

Automatic Detection of the Circulating Cell-Free Methylated DNA Pattern of *GCM2*, *ITPRIPL1* and *CCDC181* for Detection of Early Breast Cancer and Surgical Treatment Response

Sheng-Chao Wang, Li-Min Liao, Muhamad Ansar, Shih-Yun Lin, Wei-Wen Hsu and Chih-Ming Su,
Yu-Mei Chung, Cai-Cing Liu, Chin-Sheng Hung and Ruo-Kai Lin

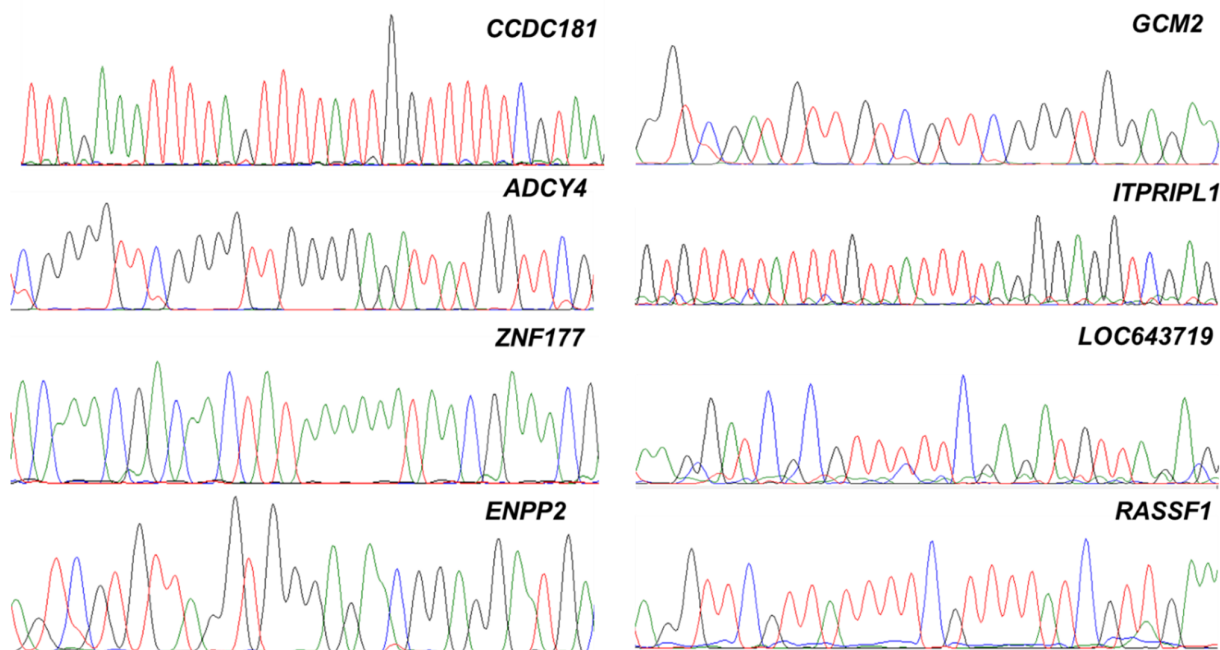


Figure S1. Representative standard sequencing diagram for bisulfite direct sequencing of the *CCDC181*, *GCM2*, *ITPRIPL1*, *ENPP2*, *LOC643719*, *ZNF177*, *ADCY4* and *RASSF1* genes.

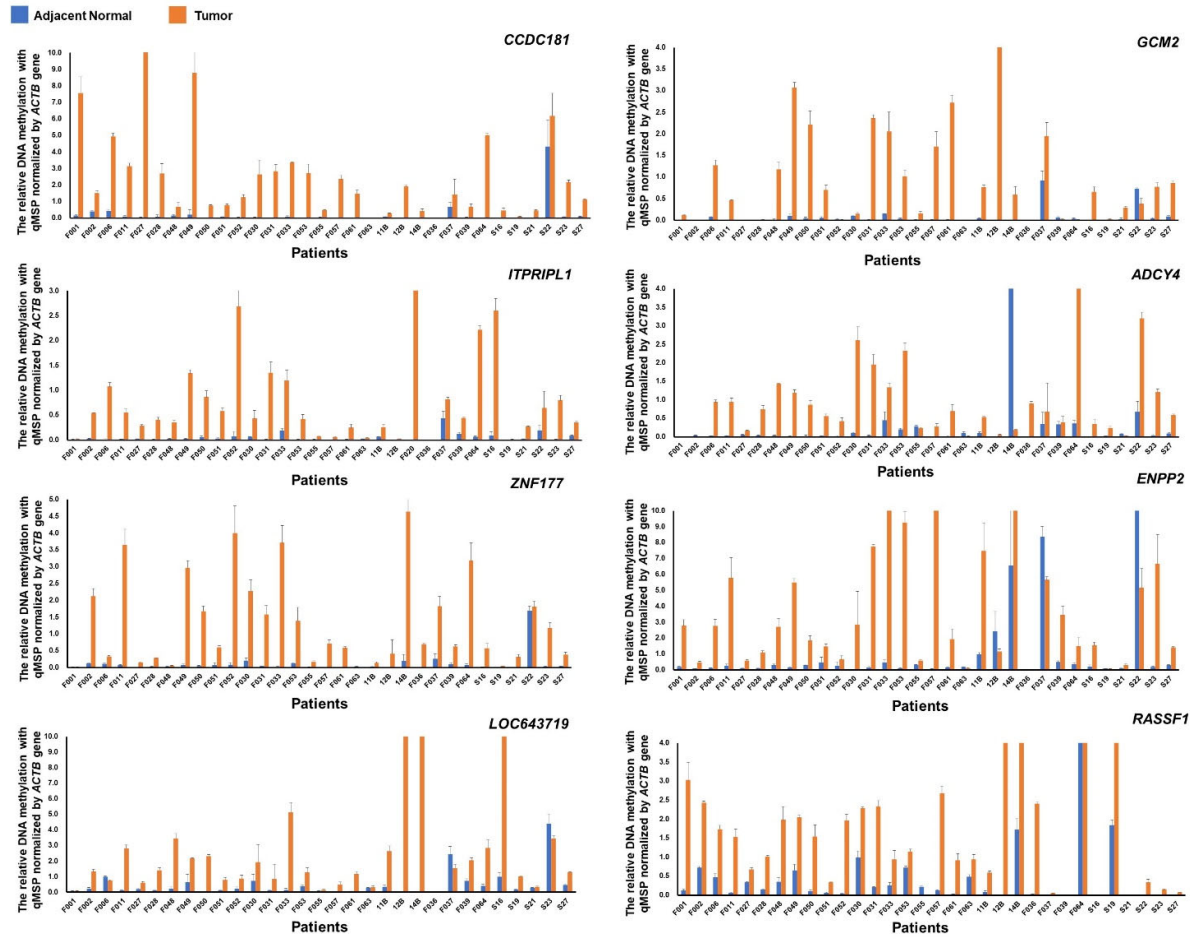


Figure S2. Representative figures showing the DNA methylation levels of the candidate genes *CCDC181*, *GCM2*, *ITPRIPL1*, *ENPP2*, *LOC643719*, *ZNF177*, *ADCY4* and *RASSF1* using qMSP in breast cancer patients.

Table S1. The clinical parameters of breast cancer patients for plasma ccfDNA analysis.

Characteristics	Manual 0.2 mL Plasma	Manual 0.5 mL Plasma	Automatic Labturbo 1.6 mL Plasma	Automatic Duo Prime 1.6 mL Plasma
	N (%)	N (%)	N (%)	N (%)
Overall	45	34	63	57
Type				
≤45	19(42.2)	13(38.2)	9(14.3)	10(17.5)
>45	26(57.8)	21(61.8)	54(85.7)	47(82.5)
Type				
DCIS	3(6.7)	10(29.4)	7(11.1)	1(1.8)
IDC	40(88.9)	22(64.7)	47(74.6)	48(84.2)
ILC	1(2.2)	2(5.9)	1(1.6)	2(3.5)
Others	1(2.2)	0	8(12.7)	6(10.5)
Tumor Stage				
0, I and II	32(71.1)	29(85.3)	52(82.5)	44(77.2)
III and IV	13(28.9)	5(14.7)	11(17.5)	13(22.8)
Tumor Size				
T0–T2	38(84.4)	31(91.2)	56(88.9)	49(86.0)
T3–T4	7(15.6)	3(8.8)	7(11.1)	8(14.0)
Lymph node				
N = 0	16(35.6)	22(64.7)	31(49.2)	30(52.6)
N > 0	29(64.4)	12(35.3)	32(50.8)	27(47.4)
ER				
Negative	21(46.7)	7(20.6)	19(30.2)	19(33.3)
Positive	24(53.3)	27(79.4)	44(69.8)	38(66.7)
PR				
Negative	20(44.4)	11(32.4)	21(33.3)	22(38.6)
Positive	25(55.6)	23(67.6)	42(66.7)	35(61.4)
HER2				
Negative	35(77.8)	26(76.5)	46(73.0)	35(61.4)
Positive	10(22.2)	8(23.5)	17(27.0)	22(38.6)
Ki-67				
High	24(53.3)	24(70.6)	39(61.9)	39(68.4)
Low	21(46.7)	10(29.4)	24(38.1)	12(21.1)
n.d.	-	-	-	6(10.5)

Table S2. List of primer sequences and conditions used in the present study.

Gene	Primer	5'→3'Sequences	Application	Size (bp)	Tm (°C)
<i>BACTIN</i>	Forward	TGGTGATGGAGGAGGTTTAGTAAGT	Input DNA control (No CpG)	132	60
	Reverse	AACCAATAAAACCTACTCCTCCCTTAA			
	Probe	ACCACCACCCAACACACAATAACAAACACA			
<i>CCDC181</i>	Forward	TTTTATTGGTTTTTCGTAAGTATCG	MSP-M	143	60
	Reverse	CATAACAACAACGTACCTCTACGTC			
	Probe	TCGGGAGGGGTCGGTGGTTTGAG			
<i>GCM2</i>	Forward	GAGATAGGGCGGAGTTTTTC	MSP-M	105	60
	Reverse	CTTAACCGCGATACTAAACGTT			
	Probe	TCCACCCGAACGACAACATCGACC			
<i>ITPRIPL1</i>	Forward	GAGTGTAGTTGATAGTAGGTACGGC	MSP-M	106	60
	Reverse	GTAAATTTACTAAAAAATAAAAAAACCGT			
	Probe	CACACTCTCCGCTACTCGACCTCCCTA			
<i>ZNF177</i>	Forward	TTTAGTTGTTGGTCGGAAGC	MSP-M	128	60
	Reverse	CGACCTCACTAATAAAACGCA			
	Probe	AACGAAACGACGAACGCCCACTTC			
<i>LOC643719</i>	Forward	CCTCTAACATCTCGAAAAACG	MSP-M	116	60
	Reverse	TGGAGTGTTATAAATTTATTATCGT			
	Probe	TC GGG TTC GT TTT TAG GAT ACG GAG TT			
<i>ENPP2</i>	Forward	TAAAAGGTTTTTTAAGAATTTTCGA	MSP-M	101	60
	Reverse	TAAAAATCAAACATATCCCCCG			
	Probe	TCCCACCTAACACGACTAAAACGAACT			
<i>ADCY4</i>	Forward	GAGAAAAAGTTTAGGTGGGGTTC	MSP-M	148	60
	Reverse	TAAATCTCGTAAAAAAAATCTTCGC			
	Probe	CCCCAACCCCGAACCCCGAAA			
<i>RASSF1</i>	Forward	GGTAGTTAAGGGGTAGCGTAGTC	MSP-M	105	60
	Reverse	TTCAACGATAAAACGAAAATAACG			
	Probe	CCCCTCTACCGCGACTTAACCCGC			

Table S3. Gene list of the 160 hypermethylated genes that were commonly found in Taiwanese and TCGA cohorts.

Status	Genes
Aberrant DNA methylated genes in cancers reported previously	AHRR, BARHL2, BOLL, C12orf68, C14orf23, C17orf64, C9orf122, CCDC36, CCDC8, CLIP4, CPXM1, CRHR2, CRYGD, CSDAP1, DPP6, GNG4, HLA-L, HOXA4, HOXD8, ILDR2, KCNK9, MIR129-2, MMP9, MYO15B, NES, NPTX2, NRXN1, NT5E, OLIG3, OTX2OS1, PGLYRP2, PHOX2A, PRDM14, RCN3, SCG5, SCRT2, SEMA6C, SKI, SLITRK1, SOX2OT, SPTBN4, TBR1, TIMP2, TTBK1, TTC28, TULP1, TXNRD1, VWC2, ZNF454, ZNF572
Breast cancer-associated genes reported previously	CPEB1, CYTL1, DOCK2, ESRRG, FLI1, FRZB, GRASP, GRIA1, HOXD9, LHX1, MAML3, MEIS2, NCALD, NKX2-1, NPAS4, OCA2, PDX1, RGS17, RGS20, SALL1, SSTR4, TMEM97, TNFAIP8L3, TRABD, VGLL4, WNT3A
Novel aberrant DNA methylated genes	ADCY4, ALX1, BCAT1, C12orf42, C1orf114 (CCDC181), CFTR, CHST11, CHST3, CLDN9, CLEC14A, COL11A2, CRYM, CSMD3, DBX1, DMRTA2, DNM3, DPF1, EBF1, EMX1, ENPP2, EPHX3, EVX2, F2RL3, FAM38B, FOXD3, FSD1, GALR1, GCK, GCM2, GJD2, GRIN1, H2AFY, HCK, HNF1B, HPCAL4, HTR6, IRX1, ITGA5, ITPRIPL1, KCNC3, LHX4, LHX8, LOC643719, LOC646999, MIR663, NID2, NR5A2, NRXN2, NXPH1, OTX1, OTX2, PITX2, POU3F3, POU4F2, PRDM13, PRKAR1B, PRKCB, PRKCE, PRRT1, PTPRN, RNF220, SEZ6L2, SIM1, SLC23A2, SNAP25, SOSTDC1, SRGAP3, SRRM3, SSPO, TAC1, TFAP2B, TLX1, TMEM145, TRH, TRIM46, TRIM71, TRIP10, VANGL2, WIT1, ZIC5, ZNF177, ZNF662, ZSCAN18, TBXT

Table S4. The functions of the candidate hypermethylated genes.

Gene	Functions
<i>C1orf114</i> (<i>CCDC181</i>)	<i>CCDC18</i> is a microtubule-binding protein and a structural component of cilia and sperm flagella [78].
<i>GCM2</i>	<i>GCM2</i> is a gene encoding a transcription factor required for parathyroid development. The C-terminal conserved inhibitory domain mutation of <i>GCM2</i> can cause primary hyperparathyroidism [80].
<i>ITPRIPL1</i>	Unknown
<i>ZNF177</i>	Unknown
<i>ADCY4</i>	<i>ADCY4</i> is a member of the family of adenylate cyclases, mediating cyclic adenosine monophosphate (cAMP) synthesis inhibition of caspase-11 inflammasome activation in macrophages [81]
<i>ENPP2</i>	Autotaxin (<i>ATX</i> , <i>ENPP2</i>) is a secreted glycoprotein that catalyzes the extracellular production of lysophosphatidic acid (LPA), a growth-factor-like phospholipid that is further regulated by phospholipid phosphatases (PLPP) [79].
<i>LOC643719</i> (<i>SCGB1B2P</i>)	Pseudogene

Table S5. The methylation level of *CCDC181*, *GCM2*, *ITPRIPL1* in different types of cancer patients

Taiwan		CCDC181			GCM2			ITPRIPL1		
Cancer Type	Avg T	Avg N	Pair ¹ T/N > 10	Avg T	Avg N	Pair T/N > 10	Avg T	Avg N	Pair T/N > 10	
Breast N = 109	1.27	0.09	68.0%	0.44	0.18	60.0%	0.55	0.07	68.0%	
Colorectal N = 24	0.62	0.10	20.8%	0.33	0.07	12.5%	0.08	<0.01	37.5%	
Esophageal N = 16	2.98	0.10	56.3%	0.67	0.02	25.0%	0.54	<0.01	50.0%	
Lung N = 33	0.31	0.06	12.1%	0.08	0.01	33.3%	0.01	<0.01	60.6%	
Endometrial N = 15	2.79	0.13	66.7%	0.40	0.22	20.0%	0.37	0.01	20.0%	
TCGA		CCDC181			GCM2			ITPRIPL1		
Cancer Type	Avg β(T)	Avg β(N)	Avg β (T–N)	Avg β(T)	Avg β(N)	Avg β (T–N)	Avg β(T)	Avg β(N)	Avg β (T–N)	
Breast	0.51	0.08	0.44	0.43	0.08	0.35	0.45	0.12	0.33	
Colon	0.58	0.19	0.39	0.50	0.13	0.37	0.23	0.08	0.15	
ESCC	0.37	0.13	0.24	0.46	0.17	0.29	0.21	0.11	0.10	
Stomach	0.35	0.17	0.19	0.37	0.28	0.09	0.23	0.08	0.15	
Liver	0.26	0.08	0.19	0.20	0.09	0.11	0.37	0.21	0.16	
Lung AD	0.31	0.09	0.22	0.37	0.14	0.24	0.30	0.18	0.12	
Lung SQ	0.42	0.05	0.37	0.28	0.08	0.19	0.33	0.13	0.20	
Pancreas	0.13	0.04	0.09	0.18	0.08	0.11	0.19	0.14	0.06	
Uterine	0.72	0.06	0.66	0.25	0.08	0.17	0.15	0.15	0.00	
Ovarian	0.16	-	-	0.10	-	-	0.11	-	-	

¹ The results of the paired T/N ratio were calculated from qMSP analysis in tumors (T) in comparison to adjacent normal tissues (N) of cancer patients.

Table S6. The methylation of candidate genes in relation to the clinical parameters in The Cancer Genome Atlas (TCGA) breast cancer patients¹.

Characteristics	CCDC181				GCM2			ITPRIPL1		
Methylation	N	High (%)	Low (%)	p	High (%)	Low (%)	p	High (%)	Low (%)	p
Age	547	52.3	47.7	0.041	50.1	49.9	0.052	50.6	49.4	0.759
>45	116	41.4	58.6		39.7	60.3		52.6	47.4	
<45	656	50.2	49.8		48.3	51.7		50.9	49.1	
Gender	7	71.4	28.6	0.451	42.9	57.1	1.000	57.1	42.9	1.000
Female	507	49.9	50.1		47.3	52.7		49.3	50.7	
Male	32	59.4	40.6		68.8	31.3		68.8	31.3	
Race	110	49.1	50.9	0.563	48.2	51.8	0.063	52.7	47.3	0.094
White	153	47.1	52.9		40.5	59.5		45.8	54.2	
Asian	426	53.1	46.9		52.6	47.4		54.2	45.8	
Black or African American	372	54.0	46.0	0.221	50.5	49.5	0.011	53.5	46.5	0.074
Menopause state	291	45.7	54.3		45.4	54.6		47.8	52.2	
Premenopause	9	66.7	33.3		66.7	33.3		66.7	33.3	
Postmenopause	654	50.2	49.8	0.505	48.0	52.0	0.326	50.8	49.2	0.506
Lymph node	479	47.8	52.2		46.1	53.9		50.3	49.7	
N > 1	174	56.9	43.1		53.4	46.6		52.3	47.7	
N = 0	479	56.8	43.2	0.042	52.6	47.4	0.111	51.8	48.2	0.659
Distant metastasis	145	29.7	70.3		35.2	64.8		49.7	50.3	
Yes	423	57.9	42.1		51.1	48.9		52.2	47.8	
No	198	34.8	65.2	<0.001	43.4	56.6	0.085	50.0	50.0	0.606
Stage	97	59.8	40.2		64.9	35.1		64.9	35.1	
I and II	566	48.8	51.2		45.4	54.6		48.6	51.4	
III and IV	455	51.6	48.4	0.048	49.9	50.1	<0.001	55.8	44.2	0.003
ER	133	50.4	49.6		47.4	52.6		38.3	61.7	
Positive	23	60.9	39.1		47.8	52.2		52.2	47.8	
Negative	13	30.8	69.2	0.160	38.5	61.5	0.491	30.8	69.2	0.003
PR										
Positive										
Negative				0.160			0.491			0.003
HER2										
High										
Low	39	35.9	64.1		35.9	64.1		43.6	56.4	
Histological Type										
IDC										
ILC										
Mix Histology										
Mucinous										
Others										