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Are dietary factors involved in the association of *CDH4* methylation and breast cancer risk?

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Abstract

DNA methylation is one of the most important epigenetic modifications in breast cancer (BC) development, and long-term dietary habits can alter DNA methylation. Cadherin-4 (*CDH4*, a member of the cadherin family) encodes Ca^{2+}-dependent cell–cell adhesion glycoproteins. We conducted a case–control study (380 newly diagnosed BC and 439 cancer-free controls) to explore the relationship of *CDH4* methylation in peripheral blood leukocyte DNA (PBL DNA), as well as its combined and interactive effects with dietary factors on BC risk. A case-only study (335 newly diagnosed BC) was conducted to analyse the association between *CDH4* methylation in breast tissue DNA and dietary factors. *CDH4* methylation was detected using quantitative methylation-specific PCR. Unconditional logistic regressions were used to analyse the association of *CDH4* methylation in PBL DNA and BC risk. Cross-over analysis and unconditional logistic regression were used to calculate the combined and interactive effects between *CDH4* methylation in PBL DNA and dietary factors in BC. *CDH4* hypermethylation was significantly associated with increased BC risk in PBL DNA ($OR_{adjusted}$ (OR_{adj}) = 2·70, (95 % CI 1·90, 3·83), $P < 0·001$). *CDH4* hypermethylation also showed significant combined effects with the consumption of vegetables (OR_{adj} = 4·33, (95 % CI 2·63, 7·10)), allium vegetables (OR_{adj} = 7·00, (95 % CI 4·17, 11·77)), fish (OR_{adj} = 7·92, (95 % CI 3·79, 16·53)), milk (OR_{adj} = 6·30, (95 % CI 3·41, 11·66)), overnight food (OR_{adj} = 4·63, (95 % CI 2·69, 7·99)), pork (OR_{adj} = 5·59, (95 % CI 2·94, 10·62)) and physical activity (OR_{adj} = 4·72, (95 % CI 2·87, 7·76)). Moreover, consuming milk was significantly related with decreased risk of *CDH4* methylation (OR = 0·61, (95 % CI 0·38, 0·99)) in breast tissue. Our findings may provide direct guidance on the dietary intake for specific methylated carriers to decrease their risk for developing BC.

Key words: **Dietary factors: Methylation: CDH4: Breast cancer: Risk**

Breast cancer (BC) is the most common malignant tumour among women, with its incidence and mortality rank first worldwide in 2018[1]. In 2020, BC cases and deaths will account for 30 % and 15 % of diagnosed female cancers and cancer-related deaths, respectively, in America, while in China, the percentages in 2015 were approximately 15 % and 7 %, respectively[2,3]. Although the cases and death proportions in China were much lower than in America, there has been an upward trend in recent years[4].

The malignant transformation of normal cells is analogous to somatic cell reprogramming, and the malignant transformation yields cells with unlimited self-renewal potential[5]. DNA methylation inhibits epigenetic reprogramming either by silencing transcription factors or by promoting oncogenesis by impeding anti-proliferation genes[6]. Studies have verified that DNA methylation as a relatively stable epigenetic modification type can maintain cell phenotype through continuous cell cycles[7]. Traditional research on the relationship between DNA methylation and cancerogenesis is often based on tumour tissues. However, considering the availability of samples and the acceptability of subjects, peripheral blood leukocytes (PBL) DNA methylation may be hall markers in recent cancer molecular

Abbreviations: BC, breast cancer; CDH4, Cadherin-4; PBL DNA, peripheral blood leukocytes DNA.

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epidemiology studies[8]. Kuchiba *et al.*[9] determined that the global methylation level of PBL DNA is low in BC cases, which may be a potential epigenetic marker for predicting the risk of developing BC.

The basic characteristic of cancer is the over proliferation and apoptosis suppression of cells[10]. Experimental studies have indicated that many natural dietary products, and their bioactive components could affect the development of cancer by mediating cell proliferation and apoptosis[11]. Theresa *et al.*[12] analysed the BC risk and dietary recommendations, proposed by the World Cancer Research Fund and the American Institute for Cancer Research, and found that meeting the World Cancer Research Fund/American Institute for Cancer Research cancer prevention recommendations of alcohol, body fatness and plant foods is associated with reduced postmenopausal BC risk. Natural dietary products could also interfere with DNA methylation by altering the methyl donor, or the activity of DNA methyltransferases. This phenomenon contributes to phenotype alteration and susceptibility to cancer[13]. Jana *et al.* confirmed that soya isoflavone daidzein specifically stimulates the expression of *BRF2* (TF III B-related factor 2, a transcription factor required for synthesis of a small group of non-coding RNAs by RNA polymerase III) through promoter demethylation and then induces the proliferation of human BC cells[14]. Thus, the potential relationship of epigenetic events associated with dietary factors may afford new BC prevention strategies[15].

Cadherin-4 (*CDH4*), a member of the cadherin family, encodes Ca^{2+}-dependent cell–cell adhesion glycoproteins[16]. *CDH4* has been confirmed to inhibit the proliferation, invasion and migration of cells in many cancers[17,18]. Georgia *et al.* reported that the overexpression of retinal cadherin (R-cad), encoded by *CDH4*, could induce glandular morphogenesis, inhibit invasiveness, tumour formation and lung colonisation in BC[19]. Elena *et al.* verified that *CDH4* methylation is observed in PBL DNA in gastrointestinal tumour patients and is an early event in carcinogenesis progression[20]. However, the relation between *CDH4* methylation and BC risk has never been studied.

Herein, we explored the correlations of *CDH4* methylation in PBL DNA, dietary factors and their combined and interactive effects with the risk of developing BC. We also explored the associations between *CDH4* methylation in breast tumour tissue DNA and PBL DNA and clinicopathological characteristics. The relations of dietary factors, lifestyle and *CDH4* methylation in BC tissues were also investigated.

Materials and methods

Study subjects

A case–control study including 380 cases and 439 controls was designed. Cases were newly diagnosed female BC patients from The Third Affiliated Hospital of Harbin Medical University from 2010 to 2014. Anyone who has undergone radiotherapy or chemotherapy was excluded. Controls were 208 female patients from the Department of Orthopaedics and Ophthalmology in The Second Affiliated Hospitals of Harbin Medical University and 231 female volunteers from Xiangfang District Centers for Disease Control and Prevention. The individuals who had a history of BC or malignancies, and benign neoplasms were excluded from the control group. All subjects who were pregnant, postpartum or breast-feeding were also excluded from cases and controls. Approximately 5 ml fasting peripheral venous blood was collected from case patients prior to surgery or from controls in enrolling.

A case-only study of 335 BC patients was also designed. A tumour tissue specimen from each patient was obtained during surgery, then immediately stored in liquid nitrogen after marking and finally frozen in a refrigerator at –80°C.

Data collection

A structured questionnaire was designed in accordance with quantitative reporting results[21,22]. For foods that are frequently ingested, such as cereal, vegetables, pork and eggs, were quantitatively collected based on the dietary habits of people in Northeast China[13,14]. Other food information with lower intake frequency, such as fish, milk and overnight foods, were collected according to the frequency. The question included the following information from each subject: demographic characteristics (age, marital status, occupation, etc.), lifestyle (drinking, smoking, physical activity, etc.) and frequency of food item consumption (vegetable, milk, fish, etc.). Lifestyle and dietary information for 1 year prior to a diagnosis or enrollment were collected. All subjects were interviewed face to face by well-trained interviewers. Clinicopathological information of each patient was obtained from medical records, including TNM stage, ER status, PR status, etc.

Genomic DNA extraction and sodium bisulphite modification

Genomic DNA was extracted from blood samples and tumour tissues by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and PureLinkTM Genomic DNA Kit (Thermo Fisher Scientific, Carlsbad, USA) and immediately stored at −80°C. Totally, 25 mg of tissue was prepared by cutting the tissue sample into small pieces in a tissue grinder before DNA extraction. Bisulfite modification was carried out using 2 μg of genomic DNA and EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) and stored at −80°C.

All experimental procedures were strictly in line with the manufacturer's guidelines. DNA quantity was determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific) before and after the genomic DNA extraction and bisulfite conversion.

Plasmid and standard curve construction

The cloning process was performed by TOPO TA Cloning for Sequencing kit (Invitrogen, America) using freshly purified specific PCR products and TOP10 competent cells based on the manufacturer's instructions. The plasmid DNA was detected by sequencing after extraction, and then diluted to serial concentrations (10 to 10^7 copy number) to establish a standard curve for quantitative methylation-specific PCR[23].

Quantification of DNA methylation with quantitative methylation-specific PCR

The quantitative methylation-specific PCR procedure can confirm the specificity of the amplified product after the PCR reaction using melting curve analysis. The methylation of samples and plasmid DNA were detected in duplicate by LightCycler 480 (Roche Applied Science, Mannheim, Germany) equipped with Gene Scanning software (version 2.0). The primers of *CDH4* and a housekeeping gene, *MyOD*, designed by Primer Premier 5.0 software were as follows: *CDH4*; forward primer, TGAGGGTGAGTGTAGATTTTTTTCG and reverse primer, CTCATTACAAAAAAAATAAAATCGCG; *MyOD*; forward primer, CCAACTCCAAATCCCCTCTCTAT and reverse primer, TGATTAATTTAGATTGGGTTTAGAGAAGGA.

The amplification reactions were performed in a total volume of 20 μl which consisted of: 10 μl SYBR Green Supermix, 0·6 μl of primers (final concentration: 600 nmol/l), 7·8 μl RNase-free water and 1 μl bisulphite converted DNA (concentration: PBL DNA, 15 ng/μl; tumour tissue DNA, 5 ng/μl). The amplification protocol was as follows: 6 min at 95°C for 1 cycle, 42 cycles at 94°C for 30 s, gene annealing temperature for 30 s, 72°C for 30 s and finally 30 s at 72°C for 1 cycle. The annealing temperature of *CDH4* and *MyOD* was 63°C and 56°C, respectively. Duplicate reactions, negative controls and blank controls were designed for each experiment, and the method of methylation detection was repeated for 10 % of the samples.

The R^2 value of a standard curve between 0·97 and 1·00 indicated a good fit[23]. The methylation level of each sample was calculated by the following formula: methylation level = (copy number of *CDH4*/copy number of *MyOD*) × 100 %.

A standard curve of *CDH4* was obtained using quantitative methylation-specific PCR detection. A serial of 10 to 10^7 copy number of plasmid DNA and corresponding bisulphite modified samples were used as templates in this process. The R^2 value of the standard curve is 0·99. All plots were logarithmically amplified until a plateau was observed, and all samples have only one lysed peak, which is consistent with the plasmid DNA (online Supplementary Fig. 1).

Statistical analysis

We analysed the statistical difference of demographic characteristics between BC patients and controls with PBL DNA, *CDH4* hypermethylated and hypomethylated BC patients with tumour tissue DNA. A χ^2 test was used in this calculation. OR and 95 % CI were calculated by univariate and multivariate unconditional logistic regression to analyse the association of *CDH4* methylation in PBL DNA, dietary factors and lifestyle with BC risk. The same statistical methods were also used to determine the effects of dietary factors and lifestyle on *CDH4* methylation in tumour tissue DNA. A cross-over study and multivariate logistic regression were used to assess the combined and interactive effects between *CDH4* methylation, dietary factors and lifestyle in BC cases[24]. The correlation between *CDH4* methylation in tumour tissue DNA, PBL DNA and clinicopathological characteristics were evaluated by a χ^2 test. All the calculations were performed using SPSS version 23.0, and *P*-value <0·05 was considered to have statistical significance.

Results

Association of Cadherin-4 methylation in peripheral blood leukocyte DNA and breast cancer risk

A total of 380 BC patients and 439 cancer-free controls with PBL DNA were included in this study. The demographic features are summarised in Table 1. The proportion of married women in the case group was significantly lower than the controls (89·74 % *v.* 94·05 %, $P = 0·023$). There were significant differences in educational level ($P < 0·001$), occupation ($P < 0·001$), family history of cancer ($P < 0·001$) and history of hormone therapy ($P = 0·024$) between the case group and controls. No significant differences were observed with regard to age ($P = 0·27$), BMI ($P = 0·19$) and reproductive history ($P = 0·81$).

Table 2 summarises the relation between *CDH4* methylation and BC risk. *CDH4* methylation was divided into

Table 1. Demographic characteristics of breast cancer patients and controls with peripheral blood leukocyte DNA (Number and percentages)

	Cases		Controls		
Characteristics†	*n*	%	*n*	%	*P*-value*
	380		439		
Age (year)					0·27
<40	41	10·79	50	11·39	
40–49	134	35·26	149	33·94	
50–59	133	35·00	134	30·52	
≥60	72	18·95	106	24·15	
Median (CV %)	51·00	18·60 %	50·00	19·33 %	
Marital status‡					0·023
Single	39	10·26	26	5·95	
Married	341	89·74	411	94·05	
Education level					<0·001
Primary and below	87	22·89	126	28·83	
Junior high school	134	35·26	143	32·72	
Senior middle school	92	24·21	131	29·98	
College and above	67	17·64	37	8·47	
Occupation					<0·001
Mental worker	95	27·46	87	23·84	
Manual worker	174	50·29	242	66·30	
Mixed	77	22·25	36	9·86	
Family history of cancer					<0·001
Yes	105	28·00	60	15·58	
No	270	72·00	325	84·42	
BMI§ (kg/m^2)					0·19
≤ 18·5	10	2·64	16	3·66	
18·5–23·0	164	43·27	163	37·30	
≥23·0	205	54·09	258	59·04	
Median (CV %)	23·23	20·79 %	23·80	15·79 %	
Reproductive history					0·81
No	6	1·59	6	1·38	
Yes	371	98·41	428	98·62	
History of hormone therapy					0·024
Yes	21	5·60	11	2·51	
No	354	94·40	427	97·49	

* We analysed the statistical difference of demographic characteristics between breast cancer patients and controls with PBL DNA by χ^2 test.

† Missing data: Marriage status: 2 controls; educational level: 2 controls; occupation: 34 cases, 74 controls; family history of cancer: 5 cases, 54 controls; BMI: 1 case, 2 controls; number of pregnancies: 3 cases, 5 controls; history of hormone therapy: 5 cases, 1 control.

‡ Marriage status: Single is a person who is unmarried, widowed (not remarried), separated (due to discord or long distance) and divorced (not remarried). Married is a person who is married or cohabitated.

§ BMI (weight/height^2).

Table 2. Association of *CDH4* methylation in peripheral blood leukocyte DNA and breast cancer risk (Numbers and percentages)

Methylation status*	Case *n*	%	Controls *n*	%	OR_{crude}	95 % CI	*P*-value	OR_{adj}	95 % CI	*P*-value
CDH4										
Hypomethylated	205	53·95	324	73·80	1·000			1·000		
Hypermethylated	175	46·05	115	26·20	2·41	1·79, 3·22	<0·001	2·70	1·90, 3·83	<0·001

OR_{crude}, odds ratio generated by univariate logistic regression; OR_{adj}, odds ratio generated by univariate logistic regression, adjustment factors included marital status, education level, occupation, family history of cancer and history of hormone therapy.
* Methylation status, the methylation values were tested from peripheral blood leukocyte DNA of fasting peripheral venous blood. The methylation cut-off value (0·778) was selected according to the maximum Youden index.

hypermethylation and hypomethylation based on the methylation cut-off value (0·778) when the Youden index is largest. Hypermethylated BC patients and controls in *CDH4* were 175 (46·05 %) and 115 (26·20 %), respectively. After adjusting by marital status, education level, occupation and family history of cancer, the hypermethylation of *CDH4* was significantly associated with increased BC risk in PBL DNA ($OR_{adjusted}$ (OR_{adj} = 2·70, (95 % CI 1·90, 3·83), $P < 0·001$).

Subgroup analyses

Supplementary Table 1 showed the demographic characteristics of luminal A patients and controls with PBL DNA. We observed significant differences between luminal A patients and controls regarding age ($P = 0·020$) and occupation ($P = 0·040$). Thus, the two factors were used as adjusted factors in the unconditional logistic regression. However, no significant differences were found between *CDH4* methylation and luminal A BC risk (online Supplementary Table 2).

Then we analysed the demographic characteristics of luminal B patients and controls with PBL DNA (online Supplementary Table 3). There were significant differences in marital status ($P = 0·046$), occupation ($P = 0·001$) and family history of cancer ($P < 0·001$). Supplementary Table 4 showed the association of *CDH4* methylation in PBL DNA and luminal B BC risk. Marital status, occupation and family history of cancer were adjusted in this calculation. *CDH4* hypermethylation was found to be associated with increased luminal B BC risk (OR = 2·93, (95 % CI 1·89, 4·56), $P < 0·001$).

Association between dietary factors, lifestyle and breast cancer risk

The results of the univariate and multivariate analyses for the association between dietary factors, lifestyle and BC risk are shown in Supplementary Table 5. Educational level, occupation, marital status, family history of cancer and history of hormone therapy patients were used as adjusted factors in these calculations. In the multivariate analyses, vegetables (≥500 g/d *v.* <500 g/d, OR_{adj} = 0·59, (95 % CI 0·41, 0·84)), allium vegetables (>3 times/week *v.* ≤3 times/week, OR_{adj} = 0·37, (95 % CI 0·26, 0·53)), fish (≥3 times/month *v.* <3 times/month, OR_{adj} = 0·45, (95 % CI 0·25, 0·81)), milk (≥1 times/week *v.* <1 time/week, OR_{adj} = 0·57, (95 % CI 0·35, 0·93)) and physical activity (≥1times/month *v.* <1 time/month, OR_{adj} = 0·57, (95 % CI 0·39, 0·83)) were significantly associated with decreased BC risk. However, overnight food (>3 times/week *v.* 0 time/week, OR_{adj} = 2·38, (95 % CI 1·44, 3·92)) and pork (≥250 g/week *v.* 0 g/week, OR_{adj} = 1·92, (95 % CI 1·14, 3·24)) were significantly associated with increased BC risk.

Combined and interactive effects of Cadherin-4 methylation and dietary factors, lifestyle in breast cancer

The combined and interactive effects between *CDH4* methylation, dietary factors and lifestyle in BC are shown in Table 3. Among those factors, vegetables (<500 g/week *v.* ≥500 g/week, OR_{adj} = 4·33, (95 % CI 2·63, 7·10)), allium vegetables (≤3 times/week *v.* >3 times/week, OR_{adj} = 7·00, (95 % CI 4·17, 11·77)), fish (<3 times/week *v.* ≥3 times/week, OR_{adj} = 7·92, (95 % CI 3·79, 16·53)) and milk (<1 time/week *v.* ≥1 times/week, OR_{adj} = 6·30, (95 % CI 3·41, 11·66)) were significantly related to increased BC risk when combined with *CDH4* hypermethylation. The same combined effects were also found between *CDH4* hypermethylation and overnight food (>3 times/week *v.* ≤3 times/week, OR_{adj} = 4·63, (95 % CI 2·69, 7·99)), pork (≥250 g/week *v.* <250 g/week, OR_{adj} = 5·59, (95 % CI 2·94, 10·62)) and physical activity (<1 time/month *v.* ≥1 times/month, OR_{adj} = 4·72, (95 % CI 2·87, 7·76)). No valuable interactive effect was found between any dietary factor, lifestyle with *CDH4* methylation.

Effects of dietary factors and lifestyle on Cadherin-4 methylation in tumour tissue DNA

The distribution of demographic characteristics among BC patients with tumour tissue DNA is displayed in Table 4. The *CDH4* methylation was defined as hypermethylated or hypomethylated by 55·5 % based on the 50 % percentile. Age, marital status, educational level, occupation, family history of cancer, BMI, reproductive history and history of hormone therapy were analysed in this calculation. No statistical association was found for any demographic characteristics ($P > 0·05$).

Table 5 displays the association of dietary factors and lifestyle exposure with *CDH4* methylation in breast tissue DNA. Based on the univariate analysis, physical activity, milk and pork were significantly related to *CDH4* methylation ($P < 0·05$). The multivariate logistic regression was calculated with these three factors. The results suggested that individuals who consumed milk ≥1 times/month (*v.* <1 times/month) have a lower probability of hypermethylated *CDH4* (30·25 %, OR = 0·61 (95 % CI 0·38, 0·99)) in tumour cells. There was no statistical difference between other dietary factors or lifestyle (such as beef and mutton, poultry and fish) and *CDH4* methylation.

Table 3. Combined and interactive effects between *CDH4* methylation and dietary factors and lifestyle in breast cancer (odd ratios and 95 % confidence intervals)

	CDH4† (OR_{adj} (95 % CI))*				Interactive effect		
Environmental factors	Hypomethylated		Hypermethylated		OR_i	95 % CI	*P*-value
Vegetables (g/d)					0·88	0·44, 1·78	0·73
≥500	1·00		2·51	1·51, 4·15			
<500	1·53	1·01, 2·30	4·33	2·63, 7·10			
Allium vegetables (times/week)					1·55	0·76, 3·18	0·23
> 3	1·00		3·61	2·07, 6·29			
≤ 3	3·01	1·94, 4·65	7·00	4·17, 11·77			
Fish (times/month)					1·80	0·57, 5·71	0·32
≥3	1·00		4·57	1·53, 13·62			
<3	3·13	1·55, 6·31	7·92	3·79, 16·53			
Milk (times/week)					1·30	0·50, 3·34	0·59
≥1	1·00		3·29	1·38, 7·81			
<1	2·49	1·41, 4·40	6·30	3·41, 11·66			
Overnight food‡ (times/week)					0·76	0·37, 1·57	0·46
≤ 3	1·00		2·95	1·88, 4·61			
> 3	2·07	1·34, 3·18	4·63	2·69, 7·99			
Pork (g/week)					0·51	0·25, 1·02	0·06
<250	1·00		3·63	1·51, 8·74			
≥250	2·19	1·20, 4·00	5·59	2·94, 10·62			
Physical activity§ (times/month)					2·05	1·00, 4·20	0·05
≥1	1·00		4·04	2·30, 7·09			
<1	2·39	1·55, 3·69	4·72	2·87, 7·76			

OR_{adj}: adjusted for marital status, education level, occupation, family history of cancer and history of hormone therapy. OR_i: $OR_{interaction}$, interactive effects.
* Bold values indicate significance after Bonferroni correction. *P* value is 0·05/14 = 0·003571.
† *CDH4*, the methylation values were tested from peripheral blood leukocyte DNA of fasting peripheral venous blood. And the methylation cut-off value (0·778) was selected according to the maximum Youden index.
‡ Overnight food, the cooked vegetables, eggs or meats that were left overnight either in the fridge or outside of the fridge.
§ Physical activity, Yes/No means exercise ≥ 70 min/<70 min of moderate-intensity aerobic physical activity; or ≥30 min/<30 min of vigorous-intensity aerobic physical activity, such as running, swimming and participating in ball games.

Correlation between clinicopathological characteristics and Cadherin-4 methylation in peripheral blood leukocyte DNA and breast tissue DNA

The association between clinicopathological characteristics and *CDH4* methylation in both PBL DNA and breast tumour tissue DNA is summarised in Supplementary Table 6. The selected clinicopathological characteristics were tumour location, TNM stage (tumour invasion, lymphnodes involved and metastasis status), ER status, PR status, HER2 status, molecular subtype, P53 status, histological type and Ki67 **proliferation marker**. No statistically significant results were observed between clinicopathological characteristics and *CDH4* methylation in PBL DNA and breast tumour tissue DNA.

Discussion

The 'mission critical' events of cancer development include sustaining proliferative signalling, evading growth suppressors, resisting cell death and other events. Cell proliferation and apoptosis play a vital role in maintaining cell populations and as a defense mechanism[10]. Natural dietary products could influence the development of cancers by mediating DNA methylation to promote or inhibit cell proliferation and apoptosis[25]. The key finding in this study is that we determined *CDH4* hypermethylation increased BC risk, and its combined effects with low intake frequencies of vegetables, allium vegetables, fish, milk, low frequencies of physical activity and high intake frequencies of overnight food and pork increased BC risk. Moreover, lower intake frequencies of milk were significantly related to *CDH4* hypermethylation in BC tissue.

The mechanism of DNA methylation in peripheral blood leukocytes and breast tumourigenesis has not been confirmed. But Marsit *et al.*[26] reported that tumours do not develop as an isolated phenomenonin their target tissue, other organ systems, including the immune system (such as peripheral blood leukocytes) is also involved in the occurrence of tumours. Moreover, carcinogenic process usually leads to immunological changes related to inflammation, causes changes in white blood cell subpopulations and their numbers, thus results to changes of epigenetic characteristics in peripheral blood leukocytes[27]. Epidemiologic studies have analysed and suggested that peripheral blood leukocytes DNA can be used as a potential tumour biomarker[28]. Increasing studies have also reported the association of gene methylation levels in PBL and BC risk, indicating that epigenetic alteration of PBL may be a potential biomarker for BC risk[29–31].

CDH4 has been reported to play anti-proliferation and pro-apoptosis roles in salivary adenoid cystic carcinoma, glioblastoma, etc.[17]. R-cad, encoded by *CDH4*, usually expresses in non-transformed breast cells but is downregulated in breast tumour cell lines. R-cad could also inhibit lung metastasis by suppressing critical regulators of metastatic seeding and growth (MMP1, MMP2 and COX2)[19]. Du *et al.* reported that *CDH4* can inhibit cell proliferation, colony formation, migration and elicit cell communication. Du *et al.* also report that *CDH4* is hypermethylated in five nasopharyngeal carcinoma cell lines, nasopharyngeal carcinoma cell xenograft lines and 94·3 % of primary

Table 4. Demographic characteristics of breast cancer patients with tumour tissues (Numbers and percentages)

	CDH4 methylation status§				
	Hypomethylated		Hypermethylated		
Characteristics*	*n*	%	*n*	%	*P*-value
	168		167		
Age (year)					0·09
<50	57	35·40	76	47·20	
50–59	63	39·13	54	33·54	
> 60	41	25·47	31	19·26	
Median (CV %)	54·00	19·07 %	51·00	17·04 %	
Marital status†					0·56
Single	2	1·27	1	0·63	
Married	156	98·73	158	99·37	
Education level					0·45
Primary and below	41	24·70	48	29·27	
Junior high school	44	26·51	50	30·49	
Senior middle school	58	34·94	45	27·44	
College and above	23	13·85	21	12·80	
Occupation					0·53
Mental worker	29	18·47	22	13·92	
Manual worker	60	38·22	66	41·77	
Mixed	68	43·31	70	44·31	
Family history of cancer					0·16
Yes	31	18·67	41	25·00	
No	135	81·33	123	75·00	
BMI‡ (kg/m^2)					0·16
≤ 18·5	7	4·22	5	3·05	
18·5–23·0	54	32·53	70	42·68	
≥23·0	105	63·25	89	54·27	
Median (CV %)	23·88	15·88 %	23·28	15·81 %	
Reproductive history					0·42
No	4	2·44	2	1·24	
Yes	160	97·56	159	98·76	
History of hormone therapy					0·56
Yes	14	8·33	11	6·67	
No	154	91·67	154	93·33	

* Missing data: Age 13; Marriage status 18; Educational level 3; Occupation 4; Family history of cancer 5; BMI 5; Reproductive history, 10; History of hormone therapy, 2.
† Marriage status: Single is a person who was unmarried, widowed (not remarried), separated (due to discord or long distance) and divorced (not remarried). Married is a person who is married or cohabitated.
‡ BMI (weight/height^2).
§ *CDH4* methylation status, the methylation values were tested from tumour tissue specimen, and its cut-off value was 55·5 %, selected according to the 50 % percentile.

tumours but not in any of twelve normal epithelial samples[32]. Elena *et al.* reported that *CDH4* methylation can be detected in 78 % of colorectal and 95 % of gastric carcinomas, and the gene expression can be restored by treatment with demethylating agent 5-aza-2-deoxycytidine[20]. In this study, we demonstrate that *CDH4* hypermethylation in PBL DNA was associated with increased BC risk ($OR_{adj} = 2·70$, (95 % CI 1·90, 3·83), $P < 0·001$), which indicated that *CDH4* hypermethylation is an independent risk factor for BC occurrence. In addition, studies on BC subtype indicated that luminal B BC has a higher expression of proliferation markers and higher histologic grade[33]. Li *et al.*[34] found that patients with luminal B BC have a higher proportion of local recurrence and single bone metastasis, and these risks presented during a 2- to 5-year period and after 5 years. In our study, *CDH4* hypermethylation was significantly associated with increased luminal B BC risk ($OR_{adj} = 2·93$, (95 % CI 1·89, 4·56), $P < 0·001$), which indicated that *CDH4* methylation may be a biomarker of luminal B BC.

Vegetables, together with allium vegetables, are essential in Chinese diet and have been shown to relate to decreased BC risk[35,36]. Vegetables contain a number of nutrients and phytochemicals with cancer chemopreventive properties, including folate, fibre, carotenoids, antioxidants, etc., and allium vegetables are rich in organosulphur compounds, such as diallyl trisulphide[35,36]. Antioxidants, such as vitamin C and vitamin E, can protect DNA from oxidative damage and help reduce oxidative stress to prevent BC[36]. In addition, dietary lycopene can inhibit MCF-7 cell proliferation by decreasing the mitogenic activity of insulin-like growth factor I. Also, all-trans-retinoic acid, a retinoid, can induce a dose-dependent MCF-7 cell death[37,38]. Diallyl trisulphide has been reported to inhibit BC stem cell proliferation by suppressing the Wnt/β-catenin pathway activation and thus inhibit the occurrence of BC[35]. The anti-cancer effects of vegetables and allium vegetables are also reflected in inflammation marker inhibition. γ-Tocopherol found in vegetables and quercetin found in onions were shown to decrease the

Table 5. Association of dietary factors and lifestyle exposures and *CDH4* methylation in breast tissue DNA (Numbers and percentages; odd ratios and 95 % confidence intervals)

	Hypomethylated*		Hypermethylated*		Univariate analysis			Multivariate analysis		
Environmental factors	*n*	%	*n*	%	OR	95 % CI	*P*-value	OR	95 % CI	*P*-value
Physical activity† (times/month)										
<1	102	62·58	119	73·46	1·00			1·000		
≥1	61	37·42	43	26·54	0·60	0·38, 0·97	0·036	0·68	0·41, 1·11	0·12
Milk (times/month)										
<1	93	58·13	113	69·75	1·00			1·000		
≥1	67	41·87	49	30·25	0·60	0·38, 0·95	0·030	0·61	0·38, 0·99	0·047
Pork (g/week)										
<250	85	52·15	101	64·33	1·00			1·000		
≥250	78	47·85	56	35·67	0·60	0·39, 0·95	0·028	0·66	0·41, 1·06	0·084
Beef and mutton (times/month)										
<1	88	53·99	96	61·15	1·00					
≥1	75	46·01	61	38·85	0·75	0·48, 1·16	0·20			
Poultry (times/month)										
<1	89	54·60	87	54·72	1·00					
≥1	74	45·40	72	45·28	1·00	0·64, 1·54	0·98			
Fish (times/month)										
<1	63	38·89	67	41·1	1·00					
≥1	99	61·11	96	58·9	0·91	0·59, 1·42	0·68			
Seafood (times/month)										
<1	139	93·92	131	92·91	1·00					
≥1	9	6·08	10	7·09	1·18	0·46, 2·99	0·73			
Refined grain (g/d)										
<150	89	55·28	96	60·00	1·00					
≥150	72	44·72	64	40·00	0·82	0·53, 1·28	0·39			
Cereal (g/week)										
≤ 50	81	51·59	81	51·59	1·00					
> 50	76	48·41	76	48·41	0·91	0·51, 1·64	0·77			
Vegetable (g/d)										
≤ 500	99	59·64	106	65·43	1·00					
> 500	67	40·36	56	34·57	0·78	0·50, 1·22	0·28			
Soybeans (times/week)										
≤ 1	56	33·53	68	41·21	1·00					
> 1	111	66·47	97	58·79	0·72	0·46, 1·13	0·15			
Fruits (g/week)										
<1500	76	46·63	75	45·45	1·00					
≥1500	87	53·37	90	54·55	1·05	0·68, 1·62	0·83			
Allium vegetable (times/week)										
<1	74	44·58	71	43·29	1·00					
≥1	92	55·42	93	56·71	1·05	0·68, 1·63	0·81			
Eggs (times/week)										
≤ 3	60	36·14	64	39·26	1·00					
> 3	106	63·86	99	60·74	0·88	0·56, 1·37	0·56			
Pickles (times/week)										
<1	86	51·81	68	41·21	1·00					
≥1	80	48·19	97	58·79	1·53	0·99, 2·37	0·054			
Fried food (times/month)										
<1	105	67·74	106	66·67	1·00					
≥1	50	32·26	53	33·33	1·05	0·66, 1·68	0·84			
Canned fruits (times/month)										
<1	142	85·54	132	79·52	1·00					
≥1	24	14·46	34	20·48	1·52	0·86, 2·71	0·15			
Canned meat (times/month)										
<1	152	92·12	150	91·46	1·00					
≥1	13	7·88	14	8·54	1·09	0·50, 2·40	0·83			
Overnight food‡ (times/week)										
≤ 3	104	62·65	102	62·20	1·00					
> 3	62	37·35	62	37·80	1·02	0·65, 1·59	0·93			
Smoke§										
No	149	90·85	142	87·12	1·00					
Yes	15	9·15	21	12·88	1·47	0·73, 2·96	0·28			
Drinking (times/month)										
<1	133	82·61	128	81·53	1·00					
≥1	28	17·39	29	18·47	1·08	0·61, 1·91	0·80			
Juice‖										
No	156	93·98	148	90·24	1·00					
Yes	10	6·02	16	9·76	1·69	0·74, 3·84	0·21			

Table 5. (*Continued*)

	Hypomethylated*		Hypermethylated*		Univariate analysis			Multivariate analysis		
Environmental factors	*n*	%	*n*	%	OR	95 % CI	*P*-value	OR	95 % CI	*P*-value
Tea¶										
No	144	85·71	148	90·24	1·00					
Yes	24	14·29	16	9·76	0·65	0·33, 1·27	0·21			

* *CDH4* methylation status, the methylation values were tested from tumour tissue specimen and its cut-off value was 55·5 %, selected according to the 50 % percentile.
† Physical activity, Yes/No means exercise ≥ 70 min/<70 min of moderate-intensity aerobic physical activity; or ≥30 min/<30 min of vigorous-intensity aerobic physical activity such as running, swimming and participating in ball games.
‡ Overnight food, the cooked vegetables, eggs or meats that were left overnight either in the fridge or outside of the fridge.
§ Smoke, Yes means smoking at least 1/d for more than 6 weeks.
‖ Juice, Yes means drinking at least 1 cup/week for more than 3 months.
¶ Tea, Yes means drinking at least 2 times/week for more than 3 months.

expression of cyclooxygenase-2 (COX-2) by inhibiting the p300 signalling and blocking the binding of multiple transactivators to COX-2 promoter and then inhibit cell proliferation in BC, which are consistent with the mechanism of *CDH4* above[19,39,40]. Similarly, this study found eating vegetables ≥500 g/d (*v.* <500 g/d) and allium vegetables >3 times/week (*v.* ≤3 times/week) decreased the risk of BC (OR_{adj} are 0·59 and 0·37 (95 % CI are 0·41, 0·84 and 0·26, 0·53)). The low intake frequencies of these two dietary factors were significantly correlated with increased BC risk when combined with *CDH4* hypermethylation (OR_{adj} are 4·33 and 7·00 (95 % CI are 2·63, 7·10 and 4·17, 11·77)), which indicated that *CDH4* hypermethylation carriers may benefit from consuming more vegetables and allium vegetables to further decrease BC risk.

Fish are rich in *n*-3 PUFA, while pork contains SFA and cholesterol[41,42]. *n*-3 PUFA can alter cell membrane phospholipid fatty acid composition and disrupt lipid rafts. This not only reduced the expression of inflammatory genes but also altered the level of epidermal growth factor receptor and induced the apoptosis of human BC cells[42,43]. SFA can stimulate the production of endogenous estrogen and induce the proliferation of breast alveolar epithelial cells, thereby promoting BC[41]. In this study, consuming fish ≥3 times/month (*v.* <3 times/month) decreased BC risk (OR_{adj} = 0·45, (95 % CI 0·25, 0·81)), while consuming pork ≥250 g/week (*v.* 0 g/week) increased BC risk (OR_{adj} = 1·92, (95 % CI 1·14, 3·24)). However, the low intake frequencies of fish and high intake frequencies of pork combined with *CDH4* hypermethylation increased BC risk (OR_{adj} = 7·92and 5·59; (95 % CI 3·79, 16·53 and 2·94, 10·62)). In view of the anti-proliferative and pro-apoptotic effects of *CDH4*[17], we recommend that *CDH4* hypermethylation carriers ingest more fish and decrease the consumption of pork to prevent BC.

Our study indicated consuming milk ≥3 times/week (*v.* <3 times/week) could decrease the risk of BC (OR_{adj} = 0·57, (95 % CI 0·35, 0·93)), and the combined effect of low intake frequencies of milk and *CDH4* hypermethylation was correlated with increased risk of BC (OR_{adj} = 6·30, (95 % CI 3·41, 11·66)). These results may be due to the presence of vitamin D. The high level of 25-hydroxyvitamin D, the form of vitamin D in serum, has been reported to decrease the activity of vascular endothelial growth factor and inhibit the migration and invasion of breast tumour tissue[44]. Finally, the activity of vitamin D receptors can induce cell cycle arrest, autophagy and apoptosis, thus decreasing the occurrence of BC[45]. We also found that consuming milk ≥1 times/month (*v.* <1 time/month) was significantly related to decreased *CDH4* methylation (OR = 0·61, (95 % CI 0·38, 0·99)) in BC tissue, which may provide an important inspiration of diet and epigenetic mechanisms in cancer. Vitamin D can induce DNA demethylation, and this demethylation effect has been shown for E-cadherin in triple-negative BC[46]. Based on the above findings, we propose that consuming milk may decrease *CDH4* methylation levels and thus decrease the risk of BC; however, this proposal still needs to be validated via *in vitro* experiments.

We also found consuming overnight food >3 times/week increased BC risk (*v.* ≤3 times/week, OR_{adj} = 2·38, (95 % CI 1·44, 3·92)), and the combination of high intake frequencies of overnight food and *CDH4* hypermethylation increased BC risk (OR_{adj} = 4·63, (95 % CI 2·69, 7·99)). Previous research has shown that the nitrate content in cooked vegetables would greatly increase when left for 24 h[47]. The nitrate can form N-nitroso compounds with amines and amides *in vivo*, which are known potent carcinogens. Maki *et al.* evaluated the interaction of dietary nitrate intake with total folate intake on BC risk and found that the interactive effect of the two dietary factors increased BC risk[48]. Therefore, we recommend *CDH4* hypermethylation carriers to lower overnight food consumption to minimise BC risk.

Dietary factors are closely related to physical activity in the cancer mechanism. For example, SFA contained in pork can stimulate the production of endogenous estrogen, which can lead to the occurrence of BC, while physical activity can decrease the risk of BC by inhibiting oestrogen levels[41,49]. Leptin has been reported to promote cell proliferation and reduce cell apoptosis, while adiponectin has the opposite effect on cells; physical activity can reduce the risk of BC by reducing leptin levels and increasing adiponectin levels[50,51]. In addition to dietary factors, we also analysed the role of physical activity and *CDH4* methylation in BC. In this study, a rate of physical activity ≥1 times/month decreased BC risk (*v.* <1 time/month, OR_{adj} = 0·57, (95 % CI 0·39, 0·83)) and the combination of low frequencies of physical activity and *CDH4* hypermethylation increased BC risk (OR_{adj} = 4·72, (95 % CI 2·87, 7·76)). *CDH4* methylation is related to low expression and its anti-proliferative and pro-apoptotic effects may be inhibited[20]. Thus, *CDH4* hypermethylation carriers are more likely to develop BC if they do not participate in higher frequencies of physical activities.

BC is a complex disease characterised by the accumulation of multiple molecular events, and each tumour phenotype shows special evolutionary potential[52]. Jacob *et al.* found a high methylation percentage of *GSTM2* in ER/PR-negative tumours compared with ER/PR-positive tumours, indicating that *GSTM2* hypermethylation may serve as a potential biomarker for aggressive tumour development[53]. However, no significant association between clinicopathological characteristics and *CDH4* methylation in PBL DNA or breast tumour tissue DNA was observed in this study.

This study does posses several limitations. First, the tissue sample from the BC patients is a very mixed population of cell types rather than only tumour cells, which may lead to a bias in estimates of the carcinogenic effects of *CDH4* methylation. Second, recall bias as a basic disadvantage of case–control studies is unavoidable. Third, we observed that *CDH4* hypermethylation is related to increased BC, but *in vitro* experiments are still needed. Finally, sample size may limit the statistical power of combination and interaction analysis, thereby larger numbers of subjects are encouraged to verify the presented conclusions.

Conclusion

In summary, we have reported a significant association between *CDH4* hypermethylation in PBL DNA and increased BC risk. The combination of *CDH4* hypermethylation in PBL DNA and lower intake frequencies of vegetables, allium vegetables, fish, milk, lower frequencies of physical activity, and higher frequencies of overnight food and pork intake may increase BC risk. Moreover, increasing milk intake significantly prevents the risk of BC, especially in *CDH4* hypermethylation carriers, and may also improve the prognosis by mediating the hypermethylation status of tumour tissue.

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The authors declare that they have no conflict of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114521002804

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