Breast cancer is associated with methylation and expression of the a disintegrin and metalloproteinase domain 33 (ADAM33) gene affected by endocrine-disrupting chemicals

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Abstract. A disintegrin and metalloproteinase domain 33 (ADAM33) gene is a transmembrane glycoprotein that mediates changes in cell adhesion and plays an important role in cancer progression. Since bisphenol A (BPA) and phthalates are epigenetically toxic, the purpose of this study was to examine whether BPA and phthalate metabolites, including monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MIBP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), have an epigenetic impact on ADAM33 and the incidence of breast cancer. CpG islands of breast cancer microarray datasets obtained from the Gene Expression Omnibus (GEO) were used to assess the ADAM33 methylation profile. We designed a case-control study including 44 cases and 22 age-matched controls to detect the methylation status of intron 1 in ADAM33 from peripheral blood mononuclear cells (PBMCs) in blood, using BSP, nested PCR, and bisulfite sequencing, and measured the *in vivo* gene expression of ADAM33 and the urinary concentrations of endocrine-disrupting chemicals (EDCs), using real-time PCR, high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). Only one dataset, GSE32393, reached significance (P=0.016). ADAM33 expression and methylation frequencies at CpG site 3 in intron 1 were higher in the control group. We found a positive association between intron 1 methylation level and ADAM33 expression as well as urinary concentrations of MEHHP, MECPP, MEOHP and Σ_4MEHP (the sum of MEHP, MECPP, MEHHP, and MEOHP) in the cases. This study suggests that metabolites of phthalate such as MEHHP, MECPP, MEOHP and Σ_4MEHP may increase the intron 1 methylation level to elevate ADAM33 gene expression and have a protective effect on reducing the risk of breast cancer.

Introduction

According to the World Cancer Report published in January 2014 by the World Health Organization (WHO) (1), breast cancer is the most commonly diagnosed cancer in women globally, and has a very high incidence compared to other cancers; thus, breast cancer is undoubtedly the world's major women's health issue. Plastics are necessary materials and widely used in modern society (2), and a variety of environmental chemicals are classified as endocrine-disrupting chemicals/substances (EDCs/EDSs) (3). Endocrine disorders are a special form of intoxication, and natural or man-made

chemicals, now known as EDCs, induce adverse health effects by destroying the endogenous hormone system (4). According to the U.S. Environmental Protection Agency (EPA) definition, EDCs refer to the artificial manufacture of foreign objects called endocrine disruptors or environmental hormones that imitate or interfere with endogenous hormones maintaining the body homeostasis, reproduction, development and behavior (5). When EDCs enter the body, they have been shown to bind to the hormone receptor, affecting the synthesis, secretion, transmission and binding activity of the original endocrine mechanism (6). EDCs are known to affect reproductive function and sex hormones primarily in humans because of their estrogenic and antiandrogenic properties. Phthalates and bisphenol A (BPA) are two well-known EDCs (7). The American Endocrinology Society says that EDCs have an impact on neuroendocrine, thyroid, metabolism, obesity, cardiovascular endocrinology, both male and female reproduction, prostate cancer, breast development and breast cancer formation (5).

The a disintegrin and metalloproteinase domain 33 (ADAM33) gene, which is located on chromosome 20p13, is a member of the ADAM family of genes, consisting of 812 amino acid residues and 22 exons (8). The ADAM proteins are transmembrane glycoproteins with a variety of different functions, including cell adhesion and proteolysis, and some members of the ADAM family are associated with extracellular matrix remodeling and cellular adhesion modifications that underlie some pathologies and the development of cancer (9). The increase of ADAM33 expression may play a critical role in the pathogenesis of gastric cancer and laryngeal carcinoma (10,11). Epigenetic regulation involves the methylation of cytosine residues in human DNA by covalent modification without altering the DNA sequence (12) and has also been associated with the regulation of gene expression and the progression of breast cancer (13,14). Only 2 studies have explored the relationship between DNA methylation of ADAM33 and breast cancer. Seniski *et al* indicated that selective DNA hypermethylation leads to the downregulation of ADAM33 expression and is likely to occur in breast carcinomas, especially in invasive lobular carcinoma (ILC); therefore, ADAM33 gene promoter methylation can differentiate ILC and invasive ductal carcinoma (IDC) (9). Furthermore, Manica *et al* showed that low ADAM33 expression is associated with shorter overall survival and metastasis-free survival and ADAM33 may be an important prognostic marker of triple-negative breast cancer (TNBC) and basal-like breast cancer (BLBC) (15). It has been demonstrated that hypermethylation of the ADAM23 promoter (a disintegrin and metalloproteinase domain 23, another ADAM family member) downregulates its expression and is associated with tumor progression and metastasis in breast cancer; consequently, epigenetic silencing of other members of the ADAM family may be associated with the development of breast cancer (16). With the exception of Seniski *et al* and Manica *et al* however, few studies have explored the association between breast cancer and the ADAM33 methylation profile (9,15).

In the present study, we utilized CpG island microarray datasets to determine the ADAM33 methylation profile in subjects. We further examined ADAM33 expression and performed bisulfite sequencing PCR (BSP), nested PCR and bisulfite sequencing to evaluate the DNA methylation status of intron 1 in ADAM33 from peripheral blood mononuclear cells (PBMCs). BPA and phthalates are epigenetically toxic and affect human health and cause disease through epigenetic mechanisms (17). The purpose of this study was to test the hypothesis that exposure to BPA and phthalate metabolites, estimated from urinary concentrations, would be associated with ADAM33 expression and methylation profile between breast cancer patients and healthy controls.

Materials and methods

Study subjects. We conducted a case-control study to examine a hypothesis concerning breast cancer risk and ADAM33 expression and methylation profile. A total of 233 newly diagnosed breast cancer patients with histologically verified disease were recruited at the Medical Center of Kaohsiung Medical University in southern Taiwan between September 2013 and June 2014. The clinical stages of the breast cancer specimens were classified according to the American Joint Commission on Cancer (AJCC) criteria (18). To avoid any effects on gene methylation associated with treatment, we selected only patients who had not received any treatment prior to their participation in this study and 71 patients were eligible eventually. Twenty-seven breast cancer patients were excluded from the study for the following reasons: i) 2 patients had a smoking habit; ii) 4 breast cancer patients did not complete questionnaires; iii) 18 breast cancer patients refused to provide blood samples; and iv) 3 patients were without detection of EDCs. Ultimately, 44 newly diagnosed female breast cancer patients were recruited and included in the analysis. Between September 2013 and June 2014, 125 healthy women from the same communities in southern Taiwan were recruited, and 95 subjects were excluded for the following reasons: i) 6 controls had benign breast diseases or a malignant tumor; ii) 6 controls had a smoking habit; iii) 3 controls refused to provide blood samples; and iv) 80 controls were without detection of EDCs. Twenty-two community controls were group-matched for age (±3 years) and paired 1:2 with the 44 breast cancer patients. All cases and controls were women between the ages of 30 and 70 years and without presentation of any other cancers (Fig. 1).

Interview questionnaires and collection of specimens. The participants provided blood and urine samples and completed questionnaires to collect information regarding smoking habit, family history of breast cancer, reproductive factors, and environmental exposure factors. The study protocol was approved by the Institutional Review Board (IRB) of Kaohsiung Medical University (IRB no. KMUHIRB-20120104). Written informed consent was obtained from the study subjects.

Methylation microarray datasets and analysis. We searched the methylation microarray datasets from the Gene Expression Omnibus (GEO) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/gds) and selected references published online prior to 15 August 2015. The selection criteria for breast cancer-related datasets were established using the following keywords: breast cancer, methylation microarray
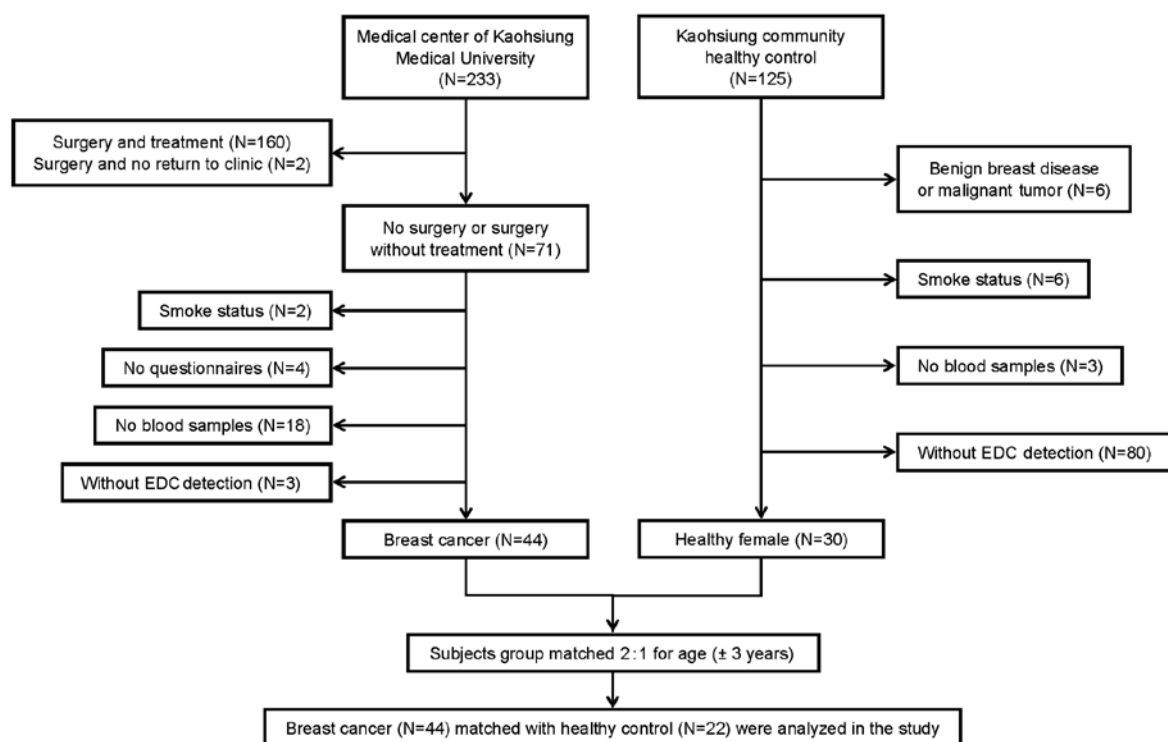
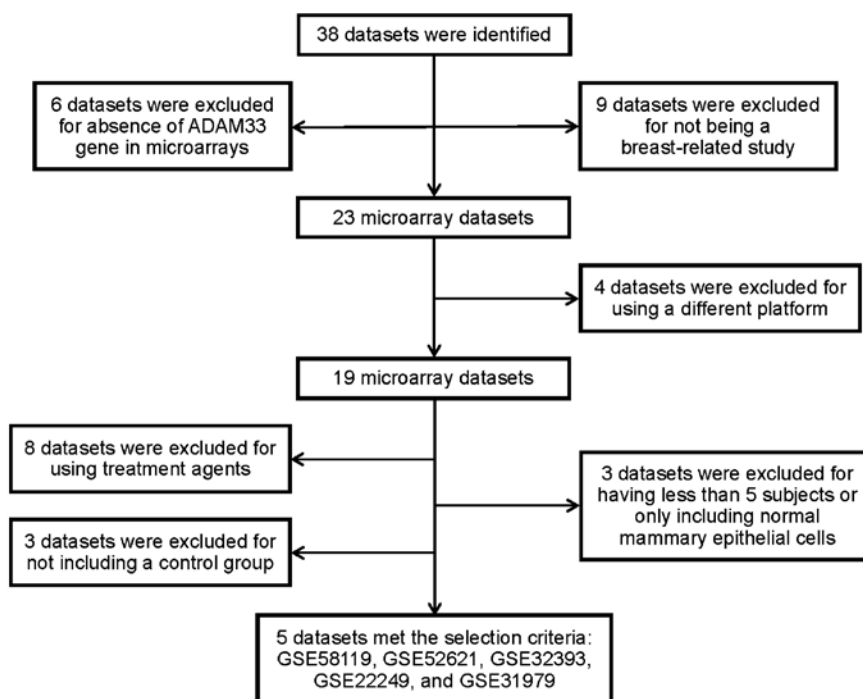Figure 1. Flowchart of participants recruited in the case-control study.

Figure 2. Flowchart of the selection process for breast cancer and healthy control microarray datasets from the GEO database. Thirty-three datasets were excluded for the following reasons: 6 datasets did not include the ADAM33 gene in the microarrays; 9 datasets were not breast-related studies; 4 datasets were not performed with the Illumina GPL8490 platform; 8 datasets were excluded because the subjects had received treatment with agents; 3 datasets lacked a control group; and 3 datasets had a sample size <5 or only included normal mammary epithelial cells.

and/or *Homo sapiens*, and pathologies associated with benign breast disease or recurrence were excluded. Thirty-eight datasets associated with breast cancer were selected, but 33 datasets were excluded. Finally, 5 datasets, including GSE58119 (19), GSE52621 (20), GSE32393 (21),

GSE22249 (22), and GSE31979 (23), [all using the GPL8490 Illumina HumanMethylation27 BeadChip (HumanMethylation27_270596_v.1.2)] met our selection criteria (Fig. 2). The β value for each CpG locus was used as a measure of methylation levels (24,25).

Screening of CpG islands and analysis of methylation levels. The identification of CpG islands in the ADAM33 gene, which was acquired from the NCBI website, was determined using the CpG Islands Searcher website (http://www.cpgislands.com) (26). Our criteria for screening the CpG islands were %GC=60%, ObsCpG/ExpCpG=0.7, and a minimum length of 500 bp. The design of the BSP primers was performed using an online biological information website: MethPrimer (http://urogene.org/methprimer/index1.html) (27). The BSP and nested PCR products included 9 CpG sites in exon 1 and intron 1 in the ADAM33 gene. However, the first 3 CpG sites could only be sequenced in less than 90% of subjects in our study, thus we presented information for only 6 CpG sites (named CpG site 1 to 6) in intron 1 in ADAM33. The nested PCR product contained the ADAM33 gene probe (cg14089692) in methylation microarray datasets. The BISMA (Bisulfite Sequencing DNA Methylation Analysis) website was used to perform the DNA methylation sequencing analysis (http://services.ibc.uni-stuttgart.de/BDPC/BISMA/manual_unique.php) (28).

Bisulfite sequencing PCR (BSP) and bisulfite sequencing. Methylation bisulfite conversion, BSP and nested PCR were performed using genomic DNA extracted from PBMC samples of study participants and PBMCs were isolated by Ficoll-Paque Plus density gradient centrifugation (Amersham Biosciences, Piscataway, NJ, USA) using the Gentra Puregene Blood Kit according to the manufacturer's instructions (Gentra Systems Inc., Minneapolis, MN, USA). Genomic DNA (400 ng) was modified with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Orange, CA, USA) prior to BSP and nested PCR. The primers of BSP did not include the CpG sites. The first set of primers for BSP included the forward primer, 5'-TTGTTGTTGTTGTTATTATTGTTGTTGT-3' and the reverse primer, 5'-AAACCAACCCAAACACACTTAAA-3'. The BSP products (266 bp) were used as templates for nested PCR amplification. The second set of BSP primers for nested PCR included the forward primer, 5'-TGTTGTTGTTATTATTGTTGTTGTTTT-3' and the reverse primer, 5'-AACTCTAAACAAAACCCATCCC-3', and the final products were 136 bp. These primers for nested PCR were designed to include the probe (cg14089692) in the ADAM33 gene from the microarray datasets. The BSP and nested PCR conditions were as follows: 95˚C for 5 min, 38 cycles of 95˚C for 30 sec, 62˚C for 1 min, 72˚C for 30 sec and 72˚C for 7 min. The Universal Methylated Human DNA Standard kit (Zymo Research Corp.) was used as a positive control DNA for bisulfite conversion. Positive primers were used for positive controls, and samples lacking DNA were used as negative controls for the BSP experiments. The nested PCR products from all case and control subjects were sequenced. Bisulfite sequencing was conducted with the ABI Reaction Kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and analyzed with an ABI Sequencer (Applied Biosystems ABI 3730xl DNA Analyzer; Thermo Fisher Scientific, Inc.).

Urinary concentrations of BPA and phthalate metabolites. The participants provided a spot first morning urine sample. We measured the urinary concentrations of bisphenol A (BPA) using high-performance liquid chromatography (HPLC). Seven phthalate metabolites including monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MIBP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), and mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) were measured with liquid chromatography-mass spectrometry (LC-MS). The urinary concentrations of bisphenol A (BPA) and phthalate metabolites were adjusted by creatinine, and these values of BPA and phthalate metabolite concentrations less than the limit of detection (LOD) were assigned a value of half the LOD (LOD/2) for the analysis.

Gene expression by RT-PCR. The ADAM33 gene probe was Hs00905552_m1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total RNA from the PBMCs was extracted using TRIzol (Life Technologies, Inc.; Thermo Fisher Scientific, Inc.). Then, glycogen (Roche Diagnostics, Indianapolis, IN, USA) was used to increase nucleic acid recovery and isopropanol was added to precipitate RNA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The quantity and quantify of RNA were determined at OD_{260nm}/OD_{280nm} using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc.). According to the manufacturer's instructions, 1000 ng of total RNA from each sample was reverse-transcribed in 20 μl reactions with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT-PCR primers were designed using the Web-based software ProbeFinder (Roche Applied Science, Indianapolis, IN, USA). Complementary DNA (20 ng) as a template with Power SYBR® Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) was assayed in a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific Inc.). Cycling conditions were 50˚C for 2 min, 95˚C for 10 min, 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The expression of each gene was measured in triplicate for each sample. The relative changes in gene expression were quantified using the $2^{-\Delta\Delta Cq}$ method relative to the GAPDH expression (Hs02758991_g1, Applied Biosystems; Thermo Fisher Scientific, Inc.) (29).

Statistical analysis. The case and control group comparisons were conducted using a two-tailed t-test for continuous data, including demographics, clinical characteristics, overall methylation status of intron 1 in ADAM33, ADAM33 expression and EDC concentrations. Nonparametric statistics were performed to analyze the difference in methylation levels (β-values) determined by the CpG island microarray datasets between cases and controls, while the β-values was calculated by M/(M+U+100), with M representing the methylated signal intensity and U representing the unmethylated signal intensity (24,25). The χ^2 test was performed to determine methylation levels of the 6 CpG sites of intron 1 in ADAM33 between 2 groups. As ADAM33 expression and the urinary concentrations of EDCs were not normally distributed, we transformed the values to logarithmic scales and then used linear regression to estimate the association of EDC concentrations, the methylation status of intron 1 in ADAM33, and ADAM33 expression in the case

Table I. β-values of the ADAM33 gene probe (cg14089692) in five datasets.

Series	Platforms	ID_REF	Cases β-value[a]			Controls β-value[a]			
			Average	Median	IQR	Average	Median	IOR	P-value[b]
GSE58119	GPL8490	cg14089692	0.130 (N=132)	0.107	0.053	0.124 (N=148)	0.107	0.057	0.286
GSE52621	GPL8490	cg14089692	0.137 (N=11)	0.086	0.048	0.083 (N=25)	0.080	0.026	0.089
GSE32393	GPL8490	cg14089692	0.098 (N=114)	0.085	0.032	0.081 (N=23)	0.074	0.021	0.016[c]
GSE22249	GPL8490	cg14089692	0.080 (N=117)	0.070	0.040	0.063 (N=8)	0.060	0.038	0.286
GSE31979	GPL8490	cg14089692	0.085 (N=103)	0.069	0.035	0.081 (N=21)	0.074	0.029	0.324

[a]β-value was calculated using the formula: M/(M+U+100), with M representing the methylated signal intensity and U representing the unmethylated signal intensity. [b]P-values were calculated for continuous variables by Mann-Whitney U test. [c]P<0.05. N, number of individuals; IQR, interquartile range (the IQR is the 1st quartile subtracted from the 3rd quartile); ADAM33, a disintegrin and metalloproteinase domain 33.
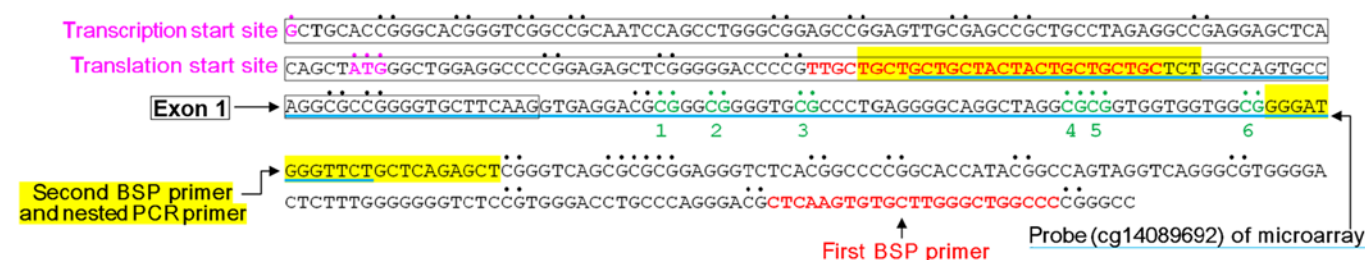

Figure 3. Schematic representation of the sequenced region of the ADAM33 gene. The blue line (bp +132 to +253) indicates the probe sequence (cg14089692). The G and ATG in pink font indicate the transcription start site (TSS) and the translation start site (as indicated by NCBI), respectively. Exon 1 is surrounded by a border, and intron1 is not surrounded by a border. The red font indicates the first BSP primer, and the yellow fluorescent font indicates the second BSP primer and the nested PCR primers. The 6 CPG sites were contained in the nested PCR product.

and control group, respectively. False discovery rate (FDR) was used to verify multiple comparisons. All P-values were 2-sided with statistical significance set at P<0.05 and were performed using the Statistical Package for Social Sciences (SPSS) software (version 20; IBM Corp., Armonk, NY, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA.

Results

Methylation microarray datasets. In the present study, the five datasets that met our selection criteria were as follows: GSE58119 (19), GSE52621 (20), GSE32393 (21), GSE22249 (22) and GSE31979 (23). The ADAM33 gene probe (cg14089692) in the microarray was located in the region between +132 bp and +253 bp, downstream of the transcriptional start site. The β-value (methylation level) between the case and control group was significantly different in the GSE32393 dataset (P=0.016) (Table I).

Participant characteristics. The basic characteristics and reproductive traits, including age, education, weight, height, BMI, menarche age, menopause age and the use of oral contraceptives were not significantly different between the case and control groups (P>0.05 for all factors) (Table II). No patient smoked or drank alcohol in either the case or control group (data not shown).

Methylation profiles. We used nested PCR to amplify a 136-bp product in the region between bp +128 and +263 using bisulfite-treated DNA as a template (Fig. 3). Bisulfite sequencing demonstrated that the methylation frequencies at CpG site 3 were significantly different between the 2 groups (P=0.005); therefore, the methylation statuses of CpG site 3 in intron 1 of the ADAM33 gene may be correlated with breast cancer (Table III).

ADAM33 expression and urinary concentrations of EDCs. ADAM33 expression was significantly higher (3.29±6.56) in the control group than that (0.83±1.04) in the case group (P=0.007). The concentration of BPA was significantly higher in the case group (P=0.033). The phthalate metabolites, including MEP, MBP, MIBP, MEHP, MEHHP, MECPP, MEOHP and Σ_4MEHP (the sum of MEHP, MECPP, MEHHP and MEOHP) were not significantly different between the case and control group (P>0.05) (Table IV). The adjusted P-values for the FDR method were not significant.

Association of urinary concentrations of EDCs, intron 1 methylation profile in the ADAM33 gene, and ADAM33 gene expression. In the case group, we found a significant positive association between the methylation level of intron 1 and ADAM33 expression (coefficient = 1.147; 95% CI: 0.268, 2.027; P=0.012) as well as EDC concentrations, including

Table II. Demographics and clinical characteristics of the participants.

Characteristics	Cases	Controls	P-value[b]
N	44	22	
Age ± SD[a] (years)	52.16±8.58	51.18±11.90	0.734
Education, n (%)			0.262
No university	28 (63.6)	17 (77.3)	
University and higher	16 (36.4)	5 (22.7)	
Weight ± SD[a] (kg)	59.22±11.25	58.26±11.86	0.749
Height ± SD[a] (cm)	157.92±5.01	157.68±6.31	0.868
BMI ± SD (kg/m^2)	23.78±4.69	23.43±4.36	0.770
Age at menarche ± SD[a] (years)	13.95±1.59	13.68±1.25	0.516
Age at menopause ± SD[a] (years)	47.83±4.69	50.30±2.95	0.064
Oral contraceptive, n (%)			1.000
No	42 (95.5)	20 (100.0)	
Yes	2 (4.5)	0 (0.0)	
Grade, n (%)			
1	6 (14.6)	-	
2	18 (43.9)		
3	17 (41.5)		
Stage, n (%)			
Stage 0/I/II	38 (90.5)	-	
Stage III/IV	4 (9.5)		
Tumor size (cm), n (%)			
≤2	26 (63.4)	-	
>2	15 (36.6)		
Invasiveness, n (%)			
Absence	10 (24.4)	-	
Presence	31 (75.6)		
ER status, n (%)			
Negative	9 (20.9)	-	
Positive	34 (79.1)		
PR status, n (%)			
Negative	16 (38.1)	-	
Positive	26 (61.9)		
Her2 status, n (%)			
Negative	18 (42.9)	-	
Positive	24 (57.1)		

[a]Values are expressed as the means ± standard deviation (SD). [b]P-values were calculated for continuous variables by t-test, and χ^2 was used for categorical variables. N, number of individuals; BMI, body mass index; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor 2; ADAM33, a disintegrin and metalloproteinase domain 33.

MEHHP (P=0.008), MECPP (P=0.013), MEOHP (P=0.028) and Σ_4MEHP (P=0.010) (Table V).

We observed an inverse correlation between ADAM33 expression and EDC concentrations, including MEP (coefficient = -0.701; 95% CI: -1.171, -0.231; P=0.006), and MBP (coefficient = -0.917; 95% CI: -1.729, -0.105; P=0.029) in the control group (Table V). The adjusted P-values were calculated by FDR, leaving only MEHHP, MECPP and Σ_4MEHP significant.

Discussion

This is the first study to explore the associations among breast cancer, endocrine-disrupting chemicals (EDCs), and methylation and expression of the ADAM33 gene. We found that ADAM33 expression was significantly higher in the controls. In cases, we also found a significant positive association between intron 1 methylation levels and ADAM33 gene expression as well as phthalate metabolites, including MEHHP, MECPP,

Table III. The methylation levels of intron 1 in the ADAM33 gene of the participants.

Methylation status[a]	Cases (N=44)	Controls (N=22)	P-value[b]
Overall	0.70±0.27	0.79±0.17	0.152
CpG 1, n (%)			
Methylated	38 (86.4)	22 (100.0)	0.167[c]
Unmethylated	6 (13.6)	0 (0.0)	
CpG 2, n (%)			
Methylated	39 (88.6)	20 (90.9)	1.000[c]
Unmethylated	5 (11.4)	2 (9.1)	
CpG 3, n (%)			
Methylated	20 (45.5)	18 (81.8)	0.005[d]
Unmethylated	24 (54.5)	4 (18.2)	
CpG 4, n (%)			
Methylated	28 (63.6)	17 (77.3)	0.262
Unmethylated	16 (36.4)	5 (22.7)	
CpG 5, n (%)			
Methylated	28 (63.6)	17 (77.3)	0.262
Unmethylated	16 (36.4)	5 (22.7)	
CpG 6, n (%)			
Methylated	31 (70.5)	10 (45.5)	0.050
Unmethylated	13 (29.5)	12 (54.5)	

[a]Values are expressed as the means ± standard deviation (SD). [b]P-values were calculated for continuous variables by t-test, and χ^2 was used for categorical variables. [c]P-values were calculated for 2-cell (50.0%) expected count <5 by Fisher's exact test. [d]P<0.01. N, number of individuals; ADAM33, a disintegrin and metalloproteinase domain 33.

Table IV. ADAM33 gene expression and urinary concentrations of EDCs in the participants.

Gene expression	Cases (N=44)	Controls (N=22)	P-value[d]	
ADAM33 gene ± SD[a]	0.83±1.04	3.29±6.56	0.007[f]	

EDCs[b]	Cases (N=44)	Controls (N=22)	P-value[d]	FDR P-value
BPA[c]	14.17 (8.75, 22.93)	5.95 (3.39, 10.46)	0.033[e]	0.297
MEP[c]	41.48 (26.53, 64.86)	40.14 (24.66, 65.35)	0.925	0.948
MBP[c]	27.43 (20.21, 37.22)	28.89 (20.67, 40.37)	0.831	0.948
MIBP[c]	20.95 (14.08, 31.18)	25.02 (16.89, 37.06)	0.566	0.948
MEHP[c]	19.07 (14.44, 25.17)	14.24 (10.46, 19.37)	0.189	0.851
MEHHP[c]	10.71 (7.47, 15.36)	10.39 (7.09, 15.23)	0.914	0.948
MECPP[c]	33.61 (23.79, 47.48)	39.82 (29.46, 53.83)	0.519	0.948
MEOHP[c]	12.13 (8.79, 16.75)	11.93 (8.20, 17.35)	0.948	0.948
Σ_4MEHP[c]	83.89 (63.47, 110.89)	78.76 (58.15, 106.69)	0.775	0.948

The urinary concentration unit of EDCs and ADAM33 gene expression were log-transformed for the analysis. [a]Values are expressed as the means ± standard deviation (SD). [b]The urinary concentration unit of BPA and phthalate metabolites was μg/g creatinine. [c]Values are expressed as the geometric means (95% confidence interval). [d]P-values were calculated for continuous variables by t-test. [e]P<0.05, [f]P<0.01. N, number of individuals; BPA, bisphenol A; EDCs, endocrine-disrupting chemicals; FDR, false discovery rate; MEP, monoethyl phthalate; MBP, mono-n-butyl phthalate; MIBP, mono-isobutyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; Σ_4MEHP, the sum of MEHP, MECPP, MEHHP, and MEOHP urinary concentrations; ADAM33, a disintegrin and metalloproteinase domain 33.

Table V. Association of urinary concentrations of EDCs, intron 1 methylation profile in ADAM33 gene, and ADAM33 gene expression.

	Cases (N=44)				Cases (N=44)			
	Univariate model				Univariate model			
	Methylation status of intron 1				ADAM33 gene expression			
EDCs[a]	Coefficient (95% CI)	Std. β	P-value[b]	FDR P-value	Coefficient (95% CI)	Std. β	P-value[b]	FDR P-value
BPA	0.003 (-0.050, 0.055)	0.017	0.912	0.912	-0.034 (-0.198, 0.131)	-0.066	0.681	0.764
MEP	0.046 (-0.012, 0.105)	0.245	0.118	0.168	-0.081 (-0.265, 0.102)	-0.146	0.375	0.680
MBP	0.048 (-0.036, 0.132)	0.177	0.256	0.288	-0.040 (-0.310, 0.229)	-0.049	0.764	0.764
MIBP	0.050 (-0.014, 0.114)	0.240	0.121	0.168	-0.060 (-0.261, 0.140)	-0.099	0.545	0.701
MEHP	0.070 (-0.022, 0.161)	0.234	0.131	0.168	0.169 (-0.116, 0.454)	0.192	0.236	0.680
MEHHP	0.091 (0.025, 0.158)	0.398	0.008[d]	0.039[c]	0.114 (-0.107, 0.335)	0.167	0.302	0.680
MECPP	0.090 (0.020, 0.161)	0.377	0.013[c]	0.039[c]	0.081 (-0.155, 0.316)	0.112	0.492	0.701
MEOHP	0.086 (0.010, 0.162)	0.335	0.028[c]	0.063	0.110 (-0.137, 0.358)	0.145	0.373	0.680
Σ_4MEHP	0.116 (0.030, 0.203)	0.391	0.010[c]	0.039[c]	0.127 (-0.161, 0.415)	0.143	0.378	0.680
Methylation status of intron 1					1.147 (0.268, 2.027)	0.389	0.012[c]	

	Controls (N=22)				Controls (N=22)			
	Univariate model				Univariate model			
	Methylation status of intron 1				ADAM33 gene expression			
EDCs[a]	Coefficient (95% CI)	Std. β	P-value[b]	FDR P-value	Coefficient (95% CI)	Std. β	P-value[b]	FDR P-value
BPA	-0.032 (-0.104, 0.040)	-0.216	0.360	0.658	-0.174 (-0.681, 0.334)	-0.185	0.477	0.537
MEP	0.056 (-0.012, 0.124)	0.359	0.101	0.658	-0.701 (-1.171, -0.231)	-0.620	0.006[d]	0.054
MBP	-0.011 (-0.117, 0.095)	-0.050	0.826	0.826	-0.917 (-1.729, -0.105)	-0.514	0.029[c]	0.131
MIBP	0.025 (-0.065, 0.115)	0.128	0.570	0.658	-0.103 (-0.873, 0.668)	-0.071	0.781	0.781
MEHP	-0.031 (-0.145, 0.084)	-0.123	0.585	0.658	-0.789 (-1.701, 0.122)	-0.417	0.085	0.255
MEHHP	0.052 (-0.038, 0.142)	0.261	0.240	0.658	-0.442 (-1.172, 0.288)	-0.305	0.218	0.366
MECPP	0.047 (-0.068, 0.163)	0.188	0.403	0.658	-0.357 (-1.327, 0.614)	-0.191	0.447	0.537
MEOHP	0.039 (-0.054, 0.133)	0.193	0.389	0.658	-0.434 (-1.182, 0.314)	-0.294	0.236	0.366
Σ_4MEHP	0.040 (-0.076, 0.156)	0.158	0.481	0.658	-0.538 (-1.480, 0.404)	-0.290	0.244	0.366
Methylation status of intron 1					-0.682 (-4.253, 2.888)	-0.101	0.691	

The urinary concentration unit of EDCs and ADAM33 gene expression were log-transformed for the analysis. [a]The urinary concentration unit of BPA and phthalate metabolites was μg/g creatinine. [b]P-values were calculated for continuous variables by linear regression. [c]P<0.05, [d]P<0.01. N, number of individuals; BPA, bisphenol A; EDCs, endocrine-disrupting chemicals; FDR, false discovery rate; MEP, monoethyl phthalate; MBP, mono-*n*-butyl phthalate; MIBP, mono-isobutyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; Σ_4MEHP, the sum of MEHP, MECPP, MEHHP, and MEOHP urinary concentrations; 95% CI, 95% confidence interval; Std. β, standardized coefficients β; ADAM33, a disintegrin and metalloproteinase domain 33.

MEOHP and Σ_4MEHP (the sum of MEHP, MECPP, MEHHP and MEOHP). We suggest that the secondary metabolites of DEHP are related to the increase in intron 1 methylation and ADAM33 expression, which is associated with a reduction in breast cancer risk. The concentration of BPA was higher in the cases, thus we suggest that BPA exposure could be associated with breast cancer. MEP and MBP were inversely correlated with ADAM33 expression in the controls, which may be correlated with breast cancer.

Cancer development is affected by environmental factors, lifestyle, and genetic mutations, the interaction between tumor cells, their surrounding stroma and transmembrane proteins altered via epigenetics. The tumor stroma primarily comprises the basement membrane, endothelial cells, extracellular

matrix (ECM), fibroblasts, immune cells, inflammatory cells and vasculature, and it plays an important role in cancer progression and metastasis (30). Yang *et al* predicted that transcriptional activity of the ADAM33 gene promoter was associated with the region between bp -550 to +87 (31). Exon 1 in ADAM33 is responsible for the translation of the signal sequence and inserts proper localization in endoplasmic reticulum during protein synthesis (8,32). If ADAM33 pre-mRNA splicing is incomplete, the mis-localization of ADAM33 may result in the loss of cell adhesion and the development of disease. Methylation of the ADAM33 gene promoter may function as a molecular marker for distinguishing invasive lobular carcinoma (ILC) from invasive ductal carcinoma (IDC) and this suggests that ADAM33 is a novel tumor-suppressor gene (9). Hypermethylation of the promoter in the ADAM23 gene is strongly associated with decreased mRNA and protein expression (16). Early growth response 2 (EGR2) functions as a tumor suppressor and its expression in human tumors and cancer cell lines is often decreased; additionally, a high level of methylation in intron 1 of EGR2 could upregulated EGR2 gene expression (33). This study also demonstrated that methylation levels at CpG site 3 in intron 1 were significantly higher in the control group, with CpG site 3 in intron 1 being located 21 bp downstream of exon 1 end. We also found that the ADAM33 expression in controls was significantly higher and positively associated with intron 1 methylation; therefore, we suggest that the overall decrease in intron 1 methylation was related to the reduction of ADAM33 expression and may be associated with the development of breast cancer.

Ligand activation of PPARγ is associated with differentiation of adipocytes, lipid accumulation, and a reduction in growth in breast cancer (34,35). One study showed that PPARα and PPARγ were induced by MBzP, MBuP and MEHP (36). MEHP activated both human PPARα and PPARγ rather than PPARβ whereas MBP could not activate any PPAR isoforms (37). López-Carrillo *et al* indicated that MBP, MBzP and MCPP were inversely associated with breast cancer (38). At present, only two studies have been found to investigate the correlation between ADAM10 and ADAM17 and BPA. ADAM17 is implicated in the shedding of membrane receptors, and as BPA and nonylphenol (NP) were found to stimulate the shedding of heparin-binding epidermal growth factor (HB-EGF) via activation of ADAM17 or ADAM10, this mechanism may be a potential target for the treatment of disease (39). Another study suggested that BPA and NP could induce germ cell apoptosis regulated by the activation of ADAM17 and p38 MAPK (40); however, no study has been conducted to investigate the relationship between ADAM33 and EDCs. According to the above-mentioned literature, another potential underlying mechanism may exist to explain the positive association between secondary metabolites of DEHP and intron 1 methylation. We suggest that MEHHP, MECPP, MEOHP and Σ_4MEHP may have a protective effect on reducing the risk of breast cancer by increasing intron 1 methylation to increase ADAM33 expression.

Phthalates and BPA may dysregulate tumor-suppressor gene (TSG) and breast cancer by epigenetics. When the gene expression of TSG is defective, it can amplify the effect of BPA on tumor induction (41). Patients with BRCA1 mutation are more sensitive to BPA exposure and show an increased

number of invasive masses (42). Furthermore, one study suggested that phthalates could active the aryl hydrocarbon receptor (AhR), upregulate HDAC6 and c-Myc oncogenes and induce proliferation of ER- breast cancer (43). Fetal BPA exposure was found to alter DNA methylation in rat mammary glands and appeared to change stromal-epithelial interactions in the fetal mammary gland, associated with development of pre-neoplastic and neoplastic lesions during adulthood (44). Breast cancer MCF7 cells treated with BBP resulted in the demethylation of estrogen receptor α (ERα) promoter, causing ERα gene re-expression (45). A review study about BPA and phthalates on epigenetic effects showed that BPA and phthalate caused variant methylation levels in different genes and species (17); therefore, the region associated with methylation is a critically important factor in breast cancer. Regardless of the relative degree of methylation, hypermethylation or hypomethylation is an abnormal methylation phenomena in different sequences such as introns or exons that are likely to cause disease (46); nevertheless, the mechanism by which these phenomena regulate gene expression remain unclear, thus the relationship between EDCs and ADAM33 methylation for breast cancer requires further exploration.

Oral administration of BPA in the human body will be quickly metabolized into monoglucuronide and excreted in the urine; thus assessing the concentration of BPA in the urine is considered an appropriate method (47,48). As phthalates in the human body are also rapidly metabolized through hydrolysis and subsequent oxidation reactions and then finally excreted as glucuronides in urine, measures of the urinary concentration of phthalate metabolite could represent the exposure to the respective parent phthalate within 24 h. Since the beginning of the millennium, studies on the investigation of phthalate exposure have increased rapidly by measuring urinary concentrations, thus the concentrations of phthalate metabolites in the urine could be a useful biomarker for phthalate exposure (49,50). The methylation level and gene expression of ADAM33 in this study were measured in the blood, while BPA and phthalate metabolites were evaluated in the urine. Although the two test items were from different samples, BPA is mainly metabolized into BPA-monoglucuronide in the intestine and liver, and then these metabolites reach the kidneys via the blood circulation system, thus PBMC exposure to BPA and its metabolites is unavoidable (51). As a result, we considered it appropriate to evaluate the urinary concentration of BPA and phthalate metabolites to assess the epigenetic effects of EDCs on ADAM33 expression in PBMCs.

Epigenetic profile of circulating white blood cells (WBCs) is directly altered by the toxic components of cigarette smoke entering the bloodstream and this may increase cancer risk (52); therefore, we excluded subjects who were smokers in both the case and control groups to control the potentially confounding effect of smoking. Other limitations of this study should be noted. Firstly, the smoking status and reproductive factors of the breast cancer subjects were evaluated by a self-reported questionnaire, which increases recall bias. Secondly, this was a case-control study; therefore, the causal relationship between the observed differences could not be determined. Thirdly, we excluded subjects with a smoking habit to control a potentially confounding effect; however, we did not consider the potential influence of nutrition on epigenetic status.

Fourthly, the microarray datasets had different criteria to recruit and exclude patients. However, we used an identical gene methylation expression platform (GPL8490 Illumina HumanMethylation27 BeadChip) to reduce any bias from inconsistent relative intensity values for a candidate gene and the quantification of different initial gene sets. Fifthly, only a first-in-the-morning urine sample from each woman was collected. One study also used a single measurement assessment of the exposure to phthalates and believed the frequency of use of these personal products containing phthalates was constant. Thus phthalate metabolites in the urine were considered to be stable concentrations (38). Two first-morning samples of phthalates for 2 consecutive days showed good reproducibility (53). A study also measured a spot urinary sample of BPA to evaluate the effects of BPA on gene expression changes (51). Nepomnaschy *et al* demonstrated that a correlation between urinary samples of BPA over 2 weeks was >0.5, which indicates that the daily exposure in a short time is similar. In addition, the first-morning urine from the same person showed a daily consistency more than a single-point urine sample, because the measurement appeared unaffected by diurnal changes (54). The detection of single-spot urinary samples of BPA and phthalates is a limitation for long-term exposure measurements, but based on the above literature, there was certainly feasibility in detecting the first-morning urinary concentration of BPA and phthalates in our study. A greater number of samples and the implementation over a longer period would be better.

Our study also had several strengths worth noting. Firstly, we used multiple available methylation microarray datasets to identify potential biomarkers, and the DNA region (probe cg14089692) identified in the microarray datasets was verified by nested PCR and bisulfite sequencing in a case-control study. Secondly, unlike other studies using breast cancer biopsy tissue, this study used PBMCs to perform nested PCR to analyze the ADAM33 methylation profile. Early detection of breast cancer can improve its cure rate, but there are currently significant limitations in detecting breast cancer in asymptomatic patients. Sharma *et al* first demonstrated that the gene expression test using peripheral blood cell samples had the potential to detect early stages of breast cancer progression (55). Thus, this non-invasive method, which is not only used in breast cancer patients, can be also used to detect the methylation level of genes to predict the chances of suffering from breast cancer. Finally, all breast cancer subjects were diagnosed by physicians, and their disease pathology was histologically verified.

To the best of our knowledge, this study is the first to explore the epigenetic effects of EDCs on methylation and expression of ADAM33 gene associated with breast cancer. We demonstrated that high methylation of intron 1 in ADAM33 may be related to the elevation in ADAM33 expression and reduced risk of breast cancer and MEHHP, MECPP, MEOHP and Σ_4MEHP may possess protective effects on reducing the risk of breast cancer. We also found that high urinary concentration of BPA may be associated with breast cancer. In addition, MEP and MBP were negatively associated with ADAM33 expression; this result deserves further evaluation.

Further investigations into the methylation of this region are required to validate the prognostic and predictive roles of the ADAM33 gene in breast cancer. In addition, a larger population must be evaluated to determine the role of ADAM33 methylation and epigenetic mechanisms by EDCs in breast cancer and to determine whether the methylation level of intron 1 in ADAM33 has the potential to serve as a diagnostic or prognostic tumor marker in breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

PJY and TNW conceived and designed the study. PJY drafted the manuscript and performed the experiments. EMT, CYP, SSL and CCC were involved in the conception of the study. MFH, FOY and JYK treated the patients and collected the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board (IRB) of Kaohsiung Medical University (IRB no. KMUHIRB-20120104).

Patient consent for publication

Written informed consent was obtained from the study subjects.

Competing interests

The authors declare that they have no competing interests.

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