Detection of aberrantly methylated tumor suppressor genes in breast cancer patients as a potential diagnostic biomarker

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Research

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Abstract

Background: Aberrant methylation of tumor suppressor genes is a common feature of breast cancer. Identifying a panel of methylated genes that are sensitive and specific for breast cancer could help to diagnose and predict prognosis of breast cancer.

Methods: We determined the methylation status of DACT1, PAX5, PLCD1, ZNF545 and TET1 in 32 benign controls, 237 cancer tissue samples and 33 paired plasma samples.

Results: PAX5 and PLCD1 showed exceedingly high methylation rates with percentages of 69.2% and 54.9%, whereas the methylation percentage of DACT1, ZNF545 and TET1 were 33.8%, 28.7% and 18.2% in cancer samples, respectively. A better survival of patients with ZNF545 methylation ($p = 0.0350$) was detected. Correlation of promoter methylation and clinicopathological features in 32 individuals with benign disease and 237 cancer patients demonstrated that methylated status of DACT1 ($p=0.012$), PLCD1 ($p=0.013$), and ZNF545 ($p=0.012$) had significant difference in age, and the methylation of PAX5 ($p=0.006$) was correlated with absence of hormone receptors, which implied an adverse outcome. PAX5 and PLCD1 both had high sensitivity (69.20% and 54.85%, respectively) and high specificity (87.50% and 100.00%, respectively). Patients with methylation of PAX5 likely to have a higher risk of breast cancer (OR=15.726, 95% CI=5.323-46.463, $p<0.001$), and statistical analysis of public online database showed the similar results.

Conclusion: PAX5, PLCD1, ZNF545 and TET1 may serve as new potential diagnostic and prognostic biomarkers for breast cancer.

Background

Breast cancer always tops the cancer morbidity chart in females worldwide (1, 2). Thanks to early diagnosis and multi-modal treatments, the prognosis of women with breast cancer has improved markedly. The relative 5 years survival rate for breast cancer is 89% at 5 years after diagnosis (3). Although ultrasound, mammography and magnetic resonance imaging (MRI) are useful in screening and detecting breast carcinoma, substantial limitations exist within these technologies, such as limited image resolution of sonography, personal discomfort and reduced sensitivity in dense breast tissue of mammographic examinations and the relatively high rate of false positives for MRI(4). For some occult and recurrent cases without image-detectable lesions or with lesions located in the viscera, biopsies cannot provide a definite diagnosis. These limitations call for the development of minimally invasive and more sensitive tests.

Aberrant methylation of CpG islands results in the transcriptional silencing of tumor suppressor genes (TSGs), including genes involved in cell cycle regulation (such as p16INK4a, p15INK4a, Rb and p14ARF), DNA repair (BRCA1, MGMT), apoptosis (DAPK, TMS1), drug resistance (MGMT, ER), detoxification (GSTP1) and metastasis (CDH1) (5). Methylation of TSGs has been found a common feature of various neoplasms(6–12). In addition to tumor tissue DNA, studies have demonstrated that gene promoter methylation may be detected in bodily fluids like blood(13–17), offering a non-invasive and repeatable “liquid biopsy”, thus providing a convenient and reliable method for identifying molecular phenotypes and assessing treatment. Determination of methylated biomarkers that are sensitive and specific for breast cancer in tumor tissue or plasma/serum is a promising avenue for the improved management of breast cancer.

In our previous studies, dishevelled-binding antagonist of beta-catenin 1 (DACT1) (18), phospholipase C delta 1 (PLCD1) (19), ZFP82 zinc finger protein (ZNF545) (20) and ten-eleven translocation methyl cytosine dioxygenase1 (TET1) (21)showed a notable reverse correlation between their expression and methylation status between breast cancer and normal tissue samples. In other words, aberrant methylation was accompanied with down-regulated gene expression in breast cancer but not in normal controls. The proportion of methylation at DACT1, PLCD1, and ZNF545 was 40/134 (29.9%), 13/25 (52.0%) and 37/128 (28.9%), in breast tumor tissue, respectively. Paired-box 5 (PAX5) which is under study presently by our group, presented the same pattern. Thus, we decided to identify the clinical significance of this methylation biomarker panel in a larger sample size.

Materials And Methods

Patients and Samples

In total, 302 samples obtained from female patients presented by the First Affiliated Hospital of Chongqing Medical University (Chongqing, China), were assessed in this study. 237 tumor tissues from primary breast cancer patients (age range, 20–88 years old; median age, 51 years old,) were collected from September 2009 to October 2015, and 33 provided corresponding blood samples. Tissues were obtained after surgical resection and snap frozen in liquid nitrogen. All cases were subject to histologic diagnosis by pathologists. Clinical information, including age, tumor size, therapeutic regimen after initial diagnosis, as well as pathological characteristics (227 invasive cases, 10 Carcinoma in situ) -- histological grade, lymph nodes metastasis, p53 mutation and ki67 level, and the expression of hormone receptors and human epithelial growth factor receptor 2 (HER2), were obtained. To investigate the diagnostic value of TSG methylation in breast cancer, 32 patients (age range, 19–67 years old; median age, 38.5 years old) with benign breast disease were set as control. Informed consents were obtained from patients before sampling.

Tissue DNA Extraction and Plasma DNA Isolation

Genomic DNA from the breast tissue was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Patient blood was drawn by venepuncture during surgery, collected in EDTA anticoagulant tubes and then centrifuged at 2000 × g for 10 min at room temperature. Finally, 500 µL plasma aliquots were stored at −80°C until use.

Circulating cell-free DNA (cfDNA) in plasma was isolated using the Glass-milk Gel Mini Purification Kit (ZOMANBIO, Beijing, China) according to the manufacturer's protocol with some modifications. Briefly, circulating DNA in 400 µL plasma was bound with 300 µL high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8 and 500 mM NaCl), supplemented with 20µL proteinase K (20 mg/mL), and incubated for 20 min at 65°C. Then, 2.5 µL RNase A (10µg/µL) was added followed by incubation for another 10 min at room temperature. Next, 250 µL buffer Q×1 (Qiagen) was added to correct the pH. Then, DNA was enriched with 10 µL glass-milk on ice, vibrating gently for 1–2 s every 2–3 min to ensure full adsorption. Afterwards, the glass-milk was rinsed twice with 250 µL wash buffer and dried at 50°C for 5 min. To elute the DNA, 30 µL TE buffer was added, followed by incubation for 5 min at 60°C and centrifugation for 1 min at 12000 × g. The supernatant was aspirated and stored at −20°C until use. Concentration of DNA was quantified with NanoDrop 2000 (Thermo Fisher, MA, USA).

Bisulfite Modification and Methylation-specific PCR (MSP)

DNA methylation status was determined by MSP analysis. DNA bisulfite modification was performed as described previously(22, 23). Bisulfite-modified DNA was amplified by MSP, with specific primers set for unmethylated and methylated promoter sequences using AmpliTaq-Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA). Detailed information about the primer sequences, annealing temperatures, amplification cycles and product sizes of each gene are listed in Supplementary Table 1. Methylated human DNA and water were used as positive and negative controls, respectively. The PCR products were then analyzed by 2% agarose gel containing 100 bp DNA markers (MBI Fermentas, Vilnius, Lithuania) and visualized under UV illumination (BIO-RAD, California, USA).

Online Databases Analyses

To further validate the correlation between methylation status and expression, we analysed promoter methylation status and gene expression of breast cancer versus normal tissues for the five candidate genes from TCGA (766 breast cancer samples and 84 normal samples). To supplement the prognostic value of the five TSGs, relapse-free survival (RFS)--similar to progression-free survival (PFS) based on gene expression was obtained from Kaplan–Meier Plotter (www.kmplot.com) (24), split patients by best cutoff : DACT1 cutoff = 244, PAX5 cutoff = 11, PLCD1 cutoff = 383, TET1 cutoff = 92, ZNF545 cutoff =

205. Metastatic Relapse (MR)-free survival was obtained from bc-GenExMiner V4.0 (http://bcgenex.centregauducheau.fr) (25), split patients by median.

Statistical Analyses

Continuous variables were reported as the mean value ± standard deviation (SD). The Shapiro-Wilk normality test were used for checking normal distribution of data. Data with a normal distribution were compared by Student's t-test, and those with unequal variance or without a normal distribution were analysed by a Mann-Whitney rank sum test. Categorical values were compared by the chi-square test. Receiver operating characteristic (ROC) curve was charted and sensitivity, specificity, and the area under the curve (AUC) were calculated to exam the discriminant validity of 5 selected genes. Odds ratios (OR) were calculated by logistic regression analysis. In consideration of the good prognosis of early breast cancer, follow-up data of patients recruited before January 2015 were included into survival analysis. Disease free survival (DFS) (n = 186) was measured from the first diagnosis of breast cancer until local and/or distant relapse (n = 22) and was censored at the last follow-up for patients alive but without evidence of relapse (n = 164). Survival analyses were conducted with the Kaplan–Meier method. Univariate analyses of DFS according to the methylation results of selected genes were performed using a two-sided log-rank test. A p value < 0.05 was considered statistically significant. Statistical analyses were performed using IBM Statistic SPSS 22.0.

Results

The promoter methylation status in breast cancer and benign control.

The promoter methylation status of DACT1, PAX5, PLCD1, ZNF545 and TET1was assayed in 32 benign control and 237 breast cancer tissues. Only PAX5 (12.5%) and TET1(6.3%) were methylated in control group. PAX5 and PLCD1 showed exceedingly high methylation rates in the cancer patients and the percentages were 69.2% (164/237) and 54.9% (130/237) respectively, whereas the percentages of DACT1, ZNF545 and TET1 are 33.8% (80/237), 28.7% (68/237) and 18.2% (43/237), respectively (Fig 1A, Table 2). Our findings were validated in the TCGA database. In general, the methylation level of those five genes were significantly higher in breast cancer tissues compared to normal tissues (Fig 1B). From these data, we can find that the methylation level of the five genes in breast cancer tissues were negatively correlated with their expression levels (Fig 1C).

To compared the methylated status of those five genes in breast cancer tissues and plasma we examined both the breast cancer tissues and paired plasma samples from 33 breast cancer patients. The result suggested that PAX5 had higher methylation frequency (78.8%) than DACT1, PLCD1, ZNF545 and TET1 (15.2%, 9.1%, 3.0% and 6.1% in plasma, respectively), which was similar to methylation status in tissue sample (Fig 1D). The concordance methylated status and gene expression analyze showed that PAX5 presented the highest concordance (81.8%) between tissue and paired plasma DNA when compared with the other four genes (DACT1 36.4%, PLCD1 51.5%, ZNF545 45.5%, TET1 0%) (Table 3). We can find that the concordance rates between tissues and plasma in patients largely differ dependently on the genes methylation.

Association between gene methylation status and clinicopathological features in breast cancer

The correlation between methylation status and patient clinicopathological parameters in tissues was lay out in Table 4. Age-dependent promoter methylation has been reported previously in other tumor suppressor genes in several human cancer types(26). In this study, we reported PAX5, PLCD1 and ZNF545 were hypermethylated in elder (> 50 years) breast cancer patients, with the p value of 0.012, 0.013 and 0.012 respectively. TET1 methylation was statistically associated with tumor size (p=0.001). In addition, we found the presence of associations between frequently methylation of PAX5 with positive hormone receptors (p= 0.006 for estrogen receptor and p = 0.032 for progesterone receptor), two elements of ER and PR regarded as

putative risk factors for early relapse in breast cancer, suggesting that the poor patient prognosis was related to the promoter methylation of PAX5. Meanwhile, DACT1 and TET1 had higher methylation rate in the HER2-positive group than HER2-negative group (p = 0.020 for DACT1, p =0.015 for TET1). Besides that, hypomethylation of DACT1 was related to progesterone receptor and p53 positive, the p value was 0.035 for progesterone receptor and 0.009 for p53. And we found that the methylation rate of ZNF545 was statistically lower in 60 patients who were given neoadjuvant chemotherapy than in patients who had mastectomy before systemic treatment (p = 0.040), which suggesting that hypomethylation of ZNF545 may related to patient chemo-response. We found that the methylation status of these five genes in patients' plasma was not associated with clinicopathological parameters except ZNF545 hypermethylated may associated with tumor size (Table S1)

PAX5, PLCD1 and ZNF545 hypomethylation were associated with better survival

Follow-up data on 194 patients enrolled before January 1, 2015 were included in a survival analysis based on DNA methylation status (Fig 2). In this small cohort with a relatively short follow-up time, the survival curves of the methylated and unmethylated groups were nearly parallel except for that of ZNF545. The test revealed a better survival of patients with methylated ZNF545 (p = 0.0350).

Considering the relatively small sample amount and short follow-up time of this study, two online databases, Kaplan–Meier Plotter and bc-GenExMiner V4.0, were employed to adduce indirect evidences to elucidate the value of the five genes on prognostic prediction in breast cancer. From bc-GenExMiner V4.0, high expression of PAX5 (HR:0.87, 95% CI: 0.78-0.98, p=0.0232), PLCD1 (HR:0.81, 95% CI: 0.72-0.91, p=0.0003) and ZNF545 (HR=0.84, 95% CI: 0.70-0.99, p=0.0407) presented better MR-free survival, whereas DACT1 expression showed no significant difference with metastatic relapse in breast cancer (HR: 1.10, 95% CI: 0.98-1.24, p=0.1120). Low expression of TET1 might plays an important role in metastatic relapse (HR:1.26, 95% CI: 1.06-1.48, p=0.0073) (Fig 3A). Analysis of RFS from Kaplan–Meier Plotter indicated that high expression of all five genes predicted showed longer RFS under individual cutoff (Fig 3B).

PAX5, PLCD1 and TET1 had statistically significance for diagnosis in breast cancer

To distinguish breast cancer from breast benign disease, we performed ROC curve analysis and calculated area under the curve (AUC), sensitivity and specificity of each gene (Table 5). According to the results, DACT1, PAX5 and PLCD1 had statistically significance for diagnosis in breast cancer. PAX5 and PLCD1 had both high sensitivity (69.20% and 54.85%, respectively) and high specificity (87.50% and 100.00%, respectively). DACT1 had low sensitivity (33.76%) but high specificity (100.00%). Next, we conducted univariate logistic regression analysis to analyze the methylation status of five genes to predict breast cancer in 237 cancer patients and 32 benign disease patients. Patients with methylation of PAX5 have a higher risk of breast cancer (OR=15.726, 95% CI=5.323-46.463, p<0.001) (Table 6).

Information on methylation level of these 5 genes of 72 pairs as case-control was obtained from TCGA database. The risk of breast cancer with DACT1, PAX5, PLCD1, ZNF454 and TET1 hypermethylation were 1.182, 20.598, 8.412, 3.130 and 12.250 times higher than that with hypomethylation, respectively, and all p values, except DACH1, were less than 0.05 (Table 7). As a result of the multiple linear regression analysis of gene methylation level and breast cancer (Table 8), the higher the methylation level of PAX5 and TET1 was correlated with higher risk of breast cancer, with the coefficients of 0.466 and 0.292, respectively.

Discussion

The promoter methylation of TSGs is a common feature of breast cancer, with aberrant methylation of promoter resulting in transcriptional silencing of TSGs. Our previous studies indicated that: DACT1 was a methylated target suppresses tumor cell

growth through antagonizing the Wnt/β-catenin signaling pathway in breast cancer(18); PAX5 was a frequently methylated TSG interfering with β-catenin signaling and GADD45G expression in lung cancer(27). PLCD1 was demonstrated frequently silenced by promoter methylation in breast cancer(19); ZNF545, has been shown to be a tumor suppressor that induces tumor cell apoptosis, represses ribosome biogenesis and targets gene transcription in multiple carcinoma types(17); The methylation level of TET1 has been proved associated with cancer pathogenesis by suppressing Wnt/β-catenin signalling via demethylation of Wnt antagonists(21). In this work, we identify the clinical utility of existing methylation biomarkers panel in an enlarged sample, including DACT1, PAX5, PLCD1, ZNF545 and TET1. The methylation rates of the five genes ranged between 18.2–69.2%. PAX5 and PLCD1 showed exceedingly high methylation rates and the percentages were 69.2% (164/237) and 54.9% (130/237), respectively. Based on previous studies, we have reason to believe that gene methylation is associated with gene expression, which validated our findings using publicly available data from the TCGA database, and demonstrated that methylation level of those five genes in breast cancer was negatively correlated with its expression level.

The correlation of promoter methylation and clinicopathological features in 32 individuals and 237 cancer patients with benign disease demonstrated that methylated status of DACT1 ($p = 0.012$), PLCD1 ($p = 0.013$) and ZNF545 ($p = 0.012$) have significant difference in age. Besides that, our results revealed that methylated PAX5 was correlated with absence of hormone receptors, which implied an adverse outcome. Of note, ZNF545 methylation was associated with better survival ($p = 0.0350$). This might be because ZNF545 methylation was significantly lower in patients receiving neoadjuvant CT (having relatively aggressive tumors). So methylated cases are more likely non-neoadjuvant treated (relatively less aggressive) cases. Implying a possible reversion of promoter methylation after chemotherapy, and the alteration of methylation of ZNF545 may be valuable to treatment evaluation. Along with the results mentioned above, online databases provided avenues to confirm the prognostic significance of the five genes, and PAX5, PLCD1 and ZNF545 showed good consistency from different database. Thus, we determined that a methylation-based biomarker panel which was consist of PAX5, PLCD1 and ZNF545 may help for diagnosis, treatment monitoring, risk stratification and outcome evaluation in breast cancer.

To confirm the diagnostic value of selected five genes, their specificity and sensitivity were calculated in this study. The sensitivity of all genes ranged from 18.14–69.20%, and specificity ranged from 87.5–100%. Combine with Univariate logistic regression analysis of methylation status in tissues, we demonstrated that the methylation of PAX5 (sensitivity: 69.2%, specificity: 87.5%, AUC: 0.627, 95% CI (0.554,0.699), $p = 0.001$; OR = 15.726, 95% CI (5.323,46.463), $p < 0.001$) may contribute to diagnosis and risk stratification for breast cancer. Given the limitation of normal sample size in this study, data from TCGA were also analysed. Univariate logistic regression analysis and Multivariate linear regression analysis of gene methylation revealed that promoter methylation of DACT1 (OR = 1.182, 95% CI (0.614, 2.274), p□0.05; β=-0.111, T=-1.405, p□0.05, R2 = 0.458), PAX5 (OR = 20.598, 95% CI (8.810, 48.155), $p < 0.001$; $\beta = 0.466$, $T = 5.157$, $p < 0.001$, $R2 = 0.458$) and TET1 (OR = 12.250, 95% CI (5.583, 26.877), $p < 0.001$; $\beta = 0.292$, $T = 4.432$, $p < 0.001$, $R2 = 0.458$) contributed most to breast cancer. Univariate logistic regression analysis and Multivariate linear regression analysis of gene expression revealed that expression of PLCD1 (OR = 8.412, 95% CI (3.975, 17.799), $p < 0.001$; $\beta = 0.091$, $T = 1.118$, p□0.05, $R2 = 0.458$) contributed most to breast cancer.

Circulating cfDNA comprises various fragments ranging from around 20 bp to 20 kbp in length depending on the mechanism of the release into circulation (28, 29), and it is released into the circulation via passive release as a result of cellular necrosis and/or active secretion from live cells. Methylation haplotyping in plasma is a promising strategy for early detection of tumor and its primary growth site, as well as a continuous monitor of tumor progression and metastasis to multiple organs(30). In this study, 33 pairs of cancerous tissue and paired plasma were used to detect methylated status. Concordance of PAX5 methylation between tissue and paired plasma is 81.25% (Table 3). Concordance of DACT1, PAX5, ZNF545 ranged from 36.4–51.5%. By contrast, TET1 methylation didn't show concordance between tissue and paired plasma. The concordance rates between plasma and tissues in patients largely differ dependently on the genes methylation indicated that blood tests may be used to monitor the absence of tissue samples after surgery. Unfortunately, correlation of promoter methylation and clinicopathological features in plasma, except ZNF545 in tumor size ($p = 0.013$, Table S1). These results could be as a pilot study of detecting methylation, and the mechanisms of this phenomenon need further research.

In conclusion, PAX5, PLCD1, ZNF545 and TET1 were closely related to breast cancer in both data from our own and public online database, which revealed that PAX5, PLCD1, ZNF545 and TET1 may serve as new potential diagnostic and prognosis

biomarkers for breast cancer.

Declarations

Author Contributions statement

DY, YW, QX, TX and GR contributed to the conception and design of the study. DY, YW, QX and DZ performed the experiments and analyzed the data. JT and BZ contributed to the RNA and DNA extraction. ZQ, ZB and XY collected the samples. DY, QX and TX prepared the figures and drafted the manuscript. XL and WP reviewed the manuscript. TX and GR finalized the manuscript. All authors reviewed and approved the final manuscript.

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Availability of data and materials

All data that support the findings of this study are available from the corresponding authors upon a reasonable request.

Ethics approval and consent to participate

This study was carried out following the rules of the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Approval number: 2016-75; Approval date: 28 April 2016).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

TSGs: tumor suppressor genes; MRI: magnetic resonance imaging; DACT1: dishevelled-binding antagonist of beta-catenin 1; PLCD1: phospholipase C delta 1; ZNF545:ZFP82 zinc finger protein; TET1: ten-eleven translocation methyl cytosine dioxygenase1; PAX5: Paired-box 5; HER2: human epithelial growth factor receptor 2; cfDNA: Circulating cell-free DNA; ROC: Receiver operating characteristic; AUC: area under the curve; OR: Odds ratios; DFS: Disease free survival.

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Tables

Table 1. List of primers used in this study.

Primer	Sequence (5'-3')	Product size (bp)	PCR cycles (Tissue/Plasma)	Annealing Temp (°C)
DACT1 mF	CGGGATAGTAGTAGTCGGC	118	41/55	60
DACT1 mR	CGCTAAAACTACGACCGCG			
DACT1 uF	GTTGGGATAGTAGTAGTTGGT	123	41/45	60
DACT1 uR	AAACACTAAAACTACAACCACA			
PAX5 mF	AAATAAAAATTCGGTTTGCGTTC	105	41/45	60
PAX5 mR	AAACATACGCTTAAAAATCGCG			
PAX5 uF	TAAAAATAAAAATTTGGTTTGTGTTT	111	41/45	58
PAX5 uR	TTAAAACATACACTTAAAAATCACA			
PLCD1 mF	AATGATAGGGTTCGCGGTTC	91	41/55	60
PLCD1 mR	CCCGAACCAACGAACGCG			
PLCD1 uF	GTAATGATAGGGTTTGTGGTTT	97	41/45	58
PLCD1 uR	CTAACCCAAACCAACAAACACA			
ZNF545 mF	TTTTTTTTAGGTTTTGTCGCGTC	177	41/55	60
ZNF545 mR	CTACTAAAAAAACCGAACGCG			
ZNF545 uF	TTTTTTTTTAGGTTTTGTTGTGTT	168	41/45	58
ZNF545 uR	CCAAACACACTCACAAAATACA			
TET1 mF	GTCGGTAGGCGTTTTTCGC	173	41/55	60
TET1 mR	CCCAACTCACCGCTAACCG			
TET1 uF	GAGTTGGTAGGTGTTTTTTGT	175	41/55	58
TET1 uR	CCCAACTCACCACTAACCA			

Table 2. Methylation results in cancer and begin breast.

Genes	Cancer (N = 237)		Control (N = 32)	
	Positive	%	Positive	%
DACT1	80	33.8	0	0
PAX5	164	69.2	4	12.5
PLCD1	130	54.9	0	0.0
ZNF545	68	28.7	0	0
TET1	43	18.2	2	6.3

Table 3. Concordance of methylation status between cancer tissue and plasma DNA.

	DACT1	*PAX5*	*PLCD1*	*ZNF545*	*TET1*
T+/P+	0	26	5	1	0
T+/P-	18	6	16	18	8
T-/P+	3	0	0	0	2
T-/P-	12	1	12	14	22
Plasma positive (%)	9.1	78.8	15.2	3.0	6.1
Concordance (%)	36.4	81.8	51.5	45.5	0

Note. T: Tissue, P: Plasma

Table 4. Correlation of promoter methylation and clinicopathological features in tissues.

	DACT1		*p*	*PAX5*		*p*	*PLCD1*		*p*	*ZNF545*		*p*	*TET1*		*p*
	M	Um		M	Um		M	Um		M	Um		M	Um	
Age			0.862			**0.012**			**0.013**			**0.012**			0.962
≤50	38	75		70	44		53	61		24	90		21	93	
>50	42	79		94	29		77	46		44	79		22	99	
Tumor			0.342			0.351			0.092			0.417			**0.001**
T1 (≤2cm)	31	69		67	34		48	53		25	76		11	90	
T2 (>2cm, ≤5cm)	43	68		83	30		66	47		37	76		31	81	
T3+T4 (>5cm)	6	17		14	9		16	7		6	17		1	21	
Pathology			0.797			0.179			0.738			0.535			0.067
CIS	3	7		5	5		6	4		2	8		4	6	
Invasive	77	150		159	68		124	103		66	161		39	188	
Grade			0.469			0.116			0.424			0.313			0.193
1+2	61	115		127	49		95	81		54	122		30	145	
3	10	14		13	12		12	13		4	21		3	22	
Unknown	9	25		24	12		23	13		10	26		10	25	
LN			0.435			0.289			0.279			0.342			0.670
Neg	44	80		81	44		67	58		35	90		24	100	
Posi	36	71		81	28		60	49		31	78		19	89	
Unknown		3		2	1		3			2	1			3	
ER			0.123			**0.006**			0.339			0.837			0.076
Neg	36	56		54	40		57	37		29	65		21	73	
Posi	43	88		102	30		67	65		36	96		18	112	
Unknown	1	10		8	3		6	5		3	8		4	7	
PR			**0.035**			**0.032**			0.359			0.918			0.117
Neg	47	67		71	45		69	47		32	84		23	93	
Posi	32	77		85	25		55	55		33	77		16	92	
Unknown	1	10		8	3		6	5		3	8		4	7	
Ki67			0.087			0.951			0.494			0.300			0.271
<14%	36	53		63	27		46	44		26	64		21	69	
≥14%	40	81		85	38		72	51		32	91		19	102	
Unknown	4	20		16	8		12	12		10	14		3	21	
p53			**0.009**			0.349			0.891			0.118			0.998
Neg	34	58		70	24		52	42		34	60		17	75	

Posi	45	75		79	42		67	54		29	92		22	99	
Unknown	1	21		15	7		11	11		5	17		4	18	
HER2			**0.020**			0.269			0.695			0.530			**0.015**
Neg	21	23		26	18		26	18		10	34		8	36	
Posi	58	119		129	51		98	82		55	125		31	147	
Unknown	1	12		9	4		6	7		3	10		4	9	
NAC			0.428			0.867			0.744			**0.040**			0.705
Yes	18	42		41	19		34	26		11	49		10	50	
No	62	112		123	54		96	81		57	120		33	142	

M, methylated; Um, unmethylated; unknown means information not well documented; CIS, carcinoma in situ; LN, lymph node metastasis status; Neg, negative; Posi, positive; ER, estrogen receptor; PR, progesterone receptor; HER2, human epithelial growth factor receptor 2; NAC, neo-adjuvant chemotherapy. Unknown data was not included in the statistical analysis.

Table 5. Diagnostic value of selected five genes.

Gene	Sensitivity	Specificity	AUC	*p*	95% CI
DACT1	33.76%	100.00%	0.585	0.028	0.515–0.655
PAX5	69.20%	87.50%	0.627	0.001	0.554-0.699
PLCD1	54.85%	100.00%	0.615	0.001	0.548–0.682
ZNF545	28.69%	100.00%	0.580	0.050	0.507–0.653
TET1	18.14%	93.75%	0.545	0.344	0.457-0.632

Table 6. Univariate logistic regression analysis of methylation status in tissues as a predictor of breast cancer in 237 cancer patients and 32 individuals with benign disease.

Gene	OR	*p*	95% CI
DACT1	329268757.8	0.997	0.000-999.999
PAX5	15.726	<0.001	5.323-46.463
PLCD1	483132663.3	0.995	0.000-999.999
ZNF454	305888727.6	0.997	0.000-999.999
TET1	3.325	0.109	0.765-14.445

*: OR and 95% CI were calculated by chi-square test, since they were infinite great when calculated by logistic regression analysis.

Table7. Univariate logistic regression analysis of gene methylation status and breast cancer

Gene	OR	*p*	95%CI	
			The lower limit	The upper limit
DACT1	1.182	0.617	0.614	2.274
PAX5	20.598	<0.001	8.810	48.155
PLCD1	8.412	<0.001	3.975	17.799
ZNF454	3.130	0.001	1.586	6.179
TET1	12.250	<0.001	5.583	26.877

Data was downloaded from the TCGA website

https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga

Table 8. Multivariate linear regression analysis of gene methylation and breast cancer

	β	T	*p*
Constant		-1.766	0.080
DACT1	-0.111	-1.405	0.162
PAX5	0.466	5.157	<0.001
PLCD1	0.091	1.118	0.266
ZNF545	0.086	1.003	0.318
TET1	0.292	4.432	<0.001

R^2=0.458; Data was downloaded from the TCGA website

https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga

Figures

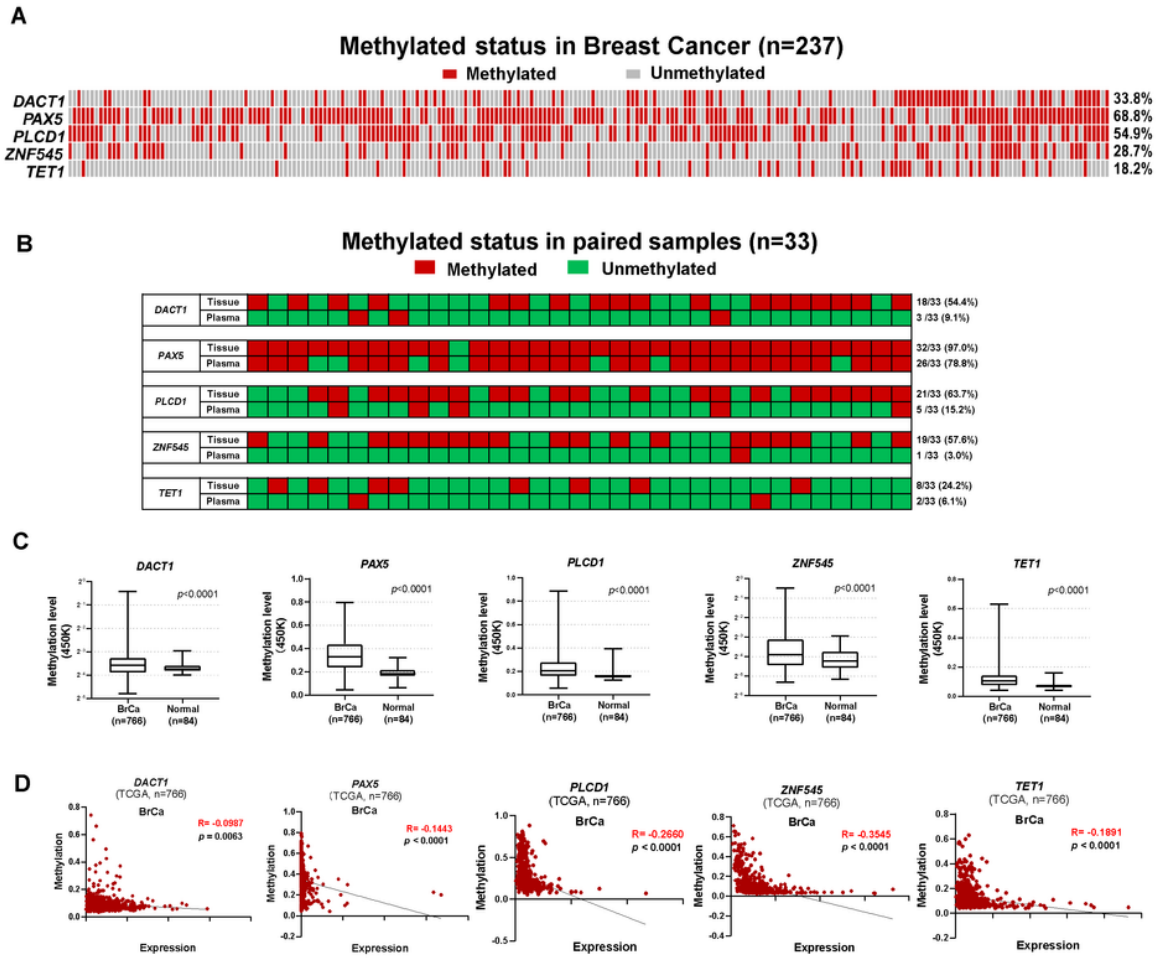

Figure 1

Comparison of methylation and expression levels of the five candidate genes. (A) An overview of the methylation status of the five genes; (B) Boxplots show comparison of average methylation and expression levels in normal and tumor tissues. (Primary data were downloaded from TCGA; p values were calculated using Mann-Whitney rank sum test). (C) The correlation between methylation and expression of in tumor tissues was performed by linear regression analysis. (D) Methylated status of five genes in breast cancer tissue and paired plasma samples.

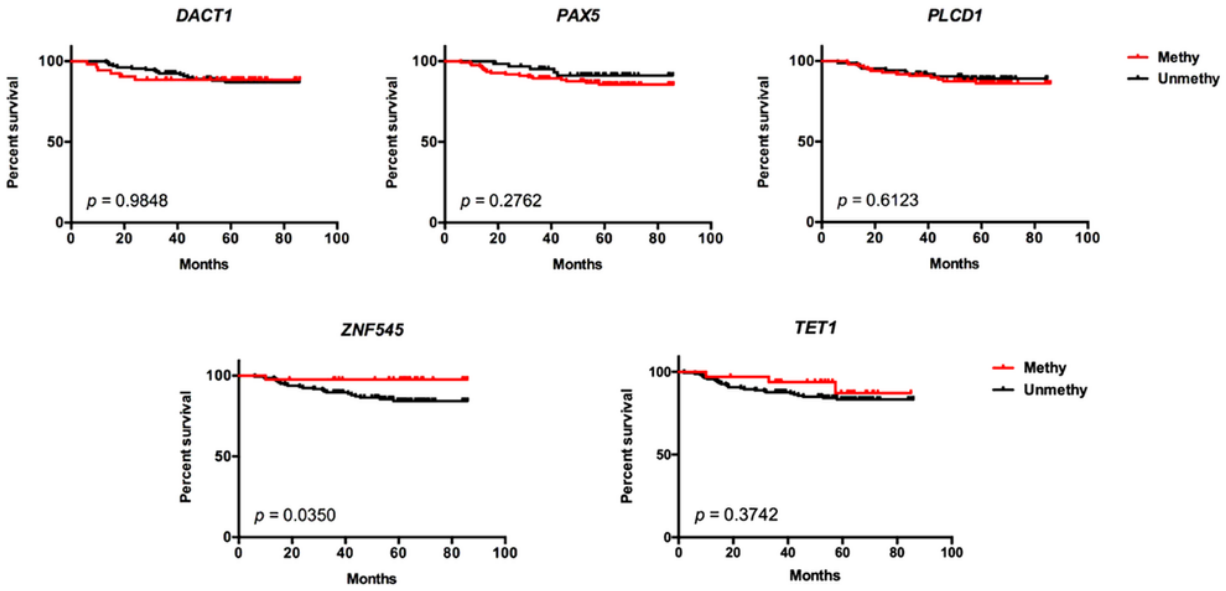

Figure 2

Survival analysis based on methylation. K-M survival analysis and log-rank test to compare PFS of patients with or without promoter methylation in tumor tissue samples of each gene. PFS: progression-free survival

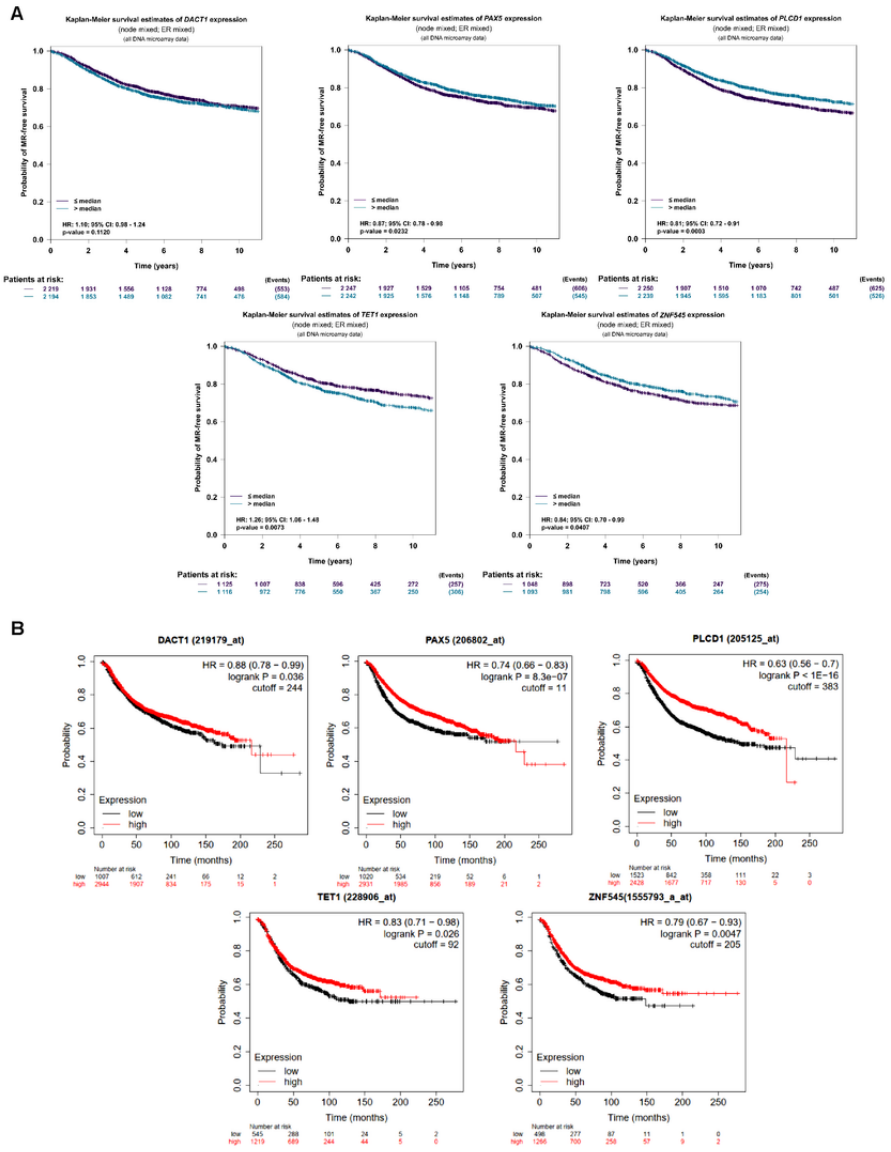

Figure 3

Survival analysis based on expression from online database. (A) MR-free survival curves plotted in bc-GenExMiner 4.0. (B) RFS curves plotted in Kaplan-Meier plotter. RFS: relapse-free survival, MR: metastatic relapse-free survival.

Supplementary Files

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

- supplementaryTable1.doc