | 14(41.1) | 119 (29.6) |
| No. of Unmethylation(%) | 348(62.5) | 40(28.4) | 34(25.6)  | 38(67.9)       | 20(58.8) | 283(70.4)  |
| No. of Methylation(%) | 1 | 1.506 (1.004–2.259) | 1.787 (1.165–2.742) | 1.270 (0.706–2.286) | 0.860 (0.425–1.740) | 1.427 (1.084–1.878) |
| Crude OR (95% CI) | 1 | 1.598 (1.042–2.451) | 2.043 (1.301–3.211) | 1.424 (0.762–2.662) | 0.939 (0.450–1.955) | 1.531 (1.144–2.051) |
| P Value | 0.048 | 0.008 | 0.002 | 0.425 | 0.674 | 0.011 |
| OR adjusted b (95% CI) | 0.032 | 1.505 (0.939–2.410) | 1.798 (1.085–2.982) | 1.366 (0.708–2.634) | 0.865 (0.461–2.198) | 1.006 (0.461–2.198) |
| P Value | 0.089 | 0.023 | 0.353 | 0.268 | 0.988 | 0.078 |
| OR adjusted c (95% CI) | 1.505 (0.939–2.410) | 1.798 (1.085–2.982) | 1.366 (0.708–2.634) | 1.354 (0.967–1.896) | 0.004 | 1.354 (0.967–1.896) |
| P Value | 0.089 | 0.023 | 0.353 | 0.268 | 0.988 | 0.078 |

a The result excluded 38 breast cancer patients with incomplete immunohistochemical records.

b Adjusted for age, BMI, ethnicity, urban and rural status and family history of breast cancer and cancer.

c Adjusted by propensity score(potential confounder included age, BMI, urban and rural status, ethnicity, education level, mammography, gynecologic surgery, breast disease history, menstrual cycle, menopause, reproduction, abortion, breast feeding, oral contraceptive, female hormone intake, fruit intake, vegetable intake, tomato intake, broccoli intake, bean products, pungent food, pork, beef and lamb consumption, chicken consumption, sea-fish, egg, diary, fungus, pickles, alcohol consumption, tea consumption, cigarette, physical activity, occupation type, family history of breast cancer and cancer).

In the subgroup analyses, after the adjustment of PS, WT1 methylation was associated with breast cancer risk in both younger (< 60-years-old) and older (≥ 60-years-old) group, with ORs of 2.640 (95% CI: 1.311–5.316, *P* = 0.007) and 4.720 (95% CI: 1.312–16.974, *P* = 0.007), respectively. CA10 methylation was
associated with breast cancer risk in younger age group (< 60-years-old) before the adjustment of PS, with OR of 1.556 (95%CI: 1.145–2.114, \( P = 0.005 \)); However, the association was not statistically significant after PS adjustment (Table 3).

### Table 3
The subgroup analysis of the associations between methylation of genes and the risk of breast cancer based on different age.

|               | Crude OR (95% CI) | \( P \) Value | OR adjusted \( ^a \) (95% CI) | \( P \) Value |
|---------------|-------------------|---------------|--------------------------------|---------------|
| \( WT1 \)    |                   |               |                                |               |
| \( \geq 60 \) | Unmethylation     | 1             | 1                              |               |
|               | Methylati on      | 1.639 (0.945–2.843) | 0.079                          | 2.640 (1.311–5.316) | 0.007 |
| \( \geq 60 \) | Unmethylation     | 1             | 1                              |               |
|               | Methylati on      | 3.155 (1.048–9.495) | 0.041                          | 4.720 (1.312–16.974) | 0.018 |
| \( CA10 \)   |                   |               |                                |               |
| \( \geq 60 \) | Unmethylation     | 1             | 1                              |               |
|               | Methylati on      | 1.556 (1.145–2114) | 0.05                           | 1.327 (0.900–1.956) | 0.153 |
| \( \geq 60 \) | Unmethylation     | 1             | 1                              |               |
|               | Methylati on      | 1.201 (0.608–2.372) | 0.598                          | 1.527 (0.692–3.371) | 0.295 |

\( ^a \) Adjusted by propensity score.

**Associations between methylation of \( WT1 \), \( CA10 \) and risk of different molecular types of breast cancer**

\( WT1 \) methylation was significantly associated with the risk of Luminal A subtype of breast cancer with multivariate adjusted OR of 2.605 (95%CI: 1.183–5.736, \( P = 0.018 \)), and PS adjusted OR of 2.620 (95%CI: 1.107–6.200, \( P = 0.029 \)). \( WT1 \) methylation was also significantly associated with the risk of Luminal B subtype breast cancer with ORs of 2.491 (95%CI: 1.126–5.507, \( P = 0.024 \)) and 3.231 (95%CI: 1.339–7.796,
\( P=0.009 \) after multivariate and PS adjustment. However, \( WT1 \) methylation was not significantly associated with the risk of HER-2 enriched and TNBC subtypes (Table 2).

The associations between \( CA10 \) methylation and the risk of Luminal B subtype breast cancer with multivariate adjusted and PS adjusted ORs were 2.043 (95%CI: 1.301-3.211, \( P=0.003 \)) and 1.798 (95%CI: 1.085-2.982, \( P=0.023 \)), respectively. However, \( CA10 \) methylation had no significant associations with the risk of Luminal A, HER-2 enriched and TNBC subtypes after the adjustment of PS.

**Association between WT1, CA10 methylation and breast cancer risk in GEO dataset**

GSE51032 dataset is a nested case control study included 233 female breast cancer cases and 340 female cancer-free controls. After the data extraction from 450K array, we found two CG locus each in our targeted \( WT1 \) and \( CA10 \) sequences (Fig. 1). ROC curves were used to calculate the cut-off values of \( \beta \), which were 0.057 and 0.226 for average \( \beta \) of probes in \( WT1 \) and \( CA10 \). The average methylation level of Cg14657517 and Cg19074340, which located within the \( WT1 \) targeted sequence, was associated with breast cancer with OR of 1.879 (95%CI: 1.247–2.830, \( P=0.03 \)). However, the average methylation level of Cg14054928 and Cg20405017, which located within the targeted sequence of \( CA10 \), was not statistically significant associated breast cancer risk (OR = 0.759, 95%CI: 0.543–1.061, \( P=0.107 \)) (Table 4).

| Hypomethylation(%) | Hypermethylation(%) | Crude OR (95% CI) | \( P \) Value |
|---------------------|---------------------|-------------------|--------------|
| **WT1**             |                     |                   |              |
| Control             | 285(83.8)           | 55(16.2)          | 1            |
| Case                | 171(73.4)           | 62(26.6)          | 1.879(1.247–2.830) | 0.03 |
| **CA10**            |                     |                   |              |
| Control             | 146(42.9)           | 194(57.1)         | 1            |
| Case                | 116(49.8)           | 117(50.2)         | 0.759(0.543–1.061) | 0.107 |

**Associations of zinc intake and its interaction with methylation of WT1, CA10 on the risk of breast cancer**
Low zinc intake (< 41.81 mg/week) was significantly associated with breast cancer risk with an adjusted OR of 2.321 (95%CI: 1.747–3.083, \(P<0.001\)) (Table S4). No significant interactions between total zinc intake and \(WT1\), \(CA10\) methylations on breast cancer risk were observed. The combination effects of lower level of total zinc intake and \(WT1\), \(CA10\) methylation on breast cancer risk were strongly significant, with ORs of 11.220 (95%CI: 4.057–31.032, \(P<0.001\)) and 4.145 (95%CI: 2.717–6.324, \(P<0.001\)), respectively (Table 5).

### Table 5

| Total Zinc Intake(mg/week) | Interaction | \(P\) |
|---------------------------|-------------|-------|
| \(\geq 41.81\) | \(< 41.81\) | \(OR_{\text{adjusted}}^\dagger (95\% \text{ CI})\) | \(OR_{\text{adjusted}}^\dagger (95\% \text{ CI})\) |
| Unmethylation | 1 | 5.227(1.516–18.029) | 1 |
| Methylation | 3.985(1.406–11.298) | 11.220(4.057–31.032) | 0.539(0.147–1.974) | 0.346 |
| \(WT1\) | | | |
| Unmethylation | 1 | 2.969(1.825–4.829) | 1 |
| Methylation | 1.488(0.961–2.305) | 4.145(2.717–6.324) | 0.938(0.519–1.695) | 0.833 |
| \(CA10\) | | | |

† Adjusted for age, BMI, ethnicity, urban and rural status and family history of breast cancer and cancer.

### Discussion

This is the first case-control study to investigate the associations between the methylation of \(WT1\), \(CA10\) in leukocyte DNA and breast cancer risk, and the risk of different molecular types of breast cancer in a Chinese female population. After PS adjustment, we found that methylation of \(WT1\) was significantly elevated breast cancer risk by 2.069-fold, \(CA10\) methylation was marginally associated with breast cancer risk with OR of 1.354. Women with \(WT1\) methylation presented a 1.620 higher risk of Luminal A and 2.231 higher risk of Luminal B subtype of breast cancer than those without methylation. \(CA10\) methylation was significantly associated with the risk of Luminal B subtype with OR of 1.798. We further used GEO-GSE51032, a nested case control study with clear temporal relationship between methylation changes and breast cancer, as an external dataset to validate our retrospective study’s results. The nested case control study’s results showed a lower but still significant association between \(WT1\) methylation and breast cancer risk, but the association between \(CA10\) methylation and breast cancer risk was not statistically significant. Furthermore, we found that low level of dietary zinc intake combined with \(WT1\), \(CA10\) methylation was strongly associated with breast cancer risk.
Breast cancer is a heterogeneous disease with different molecular subtypes, which may present different genetic and epigenetic susceptibilities. Previous studies predominantly focused on the aberrant methylation in tissue samples and its association with the risk of different molecular types of breast cancer [30, 31], few studies have focused on the gene-specific methylation in leukocyte DNA. The methylation alternation in leukocyte DNA presented a response of the hematopoietic system [32]. Leukocyte DNA methylation could represent germline methylation, which can be applied to analyze the association with cancer risk [33]. It was further reported that BRCA1 hypermethylation in peripheral blood DNA was associated with TNBC with an OR of 5.0 [34]. Our study indicated that, after adjustment of PS, WT1 methylation was associated with the risk of Luminal A and Luminal B subtype of breast cancer with ORs of 2.620 and 3.231, and CA10 methylation was significantly associated with Luminal B subtype of breast cancer with OR of 1.798.

WT1 is a zinc finger transcription factor located on 11p13, which was first identified as a tumor suppressor gene. WT1 exon displayed significantly increased methylation in cancer tissue compared to nonmalignant breast tissue [17]. WT1 methylation in the promoter and first exon region was associated with silencing WT1 mRNA expression in MCF-7 and MDA-MB-231 breast cancer cells [9]. Our investigated sequence was 160 bp downstream of the Laux et al. sequence position. Here, we reported the methylation of the CpG island in the first exon of WT1 in blood leukocyte DNA, which contains 11 CpGs in CpG island. Furthermore, we used external data of IPEC- Italy (GEO-GSE51032) with nested case control study design and found the significant association between WT1 methylation and breast cancer risk, with two CpG probes inside our sequence, with OR of 1.879.

Previous study has shown CA10 could undergo methylation during breast carcinogenesis in tumor tissue [17]. CA10 was reported to be hypermethylated among a panel of genes in urine, which may contribute to highly accurate early detection of bladder cancer [35]. Our study suggested that CA10 methylation in leukocyte DNA was marginally associated with an elevated breast cancer risk after the adjustment of PS. The amplified sequence contained 7 CpGs and located at the second exon of CA10. The external validation dataset of GEO-GSE51032 only included 2 CpG probes and did not observe statistically significant association between WT1 methylation and breast cancer risk.

To further investigate the functional relevance of the observed associations, it would be important to test whether methylation in the CpGs associated with the alteration of the expression of WT1 and CA10. Therefore, we investigated the correlations between methylation probes and expression using TCGA (http://cancergenome.nih.gov/) and Mexpress (https://mexpress.be/) The result showed that WT1 hypermethylation was negatively correlated with its expression (Cg14657517, r=-0.204, P<0.001; Cg19074340, r=-0.201, P<0.001), and CA10 hypermethylation was negatively related to its mRNA expression as well (Cg14054928, r=-0.182, P<0.001; Cg20405017, r=-0.162, P<0.001). Although discounted by different sample-derived DNA, the significant negative correlations between WT1, CA10 methylation and gene expression were consistent with our study and indicated promising potential in breast cancer risk assessment.
Recently, more researchers have focused on nutri-epigenetics, which refers to the study of nutrient with any genetic or epigenetic interaction that leads to phenotypic changes [36]. Zinc is a trace mineral that is vital for numerous cellular processes and may play an important role in cancer etiology [37]. Zinc is also an essential component of DNA-binding proteins with zinc fingers, such as \( WT1 \) [23], and carbonic anhydrase function might be influenced by the concentration of erythrocyte Zn [38]. Studies on dietary zinc intake showed that high zinc intake could reduce the risk of various cancers\([20, 39, 40]\), including breast cancer [40]. Previous studies also reported that zinc deficiency in dietary might be associated with increasing level of specific gene methylation [25, 41]. In this study, we first investigated if dietary zinc intake interacted with DNA methylation to affect the risk of breast cancer. Although we did not observe statistically significant interactions between lower dietary zinc intake and \( WT1, CA10 \) methylation on breast cancer risk, we did find that lower zinc intake combined with methylation of \( WT1, CA10 \) significantly increased the breast cancer risk by 10.220-fold and 3.145-fold, respectively.

Our previous study have tested the accuracy of MS-HRM by detecting the \( WT1 \) methylation level with both MS-HRM and pyrosequencing, and the results were highly correlated between these two methods [42]. However, the methylation level of leukocyte DNA is relatively low and the limitation of pyrosequencing is 2%. As a reliable and high sensitive technique, MS-HRM can assess the methylation level of targeted CpGs as low as 0.1% [43]. The high consistency of our test for different runs which making the non-misclassification of methylation level between case and control and the probability of higher sensitivity of MS-HRM comparing pyrosequencing can make our study result more conserved [44].

Limitations should be taken into consideration before drawing a conclusion: first, as in all retrospective analyses, our study may have some recall bias when collecting information on environmental factors including the intake of zinc. Second, the sample size of our study is not large enough for subgroup analysis, including the subgroup analyses of low frequency environmental factors, like smoking behavior, therefore their associations with DNA methylation of \( WT1, CA10 \) could not be analyzed.

In summary, our study suggested that methylation of \( WT1 \) and \( CA10 \) in blood leukocytes may associate with the risk of breast cancer. There were also associations between \( WT1 \) methylation and risk of Luminal subtypes of breast cancer; \( CA10 \) methylation and risk of Luminal B subtype of breast cancer. Lower level of dietary zinc intake combined with methylation of \( WT1, CA10 \) may be associated with breast cancer risk.

**Abbreviations**

\( WT1 \): Wilm's Tumor 1; \( CA10 \):Carbonic Anhydrase 10; MS-HRM:Methylation-Sensitive High-Resolution Melting; TNBC:Triple Negative Breast Cancer; CIs:Confidence Intervals; OR:Odds Ratio; ER:Estrogen Receptor; PR:Progesterone Receptor; HER-2:Human Epidermal growth factor Receptor-2; BMI:Body Mass Index

**Declarations**
Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interests
The authors declare that they have no competing interests

Funding
This study was supported by the National Natural Science Foundation of China (Yashuang Zhao, grant no. 81172743)

Authors’ contributions
GA and GS have been involved in drafting the manuscript. LY and ZH performed subsequent data analysis. WX, ZL and above researchers together completed experiment part of this research. Dr. ZY and Dr. PD revised the manuscript for important intellectual content.

Acknowledgements
The authors thank all the patients and healthy volunteers for providing blood samples and all the research staff for their contributions to this project.

References
1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.
2. Kamalakaran S, Varadan V, Giercksky Russnes HE, Levy D, Kendall J, Janevski A, Riggs M, Banerjee N, Synnestvedt M, Schlichting E. DNA methylation patterns in luminal breast cancers differ from non-luminal subtypes and can identify relapse risk independent of other clinical variables. Mol Oncol. 2011;5(1):77–92.

3. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet. 2007;8(4):286–98.

4. Kang HJ, Kim JM, Kim SY, Kim SW, Shin IS, Kim HR, Park MH, Shin MG, Yoon JH, Yoon JS. A Longitudinal Study of BDNF Promoter Methylation and Depression in Breast Cancer. Psychiatry Investigation. 2015;12(4):523–31.

5. Woo HD, Kim J. Global DNA Hypomethylation in Peripheral Blood Leukocytes as a Biomarker for Cancer Risk: A Meta-Analysis. Plos One. 2012;7(4):e34615.

6. Guan Z, Yu H, Cuk K, Zhang Y, Brenner H. Whole-Blood DNA Methylation Markers in Early Detection of Breast Cancer: A Systematic Literature Review. Cancer Epidemiol Biomarkers Prev. 2019;28(3):496–505.

7. Mžik M, Chmelařová M, John S, Laco J, Slabý O, Kiss I, Bohovicová L, Palička V, Nekvindová J. Aberrant methylation of tumour suppressor genes WT1, GATA5 and PAX5 in hepatocellular carcinoma. Clinical Chemistry Laboratory Medicine. 2016;54(12):1971–80.

8. Bruno P, Gentile G, Mancini R, Vitis CD, Esposito MC, Scozzi D, Mastrangelo M, Ricci A, Mohsen I, Ciliberto G. WT1 CpG islands methylation in human lung cancer: A pilot study. Biochemical Biophysical Research Communications. 2012;426(3):306–9.

9. Laux DE, Curran EM, Welshons WV, Lubahn DB, Huang THM. Hypermethylation of the Wilms’ tumor suppressor gene CpG island in human breast carcinomas. Breast Cancer Research Treatment. 1999;56(1):35–43.

10. Werner H, Re GG, Drummond IA, Sukhatme VP, Rd RF, Sens DA, Garvin AJ, Leroith D Jr. RC: Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. Proc Natl Acad Sci USA. 1993;90(12):5828–32.

11. Paik S. Expression of IGF-I and IGF-II mRNA in breast tissue. Breast Cancer Research Treatment. 1992;22(1):31–8.

12. Silberstein GB, Horn KV, Strickland P, Roberts CT, Daniel CW. Altered expression of the WT1 Wilms tumor suppressor gene in human breast cancer. Proc Natl Acad Sci USA. 1997;94(15):8132–7.

13. Nakamura J, Kitajima Y, Kai K, Hashiguchi K, Hiraki M, Noshiro H, Miyazaki K. Expression of Hypoxic Marker CA IX Is Regulated by Site-Specific DNA Methylation and Is Associated with the Histology of Gastric Cancer. Am J Pathol. 2011;178(2):515.

14. Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, et al. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol. 2001;158(3):905–19.
15. Nakamura J, Kitajima Y, Kai K, Hashiguchi K, Hiraki M, Noshiro H, Miyazaki K. Expression of hypoxic marker CA IX is regulated by site-specific DNA methylation and is associated with the histology of gastric cancer. Am J Pathol. 2011;178(2):515–24.

16. Sung HY, Ju W, Ahn JH. DNA hypomethylation-mediated overexpression of carbonic anhydrase 9 induces an aggressive phenotype in ovarian cancer cells. Yonsei Med J. 2014;55(6):1656–63.

17. Wojdacz TK, Windeløv JA, Thestrup BB, Damsgaard TE, Overgaard J, Hansen LL. Identification and characterization of locus-specific methylation patterns within novel loci undergoing hypermethylation during breast cancer pathogenesis. Breast Cancer Res. 2014;16(1):R17.

18. Gerhauser C. Cancer Chemoprevention and Nutri-Epigenetics: State of the Art and Future Challenges. Top Curr Chem. 2013;329(5):73.

19. Alder H, Taccioli C, Chen H, Jiang Y, Smalley KJ, Fadda P, Ozer HG, Huebner K, Farber JL, Croce CM. Dysregulation of miR-31 and miR-21 induced by zinc deficiency promotes esophageal cancer. Carcinogenesis. 2012;33(9):1736–44.

20. Muka T, Kraja B, Ruiter R, Lahousse L, de Keyser CE, Hofman A, Franco OH, Brusselle G, Stricker BH, Kieft de Jong JC. Dietary mineral intake and lung cancer risk: the Rotterdam Study. Eur J Nutr. 2017;56(4):1637–46.

21. Lin YS, Caffrey JL, Lin JW, Bayliss D, Faramawi MF, Bateson TF, Sonawane B. Increased risk of cancer mortality associated with cadmium exposures in older Americans with low zinc intake. J Toxicol Environ Health A. 2013;76(1):1–15.

22. Lee E, Levine EA, Franco VL, Allen GO, Gong F, Zhang Y, Hu JJ. Combined genetic and nutritional risk models of triple negative breast cancer. Nutrition Cancer. 2014;66(6):955–63.

23. Zheng D, Kille P, Feeney GP, Cunningham P, Handy RD, Hogstrand C. Dynamic transcriptomic profiles of zebrafish gills in response to zinc supplementation. Bmc Genomics. 2010;157(1):553.

24. Lukaski HC. Low dietary zinc decreases erythrocyte carbonic anhydrase activities and impairs cardiorespiratory function in men during exercise. Am J Clin Nutr. 2005;81(5):1045–51.

25. Hu YD, Pang W, He CC, Lu H, Liu W, Wang ZY, Liu YQ, Huang CY, Jiang YG. The cognitive impairment induced by zinc deficiency in rats aged 0–2 months related to BDNF DNA methylation changes in the hippocampus. Nutritional Neuroscience 2016:1.

26. Shu XO, Yang G, Jin F, Liu D, Kushi L, Wen W, Gao YT, Zheng W. Validity and reproducibility of the food frequency questionnaire used in the Shanghai Women’s Health Study. Eur J Clin Nutr. 2004;58(1):17–23.

27. Yang YX, Wang GY, Pan XC. Chinese food composition table. 2nd ed. Beijing: Medical Publishing House of Peking University; 2010.

28. Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, Charrondiere UR, Hemon B, Casagrande C, Vignat J, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr. 2002;5(6B):1113–24.

29. Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, Thurlimann B, Senn HJ, Panel M. Tailoring therapies—improving the management of early breast cancer. St Gallen
International Expert Consensus on the Primary Therapy of Early Breast Cancer 2015. Ann Oncol. 2015;26(8):1533–46.

30. Conway K, Edmiston SN, May R, Pei FK, Chu H, Bryant C, Tse CK, Swift-Scanlan T, Geradts J, Troester MA. DNA methylation profiling in the Carolina Breast Cancer Study defines cancer subclasses differing in clinicopathologic characteristics and survival. Breast Cancer Research Bcr. 2014;16(5):450.

31. Holm K, Hegardt C, Staaf J, Vallonchristersson J, Jönsson G, Olsson H, Borg Å, Ringnér M. Molecular subtypes of breast cancer are associated with characteristic DNA methylation patterns. Breast Cancer Research: BCR. 2010;12(3):R36.

32. Koestler DC, Marsit CJ, Christensen BC, Accomando W, Langevin SM, Houseman EA, Nelson HH, Karagas MR, Wiencke JK, Kelsey KT. Peripheral blood immune cell methylation profiles are associated with nonhematopoietic cancers. Cancer Epidemiol Biomarkers Prev. 2012;21(8):1293–302.

33. Wang Y, Li D, Li X, Teng C, Zhu L, Cui B, Zhao Y, Hu F. Prognostic significance of hMLH1/hMSH2 gene mutations and hMLH1 promoter methylation in sporadic colorectal cancer. Med Oncol. 2014;31(7):39.

34. Gupta S, Jaworska-Bieniek K, Narod SA, Lubinski J, Wojdacz TK, Jakubowska A. Methylation of the BRCA1 promoter in peripheral blood DNA is associated with triple-negative and medullary breast cancer. Breast Cancer Research Treatment. 2014;148(3):615–22.

35. Chung W, Bondaruk J, Jelinek J, Lotan Y, Liang S, Czerniak B, Issa JP. Detection of bladder cancer using novel DNA methylation biomarkers in urine sediments. Cancer epidemiology biomarkers prevention. 2011;20(7):1483.

36. Supic G, Jagodic M, Magic Z. Epigenetics: a new link between nutrition and cancer. Nutrition Cancer. 2013;65(6):781–92.

37. Grattan BJ, Freake HC. Zinc and Cancer: Implications for LIV-1 in Breast Cancer. Nutrients. 2012;4(7):648–75.

38. Evans DM, Zhu G, Dy V, Heath AC, Madden PA, Kemp JP, McMahon G, St Pourcain B, Timpson NJ, Golding J, et al. Genome-wide association study identifies loci affecting blood copper, selenium and zinc. Hum Mol Genet. 2013;22(19):3998–4006.

39. Hashemian M, Poustchi H, Abnet CC, Boffetta P, Dawsey SM, Brennan PJ, Pharohah P, Etemadi A, Kamangar F, Sharafkhah M. Dietary intake of minerals and risk of esophageal squamous cell carcinoma: results from the Golestan Cohort Study. Am J Clin Nutr. 2015;102(1):102–8.

40. Tinoco-Veras CM, Bezerra Sousa MS, Da SB, Franciscato Cozzolino SM, Viana PL, Coelho Pimentel JA, Do N-NN. Do N-MD: Analysis of plasma and erythrocyte zinc levels in premenopausal women with breast cancer. Nutrición Hospitalaria. 2011;26(2):293–7.

41. Kurita H, Ohsako S, Hashimoto S, Yoshinaga J, Tohyama C. Prenatal zinc deficiency-dependent epigenetic alterations of mouse metallothionein-2 gene. J Nutr Biochem. 2013;24(1):256–66.
42. Gao HL, Wang X, Sun HR, Zhou JD, Lin SQ, Xing YH, Zhu L, Zhou HB, Zhao YS, Chi Q. Methylation Status of Transcriptional Modulatory Genes Associated with Colorectal Cancer in Northeast China. *Gut & Liver* 2018, 12(2).

43. Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* 2007;35(6):e41.

44. Copeland KT, Checkoway H, McMichael AJ, Holbrook RH. Bias due to misclassification in the estimation of relative risk. *Am J Epidemiol.* 1977;105(5):488–95.

**Figures**

![Figure 1](image)

**Figure 1**

MS-HRM amplified sequence of WT1 and CA10 and the validated Cg sites in GSE51032
Figure 2

The MS-HRM based method for WT1 and CA10 methylation detection. The figures showed normalized melting curves and melting peaks for standards methylation level and of WT1(A)(B) and CA10(C)(D). The methylation status of the standards were 0%, 0.5%, 1%, 2%, 5%, 100%, respectively

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1.docx
- AdditionalFile3.docx
- AdditionalFile2.docx
- AdditionalFile4.docx