

PCDHB15 as a potential tumor suppressor and epigenetic biomarker for breast cancer

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Abstract. Breast cancer is among the most frequently diagnosed cancer types and the leading cause of cancer-related death in women. The mortality rate of patients with breast cancer is currently increasing, perhaps due to a lack of early screening tools. In the present study, using The Cancer Genome Atlas (TCGA) breast cancer dataset (n=883), it was determined that methylation of the protocadherin $\beta 15$ (*PCDHB15*) promoter was higher in breast cancer samples than that in normal tissues. A negative association between promoter methylation and expression of *PCDHB15* was observed in the TCGA dataset and breast cancer cell lines. In TCGA cohort, lower *PCDHB15* expression was associated with shorter relapse-free survival times. Treatment with the DNA methyltransferase inhibitor restored *PCDHB15* expression in a breast cancer cell line; however, overexpression of *PCDHB15* was shown to suppress colony formation. *PCDHB15* methylation detected in circulating cell-free DNA (cfDNA) isolated from serum samples was higher in patients

with breast cancer (40.8%) compared with that in patients with benign tumors (22.4%). *PCDHB15* methylation was not correlated with any clinical parameters. Taken together, *PCDHB15* is a potential tumor suppressor in cases of breast cancer, which can be epigenetically silenced via promoter methylation. *PCDHB15* methylation using cfDNA is a novel minimally invasive epigenetic biomarker for the diagnosis and prognosis of breast cancer.

Introduction

Globally, breast cancer is the second most common cancer and the most common cancer in women. In Taiwan, the age-standardized incidence rate (ASIR) of female breast cancer was 70.7 per 100,000 individuals in 2014, and the ASIR gradually increased at a rate of 3.5 per 100,000 person-years (1,2). The age of breast cancer onset is currently 10 years younger than that in Western countries (3). Thus, early detection and early treatment are important objectives for patients with breast cancer (4). At present, diagnosis and disease monitoring primarily involve tissue biopsy and imaging. Tissue biopsy is an invasive method limited to specific regions (5). Mammography is the gold standard for breast cancer detection due to its high sensitivity and specificity; however, numerous women tend to avoid this due to the exposure to radiation (6). A novel minimally invasive biomarker for breast cancer is needed.

Liquid biopsy from bodily fluid, such as circulating cell-free DNA (cfDNA), circulating tumor cells and exosomes, is minimally invasive and has proven both convenient and effective in cancer diagnosis (7,8). The quantity of cfDNA in the blood can be used to characterize tumorigenesis, inflammatory disease and stroke (9). The Epi-proColon blood test for circulating methylated DNA has been approved for the detection of colorectal cancer (10,11). It has been revealed that genomic alterations, such as human epidermal growth factor receptor 2 (HER2) amplification, can serve as a predictive

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biomarker for breast cancer (12); however, the identification of valid diagnostic biomarkers for breast cancer from liquid biopsy is needed.

DNA methylation, occurring at CpG dinucleotides, is a hallmark of cancer (13). As this epigenetic change occurs early in tumorigenesis, it can be used as a biomarker for early detection, disease prognosis and monitoring (14). Several studies have reported the presence of methylated DNA in serum samples from patients with cancer of the gastrointestinal tract, lung, head and neck, liver and breast (15-17). In cases of breast cancer, DNA methylation signature is related to the clinicopathological characteristics of the tumor, such as tumor stage and grade (18,19). It is possible that DNA methylation-mediated epigenetic silencing of tumor suppressors could affect the progression and prognosis of breast cancer (20-22). One previous study demonstrated that hypermethylation of seven biomarkers, including protocadherin $\beta 15$ (*PCDHB15*), could differentiate patients with breast cancer into the high- and low-risk groups (23).

PCDHB15 is a member of the cadherin superfamily and calcium-dependent cell-cell adhesion molecules, which encodes for PCDHB15 protein in humans (24,25). Several cell adhesion molecules, such as CDH1 (also known as E-cadherin), act as epithelial-mesenchymal transition suppressors (26). In this regard, the epigenetic silencing of *CDH1* has frequently been observed in cases of human cancer, including breast cancer (27-29). Nonetheless, the role played by the epigenetic silencing of *PCDHB15* in cases of breast cancer remains unclear. In the present study, *PCDHB15* was identified as a potential tumor suppressor gene in breast cancer, based on the observation that *PCDHB15* expression is positively correlated with the likelihood of relapse-free survival. The detection of *PCDHB15* methylation in serum samples of patients with breast cancer could be a novel minimally invasive biomarker for the diagnosis and prognosis of breast cancer.

Materials and methods

Cell culture. MCF7 and MDA-MB-231 human breast cancer cell lines were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and 50 U/ml penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were incubated under 5% CO₂ at 37°C. DNA demethylation involved treating the cells with 0.1 μ M DNA methyltransferase (DNMT) inhibitor 5'-aza-2'-deoxycytidine, (5aza; Merck KGaA) or DMSO (as control) at 37°C for 72 h. Culture media and drugs were replenished every 24 h. Following treatment, the cells were harvested for RNA analysis.

Patient samples. All patient samples were collected from the Biobank of the Ditmanson Medical Foundation Chiayi Christian Hospital, Chiayi, Taiwan (Table I). The cancer group (age range, 30-78 years) was comprised of patients with confirmed breast cancer, whereas the control group (age range, 20-53 years) was comprised of patients diagnosed with benign tumors. The inclusion criteria were patients >20 years old and who were undergoing biopsy or mastectomy; exclusion criteria were patients who could not undergo any surgery or blood

sampling. Serum samples obtained from patients with breast cancer (n=49) and patients with benign tumors (n=49) were used for quantitative methylation-specific PCR (qMSP) analysis. Briefly, blood samples were drawn into a 10-ml K₂-EDTA blood tube (BD Biosciences) and centrifuged at 1,358 x g at room temperature for 10 min, whereupon the serum was collected and stored at -80°C. The present study was approved (approval no. IRB2019006 on 2019/3/5) by the Institutional Review Board of the Ditmanson Medical Foundation Chiayi Christian Hospital (Chiayi, Taiwan) and performed in strict accordance with approved guidelines. Written informed consent was obtained from all participants.

Plasmid transfection and colony formation. The full-length human *PCDHB15* expression plasmid was a gift from Professor Jun Yu (Chinese University of Hong Kong, Hong Kong). *PCDHB15*-expressing or empty vectors (5 μ g; pCMV6-XL5) were transfected into MDA-MB-231 cells (a triple-negative breast cancer cell line with lower expression of *PCDHB15*) using TransIT-LT1 Transfection Reagent (Mirus Bio LLC) in accordance with the manufacturer's protocol. After 72 h of incubation at 37°C, the transfection reagents were removed and replaced with fresh medium. Transfected cells were cultured in fresh medium at 37°C prior to further experiments.

For the colony-formation analysis, a total of 1x10⁴ transfected cells per well were seeded in three 6-cm dishes with complete culture medium. Cells were cultured in fresh culture medium at 37°C for 5-7 days, and the culture medium was replaced at intervals of 3 days. Surviving colonies were stained with 0.4% crystal violet (solubilized in 50% methanol) at room temperature for 30 min. The number of colonies (as defined by the size of the colonies >5 pixel²) was then calculated by using ImageJ 1.53e software (National Institutes of Health).

RNA extraction and reverse transcription-quantitative PCR. Total RNA was extracted from the MCF7 and MDA-MB-231 cells using TRIzol[®] (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Briefly, 1 μ g of total RNA was treated with DNase I (Amplification grade; Invitrogen; Thermo Fisher Scientific, Inc.), before it underwent reverse transcription. First-strand cDNA synthesis was performed using MMLV Reverse Transcriptase (Epicentre; Illumina, Inc.) with oligo dT primers. Briefly, RNA was denatured and the oligo dT primers were annealed at 65°C for 2 min, then chilled on ice for 1 min. The mixture was gently mixed with dNTP, DTT, RNase inhibitor, X10 RT reaction buffer and MMLV Reverse Transcriptase. The final 20- μ l mixture was incubated at 37°C for 60 min followed by 85°C for 5 min. qPCR was performed using an ABI Step-One real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with specific primers and Power SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The primer sequences were as follows: *PCDHB15* forward, 5'-agccttcaggagaaattcgactaat-3' and reverse, 5'-gcaccttaacagagacagacatttt-3'; and *GAPDH* forward, 5'-ccccttcattgacctcaactacat-3' and reverse, 5'-cgctcctggaagatgtga-3'. Relative gene expression was calculated by comparing the quantification

Table I. Summary of clinipathological data of plasma samples.

Clinicopathological characteristic	Plasma samples	
	Cancer (n=49)	Benign (n=49)
Age, years	55.12±10.59 ^a	34.96±9.92
Histological grade ^b		
Low grade	4	
High grade	37	
Unknown	8	
Estrogen receptor		
-	18	
+	30	
Unknown	1	
Progesterone receptor		
-	20	
+	28	
Unknown	1	
Human epidermal growth factor receptor 2		
-	19	
+	28	
Unknown	2	

^aMean ± SD; ^bGrading: Low grade, G1; high grade, G2-3.

cycle (Cq) value of *PCDHB15* gene against the Cq value of *GAPDH* in a given sample (i.e., $2^{-\Delta\Delta Cq}$) (30).

Extraction and bisulphite conversion of DNA. DNA was extracted from serum samples using the QIAamp Circulating Nucleic Acid kit (Qiagen GmbH) in accordance with the manufacturer's protocol. Extracted DNA was bisulphite-modified using the EZ DNA methylation kit (ZYMO Research Corp.) in accordance with the manufacturer's protocol, as previously described (31).

MSP and qMSP. *PCDHB15* methylation in serum samples was detected by subjecting the bisulphite-modified DNA to MSP and qMSP, as previously described (32). Briefly, 4 μ l bisulphite-converted DNA was subjected to MSP within the specific promoter *PCDHB15* region. The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The PCR products were analyzed by electrophoresis in 10% polyacrylamide gel and subsequent the gel was stained with ethidium bromide. For qMSP analysis, 4 μ l bisulphite-converted DNA was subjected to qMSP within the specific promoter *PCDHB15* region using an ABI Step One real time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Power SYBR Green Master Mix. The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. The primer sequences for *PCDHB15* were as follows: Forward, 5'-acgtttttttaaggaatcg-3'

and reverse, 5'-acgaaccaatctccga-3' (130 bp). The presence of cfDNA in serum samples was detected via collagen type II α 1 chain (COL2A1) MSP using the forward primer 5'-tctacaattataaactccaaccaccaa-3' and the reverse primer 5'-gggaagatgggagtagaagggaat-3'. The quantity of methylated DNA was determined in terms of Cq value against a standard curve generated using an *in vitro* methylated DNA-MSP cloned fragment, as previously described (33).

TCGA data analysis. The Cancer Genome Atlas (TCGA) breast cancer (BRCA) Methylation450K dataset was downloaded from UCSC Xena (<http://xena.ucsc.edu>). The methylation level of CpG sites in *PCDHB15* between solid normal tissues and primary tumor tissues were compared and analyzed. Associations between the expression and methylation (cg17023770) of *PCDHB15* were analyzed.

Statistical analysis. All statistical analysis was performed using GraphPad Prism Version 5.0 software packages for Windows (GraphPad Software, Inc.). Differences between two groups were analyzed using an unpaired Student's t-test or the Mann-Whitney U test. Pearson's correlation analysis was used to analyze correlation between gene expression, methylation status of a gene or protein expression. Locoregional relapse-free survival was assessed by Kaplan-Meier analysis, and differences between groups were estimated by the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PCDHB15 is hypermethylated in breast tumor samples compared with that in normal samples. Breast cancer data from TCGA were first used to analyze DNA methylation profiles for *PCDHB15*. The present study included 785 primary tumors and 98 solid tissue normal samples. The methylation of *PCDHB15* was higher in tumor samples than that in normal solid tissue ($P < 0.001$; Fig. 1A and B). As expected, a negative association between the methylation of a particular CG site (cg17023770) in the promoter region and the expression of *PCDHB15* was observed in this TCGA cohort (Fig. 1C; $r = -0.2$, $P < 0.0001$). Kaplan-Meier Plotter (34) revealed that patients with lower *PCDHB15* expression were associated with shorter relapse-free survival times (Fig. 1D; HR, 0.74; $P = 0.00011$), but not at all with overall survival times (data not shown).

The association between promoter methylation and the expression of *PCDHB15* in breast cancer cell lines was then investigated. It was determined that the expression of *PCDHB15* was downregulated in MDA-MB-231 cells compared with that in MCF7 cells (Fig. 1E). Concomitantly, bisulphite pyrosequencing revealed that promoter methylation was higher in MDA-MB-231 cells than that in MCF7 cells at CpG sites located in the upstream promoter region of *PCDHB15* (Fig. 1F). Notably, the expression of *PCDHB15* in MDA-MB-231 cells was restored upon treatment using DNMT inhibitor (0.1 μ M 5aza; Fig. 1G).

To examine the function of *PCDHB15*, *PCDHB15* was overexpressed in MDA-MB-231 breast cancer cells, showing a lower *PCDHB15* expression, as compared with MCF7 cells. The induced overexpression of *PCDHB15*

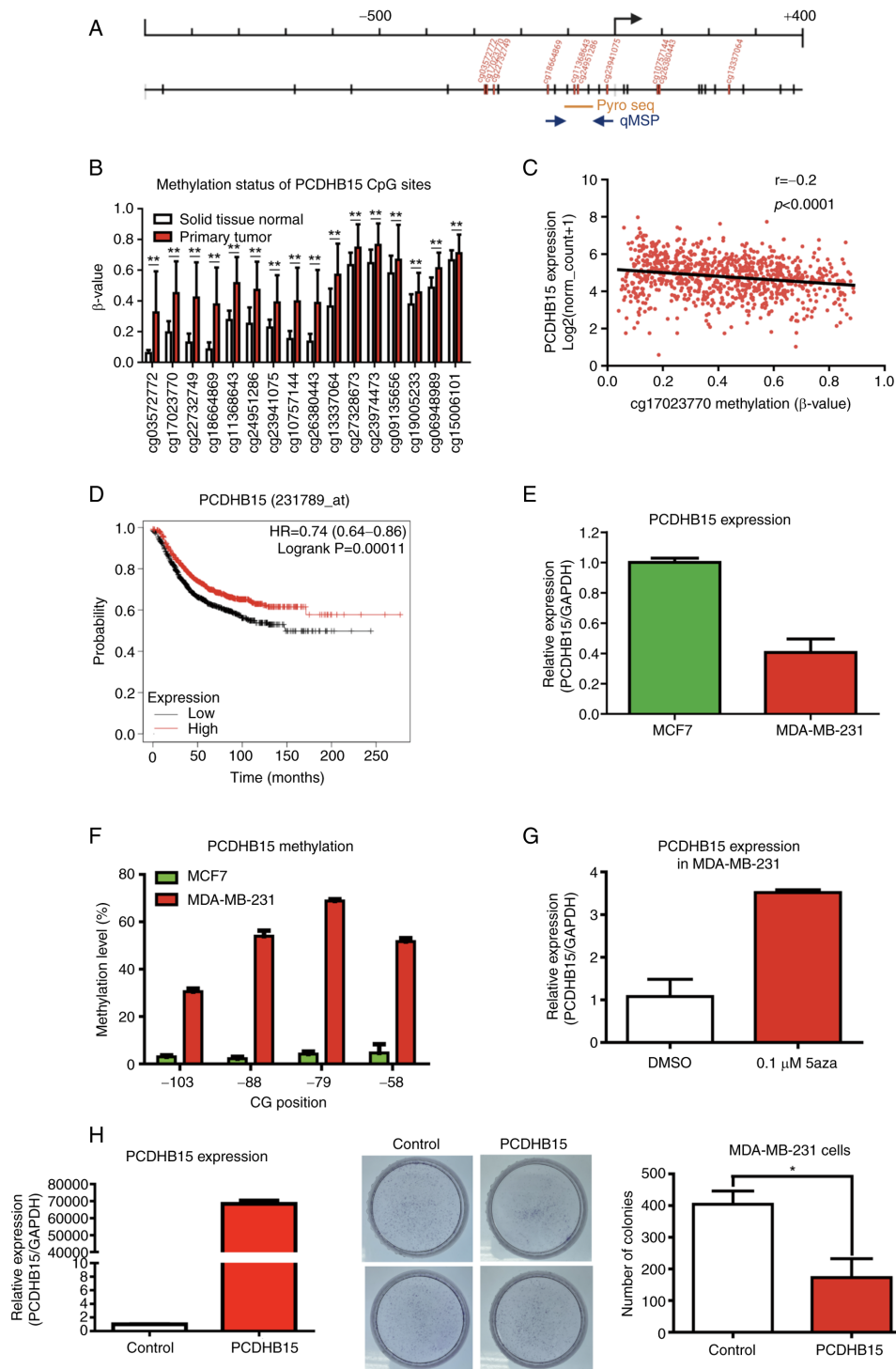


Figure 1. *PCDHB15* may be a tumor suppressor gene that is epigenetically silenced in breast cancer. (A) Schematic diagram depicting the genomic structure and position of the CpG sites (vertical dashes) in the *PCDHB15* promoter region (from -1,000 to +400 with respect to the transcriptional start site). The location of the microarray probes (red vertical dashes), bisulphite pyrosequencing (yellow horizontal line) and qMSP primers (blue solid arrows) are indicated. (B) DNA methylation level (β-value from Illumina Infinium 450 K microarray) of the *PCDHB15* CpG island from -278 (cg03572772) to +2252 (cg15006101) in solid tissue normal (white) vs. primary tumor (red) in TCGA breast cancer dataset. Primary tumor tissues (n=785) had higher methylation levels than solid tissue normal tissues (n=98). The x-axis indicates the name of the probe on the microarray. (C) Scatter plot showing correlation between *PCDHB15* promoter methylation (cg17023770; x-axis) and expression (y-axis) in TCGA breast cancer dataset (n=873). A negative association between promoter methylation and expression was observed. (D) Kaplan-Meier analysis of *PCDHB15* mRNA expression in tumor tissues for relapse-free survival of patients with breast cancer. Patients with breast cancer with lower *PCDHB15* expression demonstrated shorter relapse-free survival times than patients with higher *PCDHB15* expression (log-rank test, P=0.00011). (E) Relative expression level of *PCDHB15* mRNA in MCF7 and MDA-MB-231 breast cancer cells. (F) Methylation analysis of *PCDHB15* promoter in breast cancer cell lines using bisulphite pyrosequencing. (G) Relative expression level of *PCDHB15* in 0.1 μM 5-aza-treated MDA-MB-231 breast cancer cells, compared with DMSO control. (H) Ectopic expression of *PCDHB15* inhibited tumor proliferation by colony formation assay. MDA-MB-231 breast cancer cells were transfected with empty (control) or *PCDHB15* expression vector. Left panel, reverse transcription-quantitative PCR confirmed overexpression of *PCDHB15* in MDA-MB-231 cells transiently transfected with *PCDHB15* expression vector. Middle panel, MDA-MB-231 breast cancer cells overexpressing *PCDHB15* had significantly fewer colonies than the control. Right panel, quantitative analysis of the colony formation assay. Colony formation assay were performed in duplicate and in two independent experiments (mean ± SD). *P<0.05 and **P<0.001. PCDHB15, protocadherin β15; TCGA, The Cancer Genome Atlas; 5-aza, 5'-aza-2'-deoxycytidine; qMSP, quantitative methylation-specific PCR.

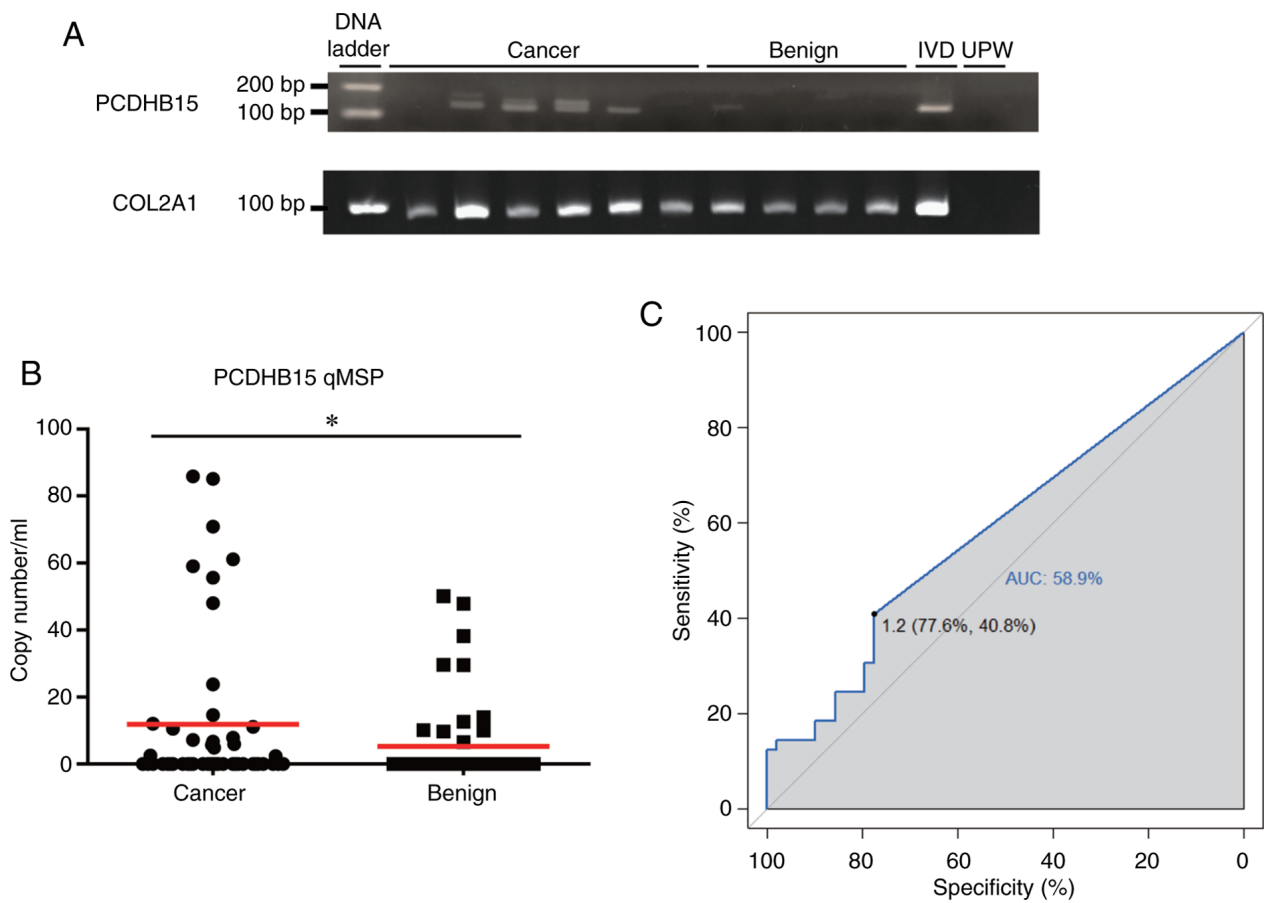


Figure 2. Methylated *PCDHB15* level is higher in cfDNA of serum samples of patients with breast cancer. (A) Gel electrophoresis image of *PCDHB15* (upper panel) and *COL2A1* (lower panel) MSP in cfDNA isolated from serum samples. IVD was a positive control for methylation and H₂O was a negative control for PCR. (B) qMSP was performed to determine the amount of methylated *PCDHB15* in cfDNA of breast cancer (n=49) and benign tumor samples (n=49). Compared with the benign tumor samples, higher amounts of *PCDHB15* were detected in cancer samples. (C) A receiver operating characteristic curve of *PCDHB15* methylation in serum samples from 49 patients with breast cancer and 49 patients with benign tumors. The original uncropped gel electrophoresis images can be found in supplementary Fig. S1. *P<0.05. *PCDHB15*, protocadherin $\beta 15$; qMSP, quantitative methylation-specific PCR; cfDNA, cell free DNA; IVD, *in vitro* methylated DNA; AUC, area under the curve; *COL2A1*, collagen type II $\alpha 1$ chain.

in MDA-MB-231 cells led to a significant decrease in the number of colonies, compared with the control vector (P<0.05; Fig. 1H). Taken together, these results suggested that *PCDHB15* may be a tumor suppressor subject to epigenetic silencing via promoter hypermethylation in breast cancer.

Measuring *PCDHB15* methylation in clinical human serum specimens. The present study also sought to determine whether *PCDHB15* methylation could be used as a serum biomarker for breast cancer. MSP revealed *PCDHB15* methylation in 66% (4/6) of cfDNA extracted from serum samples of patients with breast cancer, but only in 25% of the samples (1/4) from patients with benign tumors (Figs. 2A and S1). The presence of *COL2A1* MSP products suggested the presence of the cfDNA in serum samples that were devoid of *PCDHB15* methylation (Fig. 2A). The *PCDHB15* methylation was further examined using qMSP in serum samples from 49 patients with cancer and 49 patients with benign tumors (Table I). The quantity of methylated *PCDHB15* was higher in patients with breast cancer than that in samples from patients with benign tumors (P<0.05; Fig. 2B). Based on the cutoff value generated by the area under the receiver

operating characteristic curve (0.589; a cutoff value of 1.2 copy number/ml), *PCDHB15* methylation in serum samples provided a sensitivity of 40.8% and specificity of 77.6% in breast cancer detection (Fig. 2C; Table II). These results demonstrated the feasibility of using *PCDHB15* methylation in the cfDNA of serum samples as a minimally invasive biomarker for breast cancer.

PCDHB15 methylation is not correlated with other clinicopathological features. Several proteins, including HER2, Ki-67, estrogen receptor and progesterone receptor, are important parameters in subtyping breast cancer and pathogenesis characterization (6). Nonetheless, our analysis did not reveal any correlation between *PCDHB15* methylation and any of those clinical parameters (Fig. 3A-D).

Discussion

PCDHB15 of the protocadherin superfamily is involved in calcium-dependent cell-cell adhesion. The epigenetic silencing of other cadherins (e.g., E-cadherin) has previously been demonstrated; however, the role of *PCDHB15* in breast cancer has yet to be fully understood. In the current

Table II. Summary of protocadherin $\beta 15$ quantitative methylation-specific PCR analysis.

Diagnosis	Valid specimens (n)	Positive specimens (n)	Negative specimens (n)	Positive rate (%)
Breast cancer	49	20	29	40.8
Low grade (G1)	4	1	3	25.0
High grade (\geq G2)	37	13	24	35.1
Unknown	8	6	2	75.0
Benign	49	11	38	22.4

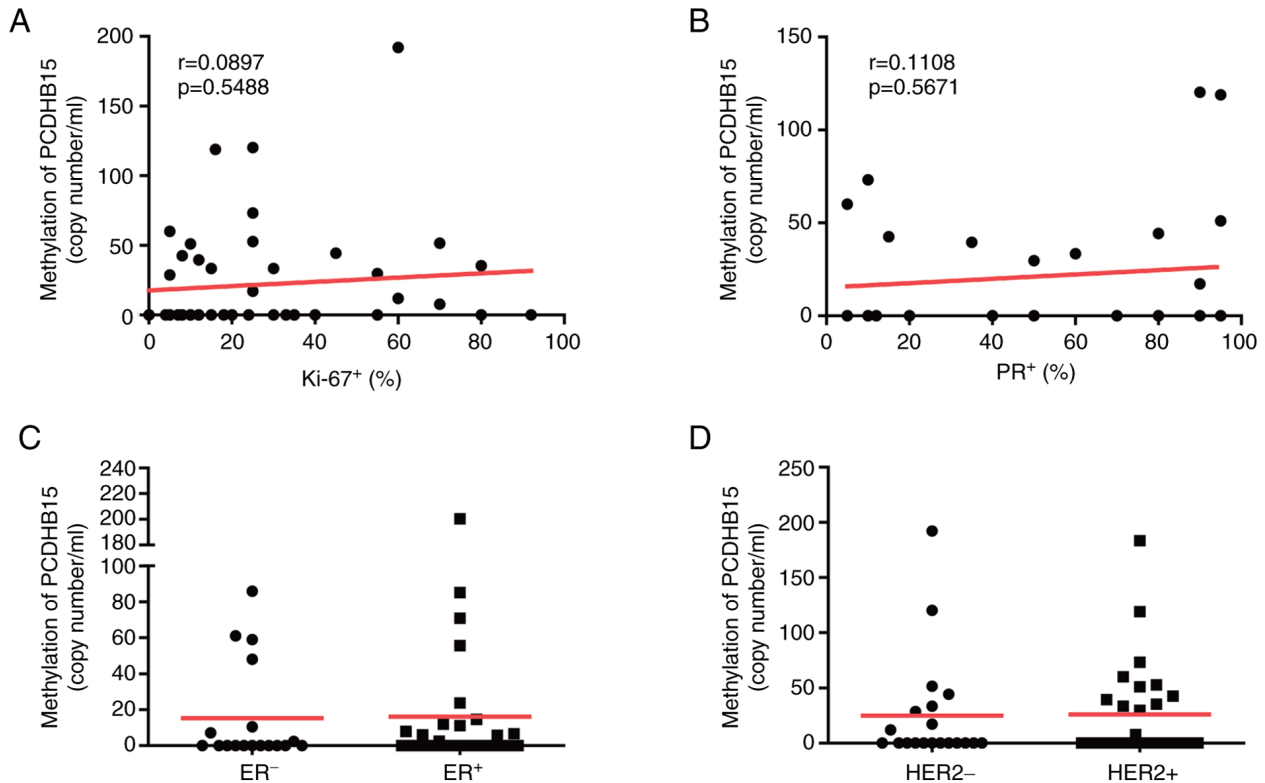


Figure 3. *PCDHB15* promoter methylation and the clinicopathological features of breast cancer are not associated. (A and B) Scatter plot showing the correlation between *PCDHB15* promoter methylation in cfDNA and expression level of (A) Ki67 and (B) PR in breast tumor tissues. (C and D) Dot plot showing the amount of methylated *PCDHB15* in cfDNA with expression status of ER (ER⁺, ER⁻) and HER (HER⁺, HER⁻). *PCDHB15*, protocadherin $\beta 15$; PR, progesterone receptor; cfDNA, cell free DNA; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

study, based on the TCGA database and in-house samples, it was determined that *PCDHB15* methylation was more pronounced in patients with breast cancer compared with that in patients with benign tumors or in normal controls. Overall, the higher expression level of *PCDHB15* was positively associated with relapse-free survival. Further analysis on specific cell lines revealed that *PCDHB15* may be a tumor suppressor downregulated via promoter hypermethylation. It is also noteworthy to point out that the correlation between *PCDHB15* methylation and relapse-free survival could not be determined, as DNA methylation data was not available from the Kaplan-Meier Plotter. The prognostic significance of *PCDHB15* methylation requires further investigation. It was recently reported that *PCDHB15*, acting as a tumor suppressor through the inhibition of WNT/ β -catenin signaling, was epigenetically silenced in KRAS-mutated colorectal cancer (35). This

phenomenon can perhaps be attributed to the overexpression of mitochondria glutamate transporter, *SLC25A22*, in KRAS-mutated colorectal cancer. Nonetheless, determining whether the overexpression of *SLC25A22* is responsible for *PCDHB15* methylation in breast cancer is worthy of further investigation.

In the present study, *PCDHB15* methylation was higher in cfDNA from serum samples of patients with breast cancer than that from patients with benign tumors. Nonetheless, *PCDHB15* methylation was not associated with any clinical parameters, thereby suggesting that *PCDHB15* methylation may occur early in the carcinogenesis. It also suggested that *PCDHB15* methylation is common to all molecular subtypes of breast cancer. The fact that 22.4% (11 out of 49) of the benign tumor samples tested positive for *PCDHB15* methylation supports these hypotheses; however, further clinical analysis using a larger sample size and different molecular subtypes will be

required to demonstrate whether *PCDHB15* methylation is involved in the field defect of breast cancer (36).

The sensitivity of *PCDHB15* methylation (40.8%) in breast cancer detection in serum would not allow its use as a sole biomarker; however it could potentially serve as one epigenetic biomarker in a 'methylation signature panel' for the diagnosis and/or prognosis of breast cancer (37-39). Previous studies have identified distinct methylation biomarkers indicating the signaling-mediated epigenetic silencing of tumor suppressors, regardless of different subtypes of breast (40-42). Multiple research groups are currently evaluating the methylation signature panel of *GSTPI*, *RASSF1* and *RARB* for the detection of breast cancer (29). The *PITX2* methylation assay, which has been already certified for *in vitro* diagnosis, has proven effective as a prognostic and predictive biomarker for breast cancer (43). It is possible that methylation of *PCDHB15* could be used in conjunction with these markers to form a novel epigenetic panel by which to characterize the progression of breast cancer.

In conclusion, *PCDHB15* is a potential tumor suppressor subject to epigenetic silencing via promoter methylation in breast cancer. *PCDHB15* methylation in serum cfDNA may provide a novel minimally invasive epigenetic biomarker for the diagnosis and prognosis of breast cancer. Determining whether *PCDHB15* methylation is involved in the early carcinogenesis of breast cancer warrants further investigation.

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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

CCC, GLL, CWT, CFW and YCH collected patient samples and analyzed clinical data. GLL, WLH, FCW, PJJ, WHH, YMC and YTL performed experiments. YMC, SY Y and MWYC performed the bioinformatic analysis. GLL, SY Y and MWYC wrote the manuscript. CCY, YCH and MWYC designed the experiments. GLL, WLH and MWYC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. IRB2019006 on 2019/3/5) by the Institutional Review Board of the Ditmanson Medical Foundation Chiayi Christian Hospital (Chiayi, Taiwan). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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