

Downregulated ZNF132 Predicts Unfavorable Outcomes in Breast Cancer via Hypermethylation Modification

zhao liu

Xi'an Jiaotong University Medical College First Affiliated Hospital

Jiaxin Liu

Xi'an Jiaotong University Medical College First Affiliated Hospital

Ruimiao Liu

People's Hospital of Xi'an

Man Xue

Hospital Central

Weifan Zhang

Xi'an Jiaotong University Medical College First Affiliated Hospital

Xinhui Zhao

Xi'an Jiaotong University Medical College First Affiliated Hospital

jiang Zhu

The Tumor Hospital of Shannxi Province

Peng Xia (✉ peng_xia666@126.com)

<https://orcid.org/0000-0001-7448-4464>

Research article

Keywords: ZNF132, Breast cancer, Bioinformatic, Methylation, Diagnosis, Prognosis

Posted Date: July 6th, 2020

DOI: <https://doi.org/10.21203/rs.2.15590/v2>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at BMC Cancer on April 7th, 2021. See the published version at <https://doi.org/10.1186/s12885-021-08112-z>.

Abstract

Background: An important mechanism that promoter methylation-mediated gene silencing for gene inactivation, is identified in human tumorigenesis. Although a series of methylated genes have been found in breast cancer (BC), beneficial biomarkers for early diagnosis and prognostic assessment of this cancer remain little known. ZNF132 has been reported to be downregulated by promoter methylation in prostate cancer and esophageal squamous cell carcinoma. However, no study provides information on the status of ZNF132 as well as analyzes diagnosis and prognostic significance of ZNF132 in BC.

Methods: In the present study, we investigated the expression of ZNF132 mRNA and protein level based on the Cancer Genome Atlas (TCGA) RNA-Seq database and clinical samples analysis as well as multiple cancer cell lines verification. Besides, prognostic significance of ZNF132 in BC was assessed by the Kaplan-Meier plotter. Importantly, a molecular mechanisms exploration of ZNF132 in BC was performed using the multiple bioinformatic tools. Simultaneously, hypermethylated status of ZNF132 in BC cell lines was confirmed via MSP analysis.

Results: A consistent results, the expression of ZNF132 both the mRNA and protein levels was significantly downregulated in BC tissues, were obtained based on TCGA database and clinical sample analysis. Survival analysis from the Kaplan-Meier plotter revealed that the lower level of ZNF132 was associated with a shorter Relapse Free Survival (RFS) time. Besides, a ROC of 0.887 confirmed ZNF132 had powerful sensitivity and specificity to distinguish between BC and adjacent normal tissues. Importantly, bioinformatic analysis brought us into a deep insight that 6% ((58/960)) alterations of ZNF132 were identified from cBioPortal. ZNF132 participated in multiple biological pathways based on the GSEA database, including the regulation of cell cycle and glycolysis. Finally, MSP analysis demonstrated that ZNF132 was hypermethylated in a panel of breast cancer cell lines and 5-aza-2'-deoxycytidine (5-Aza-dC) treatment significantly restored ZNF132 expression in partial cell lines.

Conclusions: Our results revealed that hypermethylation of ZNF132 contributed to its downregulated expression and could be identified as a new diagnostic and prognostic marker in BC.

Background

Breast cancer (BC) is one of the most frequent malignant tumors in females and the fifth leading cause of cancer-associated mortality in worldwide[1, 2]. Advancement in early detection and treatment has improved 5 year-survival rates of BC patients[3-8]. In 2018, it is estimated that a larger number of patients (62%) with no distant metastasis are diagnosed and these cases displayed a favorable 5-year survival rate of 99%[9]. In comparison, the 5-year survival rate of the patients with distant metastases decreases to 23%[10]. Thus, early diagnosis and treatment can significantly improve survival time for BC patients.

Until now, multiple mechanisms were reported for breast carcinogenesis, including overactivation of oncogenes, overexpression of growth factors, and receptors, as well as the silence of tumor suppressor[11-13]. Besides, an increasing number of studies confirmed epigenetic alterations were also

involved in tumor progression[12, 14], especially, aberrant DNA methylation of CpG islands was considered to be a vital mechanism to silence anti-tumor genes[15, 16]. Given its role in tumor cell differentiation, proliferation, and survival, promoter methylation was assessed as an important marker of tumor cells[17, 18]. Zinc finger proteins are an important member of transcription factor family and play a vital role in human diseases including cancers [19]. The majority of zinc finger proteins contain Kruppel associated box (KRAB) domains, which has been reported to induce inhibition of the transcription of downstream genes [20]. Zinc Finger Protein 132 (ZNF132), located at chromosome 19q13.4, belongs to the zinc finger protein family. To date, ZNF132 was only found to be methylated in prostate cancer and esophageal squamous cell carcinoma. However, its role in BC remains unknown.

In the present study, we investigated the expression and clinical significance of ZNF132 based on the TCGA database and clinical samples analysis. Besides, further exploration was performed to assess the status of ZNF132 in BC using multiple bioinformatics tools. Importantly, we confirmed the epigenetic alteration of ZNF132 was associated with its downregulated expression. Collectively, our findings demonstrated ZNF132 could be a new potential prognostic factor for BC and may serve as a promising therapeutic target for BC patients.

Methods

Extraction of Multiple platforms data

Multiple databases, including TCGA database(<http://cancergenome.nih.gov/>), the Oncomine™ database (www.oncomine.org) and UALCAN platform (<http://ualcan.path.uab.edu/>) were used to identify the expression of ZNF132 in BC. Additional clinical variables from the TCGA database, including age, gender, ER, PR, HER2, metastasis, and clinical stage, were analyzed to assess the association between the expression of ZNF132 and these parameters.

Clinical samples and cell lines analysis

To verify the expression of ZNF132 in mRNA and protein level, a total of 19 clinical samples were collected from the First Affiliated Hospital of Xi'an Jiaotong University from January 2019 to March 2019. These patients did not receive any therapeutic intervention and signed an informed consent before surgery. All patients were finally histologically diagnosed by two pathologists. Ethical approval was provided by the First Affiliated Hospital of Xi'an Jiaotong University Ethics committee. RNA was extracted according to a previous protocol[21], qRT-PCR was performed using the Bio-Rad CFX Manager detection system to assess the mRNA expression of ZNF132 between BC and normal tissues. The SYBR protocol followed the parameters: 95°C for 30 sec, 38 cycles of 5 sec at 95°C and 30 sec at 55°C. The primers used are as follow: ZNF132: forward: 5'- CCACAGTGTGATGCTGGAAAACC-3', reverse: 5'- GCTTTCTTGGTGAAGGATCTGC-3'; 18s rRNA: forward: 5'-CGCCGCTAGAGGTGAAATTC-3', reverse: 5'-CTTTCGCTCTGGTCCGTCTT-3'.

In addition, a protein level analysis was performed according to IHC assay using clinical samples and western blot assay using breast cell lines. All tissues were fixed in 4% formaldehyde at room temperature for 48h in preparation. The ZNF132 antibody (BIOSS, Beijing, China; cat. no. bs-7150R, 1:1000 dilution) was used for IHC detection. The staining intensity was defined based on Image-pro plus software. Besides, cellular protein, including MCF10A, MDA-MB-231, MCF7, MDA-MB-453, HCC1937, T47D and DU4475 was extracted by the previous description[22], Anti-ZNF132 antibody (1:2000 dilution) were purchased from BIOSS, Inc. Anti-GAPDH antibody (1:40000 dilution) was purchased from Abgent, Inc. the protein bands were visualized using Western Bright ECL detection system (Advansta, CA).

Diagnostic and prognostic significance of ZNF132

The Kaplan-Meier plotter (<http://kmplot.com/>) was used to assess the prognosis value of ZNF132 in BC patients, including Relapse Free Survival (RFS) and Overall Survival (OS). Besides, univariate and multivariate analysis based on a Cox proportional hazard regression model were performed to evaluate the independent prognostic significance of ZNF132, Furthermore, a receiver operating characteristic (ROC) curve was plotted to determine whether ZNF132 expression could distinguish the difference between BC tissues and adjacent normal tissues.

Bioinformatic exploration

To further investigate the molecular mechanisms of ZNF132 in BC, we firstly evaluated the status of ZNF132 that included ZNF132 alteration and its impact on the prognosis of BC patients by the cBioPortal OncoPrint (<http://www.cBioPortal.org/index.do>). Next, Gene Set Enrichment Analysis (GSEA, <http://www.linkedomics.org/>) was used to predict potential biological processes and pathways. In addition, DNA methylation expression and different methylation sites analysis from the TCGA database were evaluated to identify the downregulated mechanisms of ZNF132 in BC.

Methylation analysis

To explore the epigenetic mechanism of ZNF132 inactivation, genomic DNA from 6 BC cell lines was extracted according to standard phenol/chloroform protocol. Then, DNA was treated using sodium bisulfite. Briefly, a mixture, including 4 μ g genomic DNA, 10 μ g salmon sperm DNA, and 0.3M NaOH, was collected. Next, supply a certain volume of water to a final volume of 20 μ l, incubated at 50°C for 20min to denature the DNA. Finally, transfer the mixture into 500 μ l of solution containing 3M sodium bisulfite and 10mM hydroquinone (Sigma, Saint Louis, MO), incubated at 70°C for 4h. DNA was subsequently purified using the Wizard DNA Clean-Up System (Promega Corp., Madison, WI) and dissolved in distilled water. Importantly, a methylation-specific PCR (MSP) was performed in our study. The PCR procedure was as follows: 4 min denaturation at 95°C, then 45s denaturation at 95°C, 45s anneal at 55°C, and 45s extension at 72°C, repeat this step with 35 cycles, finally an extension at 72°C for 5 min. The reaction products were presented on a 1.2% agarose gel and visualized under UV illumination using an ethidium bromide stain along with a positive control and negative control.

In addition, we want to know whether 5-aza-2'-deoxycytidine (5-Aza-dC), DNA methyltransferase (DNMT) inhibitor, could restore the expression of ZNF132 both mRNA and protein level. Therefore, the test groups were treated with 5 μ M 5-Aza-dC (Sigma-Aldrich) and control groups were treated with the vehicle. When the cell density was up to 80%, RNA was extracted using TRIzol® protocol. qRT-PCR was performed using the Bio-Rad CFX Manager detection system based on the previous description. Besides, cellular protein was also extracted by the previous description to detect the restoration of ZNF132 in the protein level.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 5.0 software. The association between ZNF132 expression and clinical characteristics was analyzed using the Chi-square test. Univariate and Multivariate analyses based on the COX regression model were performed to detect the association between clinical variables and the prognosis of BC. Moreover, the ROC curve was identified to evaluate the diagnostic capability between BC and adjacent normal tissue. Student's t-test was used to assess methylation differences of CpG island sites between BC and adjacent normal tissue. P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of ZNF132 is downregulated in BC

As shown in Fig. 1, a consistent results, the expression of ZNF132 was significantly downregulated in BC tissues, were obtained based on multiple bioinformatic tools, including TCGA database, Oncomine database and UALCAN platform (Fig. 1A-C), which was further supported by the qRT-PCR and immunohistochemical analysis, as well as western blotting verification (Fig. 1D-F). Besides, chi-square test showed that expression of ZNF132 was significantly associated with HER2 status (P =0.001), ER status (P =0.000), PR status (P =0.000), tumor size (P =0.006), and lymph node metastasis (P =0.003) (Table 1).

ZNF132 was a prognostic factor and diagnostic marker in BC

The result from the Kaplan-Meier plotter revealed low ZNF132 expression was significantly associated with a shorter RFS time in BC (Fig. 2A, P =2.3E-14). Besides, univariate COX regression analysis confirmed that some clinical features, including age, tumor size, lymph node metastasis, distant metastasis, and clinical stage were significantly associated with OS in BC patients (Table2). However, there was no statistical significance between the expression of ZNF132 and the prognosis of BC (HR =1.129, P =0.587) using multivariate analysis (Table3). Moreover, an area under the curve (AUC, representing the accuracy of differentiation) of 0.887 suggested that the level of ZNF132 has sufficient sensitivity and specificity to identify the difference between BC and adjacent normal tissues (Figure. 2B).

Bioinformatic analysis of ZNF132

The result from cBioPortal revealed that 6% (58/960) of BC exhibited ZNF132 alteration, including missense mutation (2/960), amplification (19/960), deep deletion (6/960), mRNA upregulation (13/960) and mRNA downregulation (18/960) (Fig. 3A). Besides, prognosis analysis was performed to explore the influences with and without ZNF132 alteration. The result showed a statistically significant difference existed for OS, but not for DFS (Fig. 3B). Subsequently, the analysis from GSEA demonstrated that ZNF132 participated in mediating multiple biological processes, including cilium organization, cilium or flagellum-dependent cell motility, synaptic transmission, glutamatergic, microtubule bundle formation, mitochondrial gene expression, mitochondrial respiratory chain complex assembly, ribonucleoprotein complex biogenesis, translational initiation, etc (Fig. 3C). The biological pathways of ZNF132 contained the regulation of cell cycle, glycolysis, cholesterol biosynthesis, ubiquitin proteasome pathway, TCA cycle (Fig.3D). Importantly, ZNF132 was negatively associated with the cyclin E1 (Fig. 4A) and ENO1 (Fig. 4B) level.

Hypermethylation of ZNF132 in BC

To explore the downregulated mechanism of ZNF132 expression, we firstly analyzed the expression of DNMT1, DNMT3A, and DNMT3B in the BC tissues. Compared to ZNF132^{high}, the 3 DNA methyltransferases showed significantly higher expression in the ZNF132^{low} group (Fig. 5A). Importantly, the result from MethHC (<http://methhc.mbc.nctu.edu.tw/php/index.php>) also demonstrated that the methylation level of ZNF132 in BC tissues was significantly higher than the normal sample (Fig. 5B). Besides, the analysis of different CpG island methylation sites based on the TCGA database also revealed the same trend (Fig. 5C). In sum, the ZNF132 methylation level was negatively correlated with its gene expression (Fig. 5D). Importantly, we also detected ZNF132 methylation in a panel of breast cancer cell lines using MSP assay. As shown in Fig. 5F-G, compared to the untreated normal controls, 5-Aza-dC could restore the mRNA and protein levels of ZNF132 in some cell lines, including MDA-MB-231, MCF7, and HCC1937. However, 5-Aza-dC could not restore the expression of ZNF132 between the mRNA and protein levels in the T47D cell. Besides, the MDA-MB-453 cell did not restore ZNF132 expression at the protein level. Moreover, an opposite trend was obtained in DU4475.

ZNF132 methylation was correlated with prognosis and clinicopathological features of BC

The investigation from MethSurv (<https://biit.cs.ut.ee/methsurv/>) showed that BC patients with higher ZNF132 methylation had a shorter survival time (Fig. 6A; $P = 1.802E-04$). Importantly, survival analyses of different methylated regions also demonstrated a similar trends (Fig. 6A; cg12042695, $P = 0.00038$; cg19776201, $P = 0.041$; cg24366702, $P = 0.029$; cg00868383, $P = 0.0023$; cg03735888, $P = 0.00024$). Besides, UALCAN was used to evaluate the impact of aberrant methylation on the clinicopathological features of BC patients. Compared to the patients without or lower methylation of ZNF132, the BC patients with higher methylation of ZNF132 were associated with age, gender, ethnic, and tumor stage (Fig. 6B).

Discussion

Breast cancer is an aggressive malignancy tumor in females, the common metastasis locations in clinical included the lung, bone, and brain, leading to approximately 522,000 deaths yearly [1]. Until now, the specific mechanism of BC is still unclear. Currently, the causes of BC were considered to be involved in a variety of events, including genetics and epigenetics modification, especially for epigenetic change, such as DNA promoter methylation, gene mutation and deletion in tumorigenesis. In the past decades, DNA methylation has been demonstrated to be a promising early diagnostic biomarker for BC, however, useful markers in practice have not been completely identified.

Zinc finger protein is an important family of transcription factors and the majority of human zinc finger proteins contain the KRAB domains, which has been proved to act as a transcriptional repressor by interacting with KAP1 and subsequently recruiting histone-modifying proteins [20, 23, 24]. ZNF132, a member of the zinc finger protein family, was only reported to be downregulated by promoter methylation in ECSC and PC [25, 26]. However, its diagnostic and prognostic values have not been elucidated in BC until now. The present study, to the best of our knowledge, is the first one to systematically explore the clinical significance of ZNF132 in BC.

In agreement with the study of ZNF132 in ECSC and PC, our results indicated that ZNF132 had a significantly lower expression in BC tissues than adjacent normal tissues both in mRNA and protein level, which implied that ZNF132 might serve as a tumor suppressor in BC. Without a doubt, the larger samples need to be collected to provide more powerful evidence to verify the role of ZNF132 in BC. In addition, the ROC curve revealed that ZNF132 displayed a significant diagnostic value for BC (AUC = 0.887, $P < 0.001$). Significantly, lower ZNF132 expression was correlated with the worse prognosis of BC. Consequently, ZNF132 might be served as a promising diagnostic and prognostic marker for BC. However, as demonstrated in Table 2 and Table 3, univariate and multivariate analysis did not provide independent prognostic information for ZNF132 expression. The possible reasons we summarized are as follows: a. A series of mixed factors were involved in the prognosis of BC, the common factors were only listed in the present study. b. Samples in our study based on the TCGA database were not enough to clarify the correlation between ZNF132 expression and prognostic value, so large-scale prospective study would be necessary to confirm the value of ZNF132 in future. Moreover, analysis from Kaplan-Meier plotter revealed that low ZNF132 expression was significantly associated with a shorter RFS for patients of BC, but not with OS. Therefore, we speculated that ZNF132 expression can detect BC recurrence. Owing to tumor heterogeneous characteristics, BC has usually been classified several molecular subgroups: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2), normal, and basal-like based on immunohistochemical evaluation of estrogen receptor (ER), progesterone receptor (PR), HER2 and proliferation marker Ki-67. Analysis based on TCGA database revealed that downregulated ZNF132 was significantly correlated with the malignant phenotype of BC, including positive HER2 status, larger tumor sizes, distant metastasis and advanced clinical stage, which suggested that ZNF132 might inhibit the progression of BC by inhibiting the growth, invasion, and metastasis of tumor cells. Simultaneously, ZNF132 expression was positively associated with ER and PR status, further investigation is called for to identify whether combined detection of ZNF132 together with some of these other molecules would be valuable in improving prognosis assessment.

To recognize the potential mechanisms of ZNF132 in BC, the first investigation from cBioPortal showed that approximately 6% BC patients exhibited ZNF132 alterations, among which, the mRNA downregulation was the predominant type of alteration, which could contribute to the downregulation of ZNF132 in BC. In addition, the analysis based on GSEA demonstrated that ZNF132 participated in a variety of important biological processes and pathways. Significantly, ZNF132 expression was negatively correlated with CCNE1 and ENO1. Cyclin E1 (CCNE1), belongs to the highly conserved cyclin family, forms a complex with CDK2, whose activity is required for cell cycle G1/S transition. CCNE1 has been reported to upregulated in various human cancer, including breast[27], bladder[28], and ovarian[29], by mediating premature S-phase entry, ineffective DNA replication, and genomic instability. Alpha-enolase (ENO1), as a prominent glycolytic enzyme, was upregulated in multiple cancers and its overexpression was involved in tumor cell proliferation and metastasis, such as glioma[30], gastric[31], pancreatic[32], colorectal[33], BC[34]. So we speculated that ZNF132 might inhibit the progression of BC by regulating the expression of ENO1 and CCNE1.

Aberrant promoter methylation can permanently inactivate tumor-associated genes, particularly tumor suppressor genes. In the present study, we found that the ZNF132 methylation level was higher in BC tissue than that in normal tissues.. Besides, 3 DNA methyltransferases were also overexpressed in the ZNF132^{low} group. Simultaneously, methylated modification of ZNF132 was detected in 6 BC cell lines and 5-Aza-dC treatment could restore the mRNA and protein levels of ZNF132 in some cell lines, including MDA-MB-231, MCF7, and HCC1937. However, 5-Aza-dC could not restore the expression of ZNF132 between the mRNA and protein levels in the T47D cell. Besides, the MDA-MB-453 cell did not restore ZNF132 expression at the protein level. Moreover, an opposite trend was obtained in DU4475. The most likely reason is that the ZNF132 gene may undergo other epigenetic modifications, including transcription and post-transcriptional regulation. Finally, methylation analysis in 10 CpG island sites, including cg169294963, cg11618529, cg07878486, cg00868383, cg24366702, cg00547077, cg13877915, cg03735888, cg12042659, and cg19776201, indicated that these sites were hypermethylated in BC sample, suggesting that DNA methylation in these sites might inactivate ZNF132 gene transcription. Importantly, clinical samples analysis provided us a strong evidence between promoter methylation status and expression of ZNF132 in BC. Therefore, ZNF132 hypermethylation may act as an independent risk factor in BC patients.

Conclusions

In conclusion, our research firstly demonstrated the expression, diagnostic ability, and prognostic significance of ZNF132 based on the TCGA database. Importantly, aberrant hypermethylation of ZNF132 mediated its silence in BC. Therefore, ZNF132 could be used as a potential target for diagnosis and prognostic evaluation in BC.

Declarations

Ethics approval and consent to participate

The present study was approved by the First Affiliated Hospital of Xi'an Jiaotong University Ethics committee. Written informed consent was obtained from all patients.

Consent for Publication

All patients consented to the publication of data and any associated images.

Availability of Data and Materials

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

Competing interests

The authors declared that they had no competing interest

Funding

This study was supported by the Open Project Program of Key Laboratory for Tumor Precision Medicine of Shaanxi Province, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, People's Republic of China (KLTPM-SX2024-C1).

Authors' contributions

All authors have read and approved the manuscript. ZL, JL and PX conceived and designed the experiments; RML, WFZ and XHZ performed the experiments; MX and JZ analyzed the data. In addition, PX provided BC specimens, as well as giving final approval of the version to be published.

Acknowledgments

Not applicable.

Abbreviations

TCGA: The Cancer Genome Atlas; ZNF132: Zinc Finger Protein 132; AUC: An area under the curve; ROC: receiver operating characteristic; OS: Overall survival; RFS: Relapse-free survival; DFS: Disease-free survival; BC: BC; GSEA: Gene Set Enrichment Analysis; MSP: Methylation-specific PCR;

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F: **Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012**. *International journal of cancer* 2015, **136**(5):E359-386.

2. DeSantis C, Ma J, Bryan L, Jemal A: **Breast cancer statistics, 2013**. *CA: a cancer journal for clinicians* 2014, **64**(1):52-62.
3. Kolacinska A, Herman K, Morawiec J, Paszek S, Zawlik I, Sliwczynski A: **Improvement in outcomes of breast cancer patient treatment in Poland in the 21st century**. *The breast journal* 2019, **25**(3):474-478.
4. Koroukian SM, Bakaki PM, Htoo PT, Han X, Schluchter M, Owusu C, Cooper GS, Rose J, Flocke SA: **The Breast and Cervical Cancer Early Detection Program, Medicaid, and breast cancer outcomes among Ohio's underserved women**. *Cancer* 2017, **123**(16):3097-3106.
5. Howard DH, Tangka FK, Royalty J, Dalzell LP, Miller J, O'Hara B, Joseph K, Kenney K, Guy G, Hall IJ: **Breast cancer screening of underserved women in the USA: results from the National Breast and Cervical Cancer Early Detection Program, 1998-2012**. *Cancer causes & control : CCC* 2015, **26**(5):657-668.
6. Miller JW, Hanson V, Johnson GD, Royalty JE, Richardson LC: **From cancer screening to treatment: service delivery and referral in the National Breast and Cervical Cancer Early Detection Program**. *Cancer* 2014, **120 Suppl 16**:2549-2556.
7. Miller JW, Plescia M, Ekwueme DU: **Public health national approach to reducing breast and cervical cancer disparities**. *Cancer* 2014, **120 Suppl 16**:2537-2539.
8. Plescia M, Wong F, Pieters J, Joseph D: **The National Breast and Cervical Cancer Early Detection Program in the era of health reform: a vision forward**. *Cancer* 2014, **120 Suppl 16**:2620-2624.
9. Siegel RL, Miller KD, Jemal A: **Cancer statistics, 2018**. *CA: a cancer journal for clinicians* 2018, **68**(1):7-30.
10. Howlader NNA KM, Neyman N, Aminou R, Waldron W, Altekruse SF, Kosary CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA, Edwards BK: **SEER cancer statistics review, 1975–2008**. . *National Cancer Institute, Bethesda* 2011
11. Gyorffy B, Bottai G, Fleischer T, Munkacsy G, Budczies J, Paladini L, Borresen-Dale AL, Kristensen VN, Santarpia L: **Aberrant DNA methylation impacts gene expression and prognosis in breast cancer subtypes**. *International journal of cancer* 2016, **138**(1):87-97.
12. Jovanovic J, Ronneberg JA, Tost J, Kristensen V: **The epigenetics of breast cancer**. *Molecular oncology* 2010, **4**(3):242-254.
13. Polyak K: **Breast cancer: origins and evolution**. *The Journal of clinical investigation* 2007, **117**(11):3155-3163.
14. Rodriguez-Paredes M, Esteller M: **Cancer epigenetics reaches mainstream oncology**. *Nature medicine* 2011, **17**(3):330-339.
15. Tapia T, Smalley SV, Kohen P, Munoz A, Solis LM, Corvalan A, Faundez P, Devoto L, Camus M, Alvarez M *et al*: **Promoter hypermethylation of BRCA1 correlates with absence of expression in hereditary breast cancer tumors**. *Epigenetics* 2008, **3**(3):157-163.
16. Esteller M, Corn PG, Baylin SB, Herman JG: **A gene hypermethylation profile of human cancer**. *Cancer research* 2001, **61**(8):3225-3229.

17. Widschwendter M, Jones PA: **DNA methylation and breast carcinogenesis.** *Oncogene* 2002, **21**(35):5462-5482.
18. Wittenberger T, Sleigh S, Reisel D, Zikan M, Wahl B, Alunni-Fabbroni M, Jones A, Evans I, Koch J, Paprotka T *et al*: **DNA methylation markers for early detection of women's cancer: promise and challenges.** *Epigenomics* 2014, **6**(3):311-327.
19. Jen J, Wang YC: **Zinc finger proteins in cancer progression.** *Journal of biomedical science* 2016, **23**(1):53.
20. Fedotova AA, Bonchuk AN, Mogila VA, Georgiev PG: **C2H2 Zinc Finger Proteins: The Largest but Poorly Explored Family of Higher Eukaryotic Transcription Factors.** *Acta naturae* 2017, **9**(2):47-58.
21. Rio DC, Ares M, Jr., Hannon GJ, Nilsen TW: **Purification of RNA using TRIzol (TRI reagent).** *Cold Spring Harbor protocols* 2010, **2010**(6):pdb.prot5439.
22. Shi J, Liu W, Sui F, Lu R, He Q, Yang Q, Lv H, Shi B, Hou P: **Frequent amplification of AIB1, a critical oncogene modulating major signaling pathways, is associated with poor survival in gastric cancer.** *Oncotarget* 2015, **6**(16):14344-14359.
23. Margolin JF, Friedman JR, Meyer WK, Vissing H, Thiesen HJ, Rauscher FJ, 3rd: **Krüppel-associated boxes are potent transcriptional repression domains.** *Proceedings of the National Academy of Sciences of the United States of America* 1994, **91**(10):4509-4513.
24. Vissing H, Meyer WK, Aagaard L, Tommerup N, Thiesen HJ: **Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins.** *FEBS letters* 1995, **369**(2-3):153-157.
25. Jiang D, He Z, Wang C, Zhou Y, Li F, Pu W, Zhang X, Feng X, Zhang M, Yecheng X *et al*: **Epigenetic silencing of ZNF132 mediated by methylation-sensitive Sp1 binding promotes cancer progression in esophageal squamous cell carcinoma.** *Cell death & disease* 2018, **10**(1):1.
26. Abildgaard MO, Borre M, Mortensen MM, Ulhøi BP, Tørring N, Wild P, Kristensen H, Mansilla F, Ottosen PD, Dyrskjot L *et al*: **Downregulation of zinc finger protein 132 in prostate cancer is associated with aberrant promoter hypermethylation and poor prognosis.** *International journal of cancer* 2012, **130**(4):885-895.
27. Zhao ZM, Yost SE, Hutchinson KE, Li SM, Yuan YC, Noorbakhsh J, Liu Z, Warden C, Johnson RM, Wu X *et al*: **CCNE1 amplification is associated with poor prognosis in patients with triple negative breast cancer.** *BMC cancer* 2019, **19**(1):96.
28. Matsushita R, Seki N, Chiyomaru T, Inoguchi S, Ishihara T, Goto Y, Nishikawa R, Mataka H, Tatarano S, Itesako T *et al*: **Tumour-suppressive microRNA-144-5p directly targets CCNE1/2 as potential prognostic markers in bladder cancer.** *British journal of cancer* 2015, **113**(2):282-289.
29. Nakayama N, Nakayama K, Shamima Y, Ishikawa M, Katagiri A, Iida K, Miyazaki K: **Gene amplification CCNE1 is related to poor survival and potential therapeutic target in ovarian cancer.** *Cancer* 2010, **116**(11):2621-2634.
30. Song Y, Luo Q, Long H, Hu Z, Que T, Zhang X, Li Z, Wang G, Yi L, Liu Z *et al*: **Alpha-enolase as a potential cancer prognostic marker promotes cell growth, migration, and invasion in glioma.** *Molecular cancer* 2014, **13**:65.

31. Sun L, Lu T, Tian K, Zhou D, Yuan J, Wang X, Zhu Z, Wan D, Yao Y, Zhu X *et al*: **Alpha-enolase promotes gastric cancer cell proliferation and metastasis via regulating AKT signaling pathway.** *European journal of pharmacology* 2019, **845**:8-15.
32. Shen J, Person MD, Zhu J, Abbruzzese JL, Li D: **Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry.** *Cancer research* 2004, **64**(24):9018-9026.
33. Zhan P, Zhao S, Yan H, Yin C, Xiao Y, Wang Y, Ni R, Chen W, Wei G, Zhang P: **alpha-enolase promotes tumorigenesis and metastasis via regulating AMPK/mTOR pathway in colorectal cancer.** *Molecular carcinogenesis* 2017, **56**(5):1427-1437.
34. Tu SH, Chang CC, Chen CS, Tam KW, Wang YJ, Lee CH, Lin HW, Cheng TC, Huang CS, Chu JS *et al*: **Increased expression of enolase alpha in human breast cancer confers tamoxifen resistance in human breast cancer cells.** *Breast cancer research and treatment* 2010, **121**(3):539-553.

Tables

Table1. Clinical association between ZNF132 expression and clinicopathological variables in BC patients

Variable	Number	ZNF132 expression		c ² test P- value	Correlation	
		Low	High		r	P-value
Age						
≤60	611	289	321	0.053	-0.046	0.123
>60	493	263	231			
HER2				0.001	-0.135	0.000
Negative	652	292	360			
Positive	114	70	44			
ER				0.000	0.209	0.000
Negative	179	111	68			
Positive	601	266	335			
PR				0.000	0.236	0.000
Negative	255	157	98			
Positive	522	216	306			
Tumor size				0.006	-0.125	0.000
T1	282	121	161			
T2-T4	819	429	390			
Lymph node metastasis				0.003	-0.056	0.064
No	516	420	465			
Yes	568	118	81			
Distant metastasis				0.233	-0.044	0.170
No	964	464	450			
Yes	22	14	8			
Clinical stage				0.058	-0.084	0.006
I	183	80	103			
II-IV	899	462	437			

Table 2. Univariate analysis of prognostic factors of BC

Variable	Hazard ratio	95%CI	P-value
Age($\leq 60 / > 60$)	1.928	(1.402,2.652)	0.000
HER2(Positive / Negative)	1.402	(0.592,1.834)	0.886
ER(Positive/Negative)	1.018	[0.659,1.573]	0.937
PR(Positive/Negative)	0.938	[0.637,1.379]	0.744
Tumor size(T2-T4/T1)	1.500	(1.020,2.206)	0.040
Lymph Node metastasis(Yes/No)	2.200	(1.542,3.140)	0.000
Distant metastasis(Yes/No)	4.749	(2.840,7.940)	0.000
Clinical stage(II-IV/I)	2.249	(1.337,3.783)	0.002
ZNF132 expression(High/Low)	0.829	(0.602,1.143)	0.252

Table 3. Multivariate analysis of prognostic factors of BC.

Variable	Hazard ratio	95%CI	P-value
Age($\leq 60 / > 60$)	2.278	(1.453,3.571)	0.000
HER2(Positive / Negative)	0.804	(0.405,1.597)	0.533
ER(Positive/Negative)	1.015	[0.507,2.032]	0.966
PR(Positive/Negative)	0.618	[0.330,1.154]	0.131
Tumor size(T2-T4/T1)	1.238	(0.548,2.800)	0.608
Lymph Node metastasis(Yes/No)	1.630	(0.959,2.770)	0.071
Distant metastasis(Yes/No)	3.427	(1.665,7.055)	0.001
Clinical stage(II-IV/I)	1.140	(0.386,3.369)	0.813
ZNF132 expression(High/Low)	1.129	(0.728,1.753)	0.587

Figures

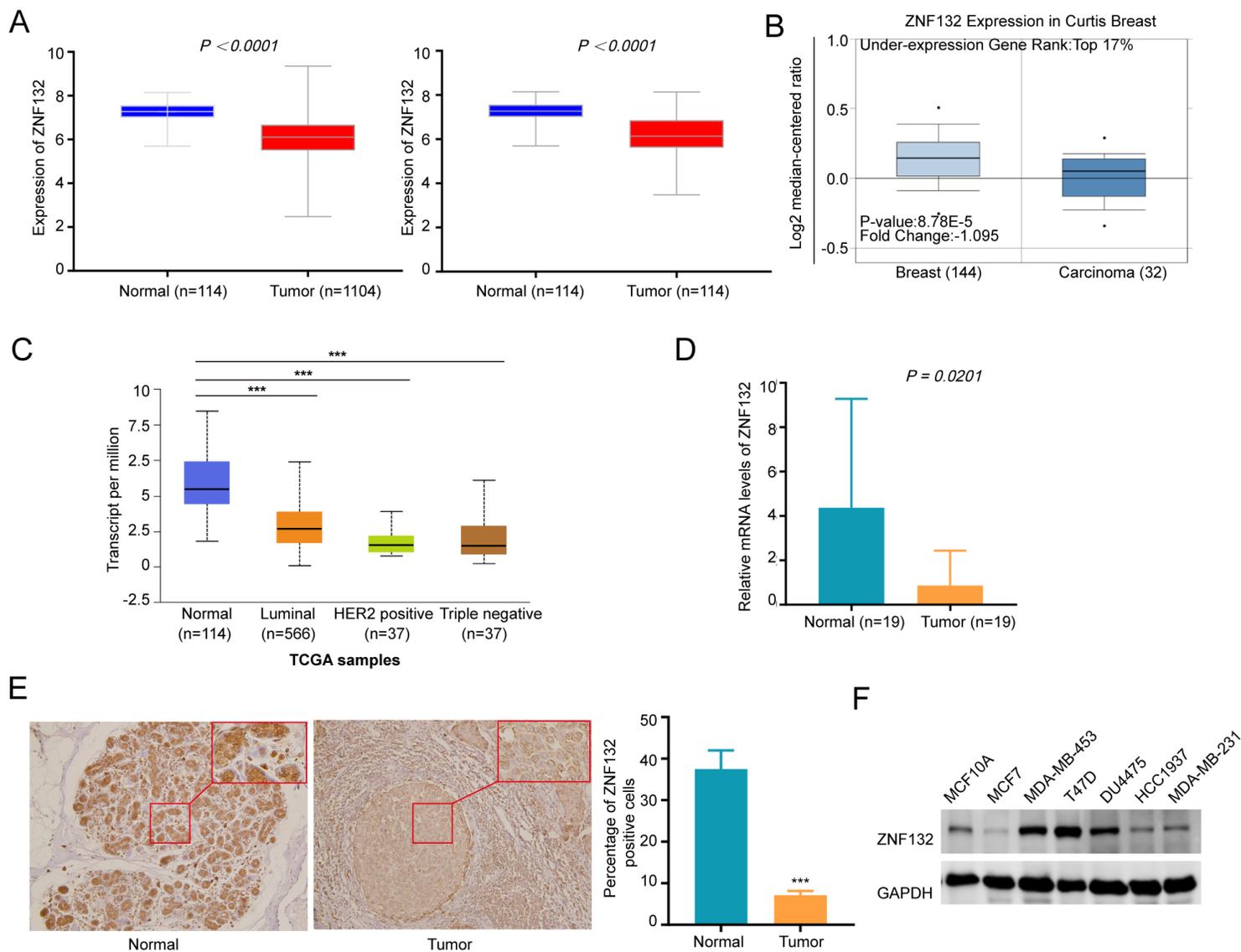


Figure 1

Expression analysis of ZNF132 in BC. (A) ZNF132 was downregulated in 1104 BC tissues and 114 matched pairs of BC tissues compared with adjacent normal tissues based on the TCGA database. (B) Data from the Oncomine 4.5 database also revealed that mRNA expression of ZNF132 was significantly reduced in BC tissues. (C) ZNF132 expression was reduced in different BC molecular subgroups, including luminal A, luminal B, HER2-positive, Triple negative BC. Data were presented as mean \pm SD. ***, $P < 0.001$ for comparison with the control. (D) qRT-PCR assay was performed to assess the mRNA expression of ZNF132 in 19 cases of BC tissues. (E) Immunohistochemistry analysis of ZNF132 in BC and adjacent normal tissues ($\times 20$). (F) ZNF132 expression was determined by Western blot analysis in six BC cell lines and normal breast epithelial cells. GAPDH was used as internal control.

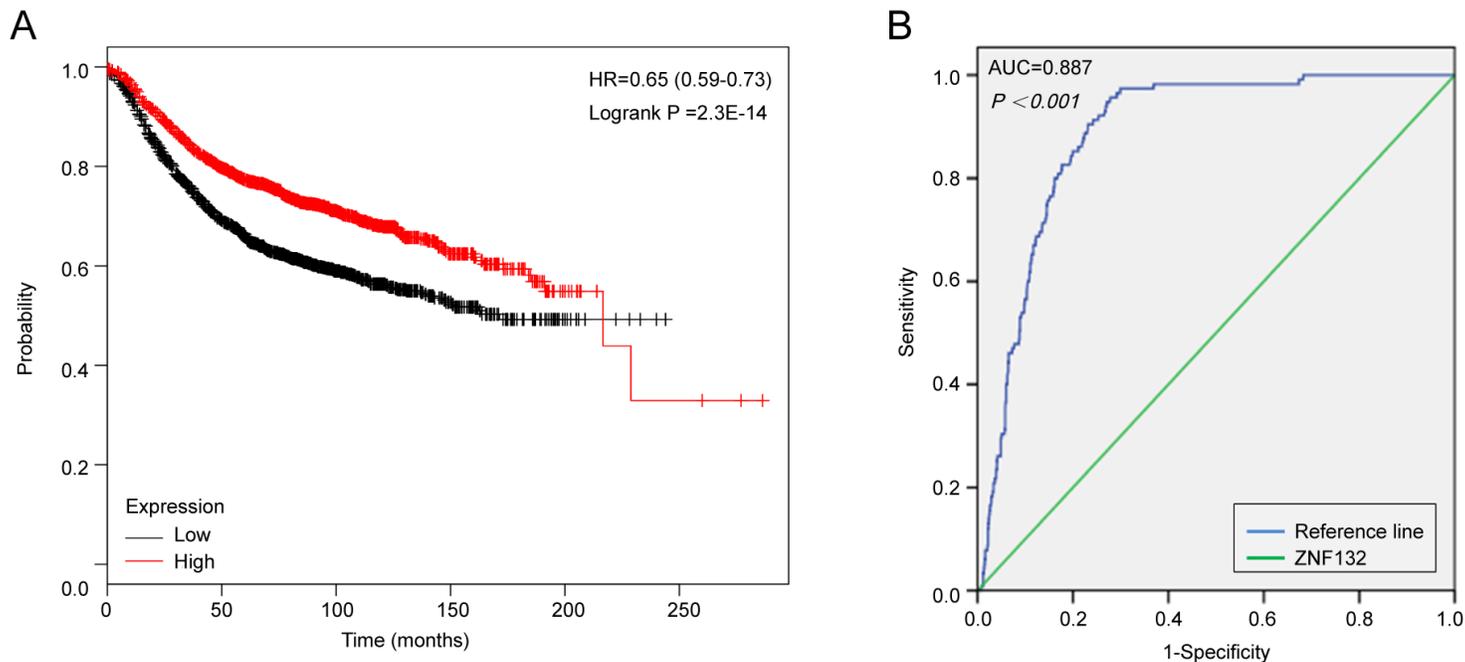


Figure 2

Prognostic and diagnostic value of ZNF132. (A) Survival analysis of ZNF132 in BC demonstrated that low ZNF132 expression was significantly associated with a reduced RFS using Kaplan-Meier Plotter. (B) The ROC curve indicated that ZNF132 possessed an adequate diagnostic ability for BC (AUC =0.887, P <0.001).

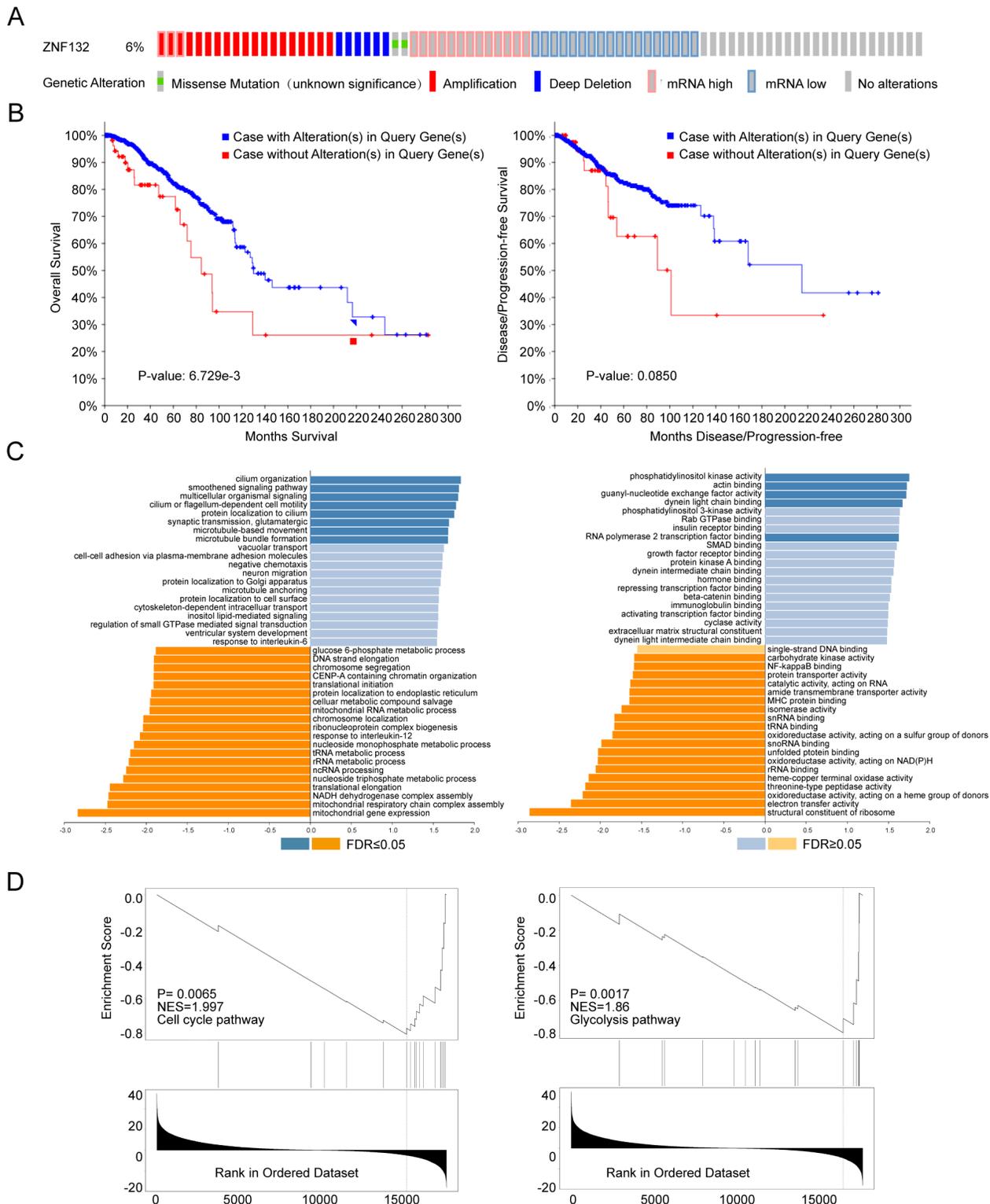


Figure 3

Bioinformatic analysis of ZNF132. (A) A total of 6% (58/960) of GC cases exhibited ZNF132 alteration. (B) OS analysis of BC patients with and without ZNF132 alteration. (C) Potential biological processes and biological pathways in BC were identified by GSEA analysis. (D) ZNF132 expression was negatively correlated with the cell cycle and glycolysis pathway.

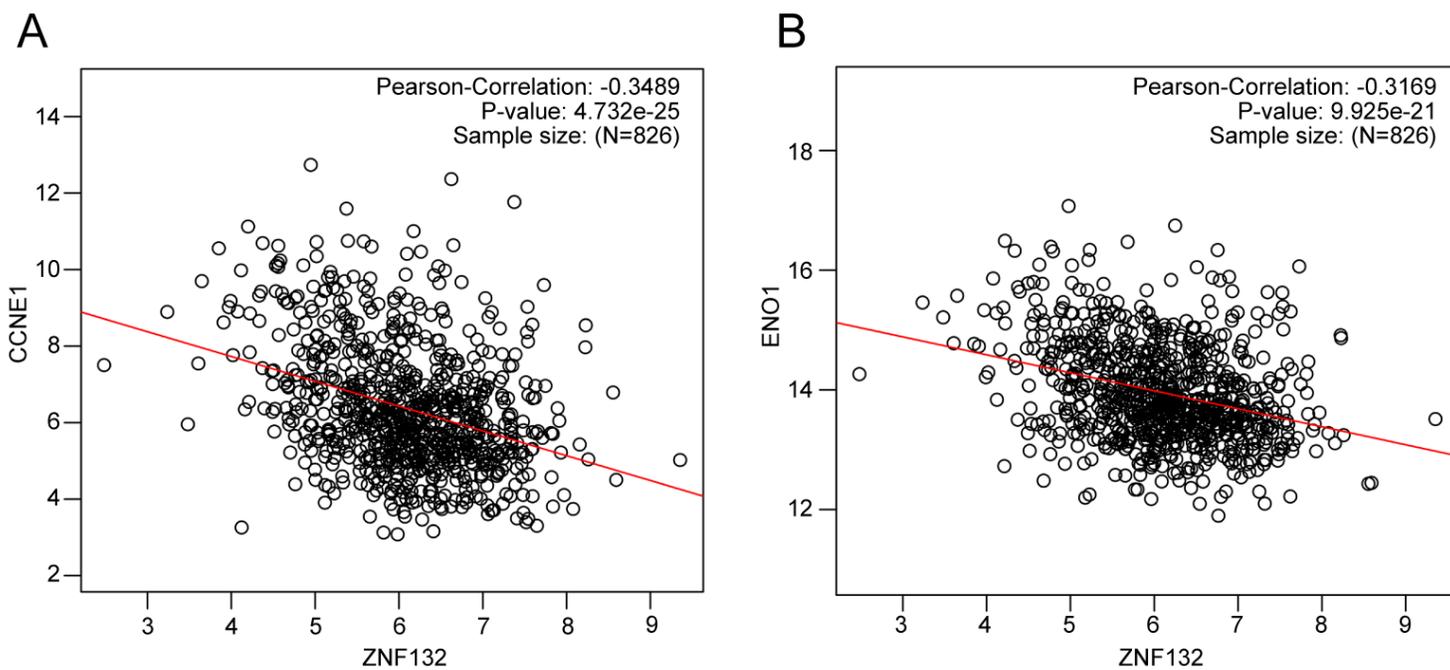


Figure 4

Genes involved in the cell cycle and glycolysis were associated with ZNF132 expression. (A) ZNF132 was negatively correlated with the expression of CCNE1 in cell cycle and (B) the expression of ENO1 in glycolysis.

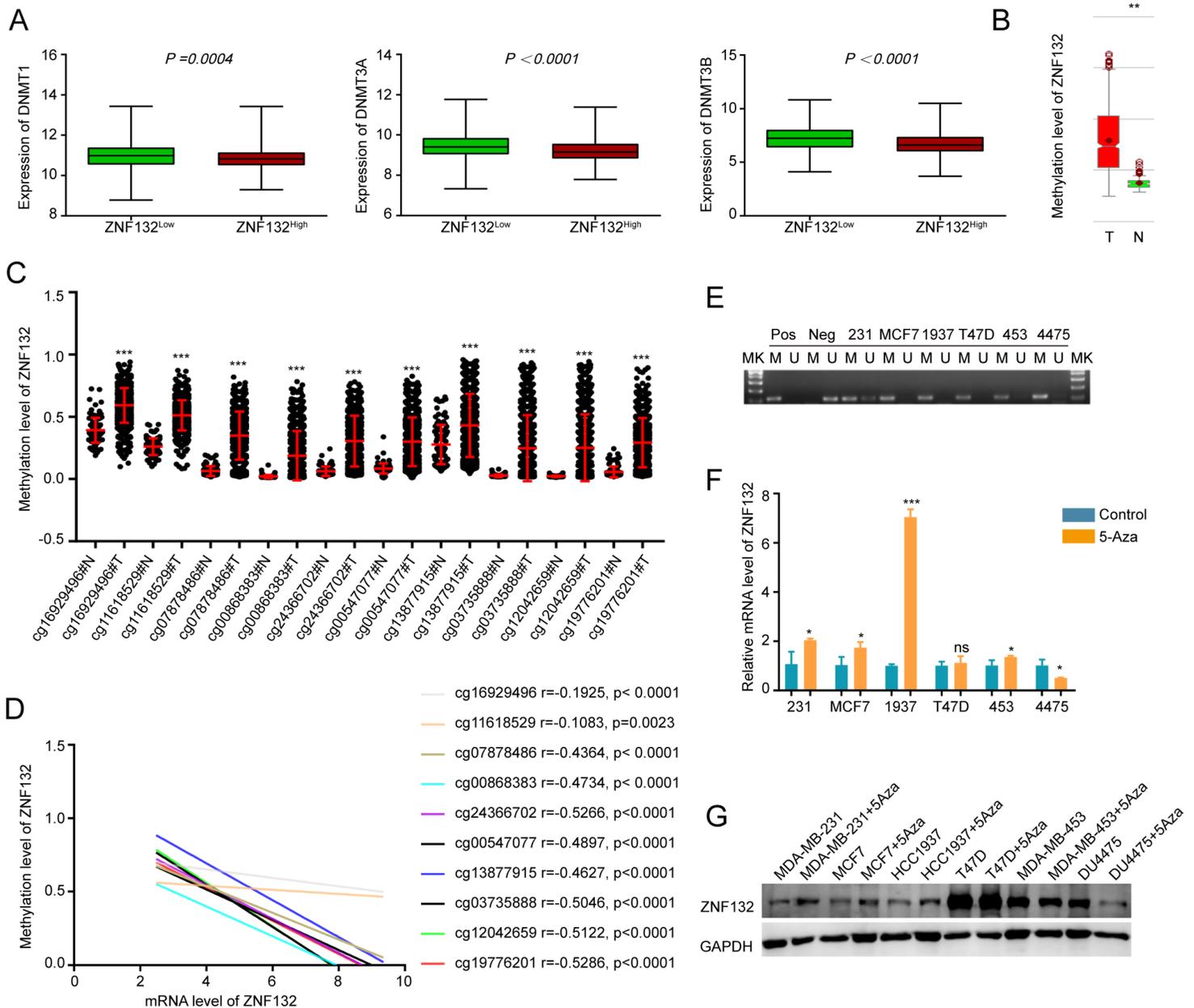


Figure 5

Aberrant methylation of ZNF132 in BC. (A) Expression of 3 DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) in ZNF132^{High} and ZNF132^{Low} group. (B) MethHC analysis demonstrated that the methylation level of ZNF132 in BC tissues was significantly higher than the normal sample. (C) Methylation level of ZNF132 in different CpG sites between BC tissues and matched normal tissues. (D) ZNF132 methylation level was negatively correlated with its gene expression. (E) Promoter methylation of ZNF132 in BC cell lines was determined by the MSP assay. In vitro methylated DNA as a positive control for methylated gene (Pos); Bisulfite-modified normal leukocyte DNA as a positive control for unmethylated gene (Neg); Mk, DNA marker; M, methylated gene; U, unmethylated gene; 231, MDA-MB-231; MCF7; 1937, HCC1937; T47D; 453, MDA-MB-453; 4475, DU4475. (F) mRNA level of ZNF132 was partially restored by 5-Aza-dC including MDA-MB-231, MCF7, HCC1937, and MDA-MB-453. (G) The protein level of

ZNF132 was partially restored by 5-Aza-dC, including MDA-MB-231, MCF7, and HCC1937. Ctr, Control; 5-Aza, 5-Aza-Dc. Statistically significant differences were indicated: *P <0.01, **P <0.001, ***P <0.0001.

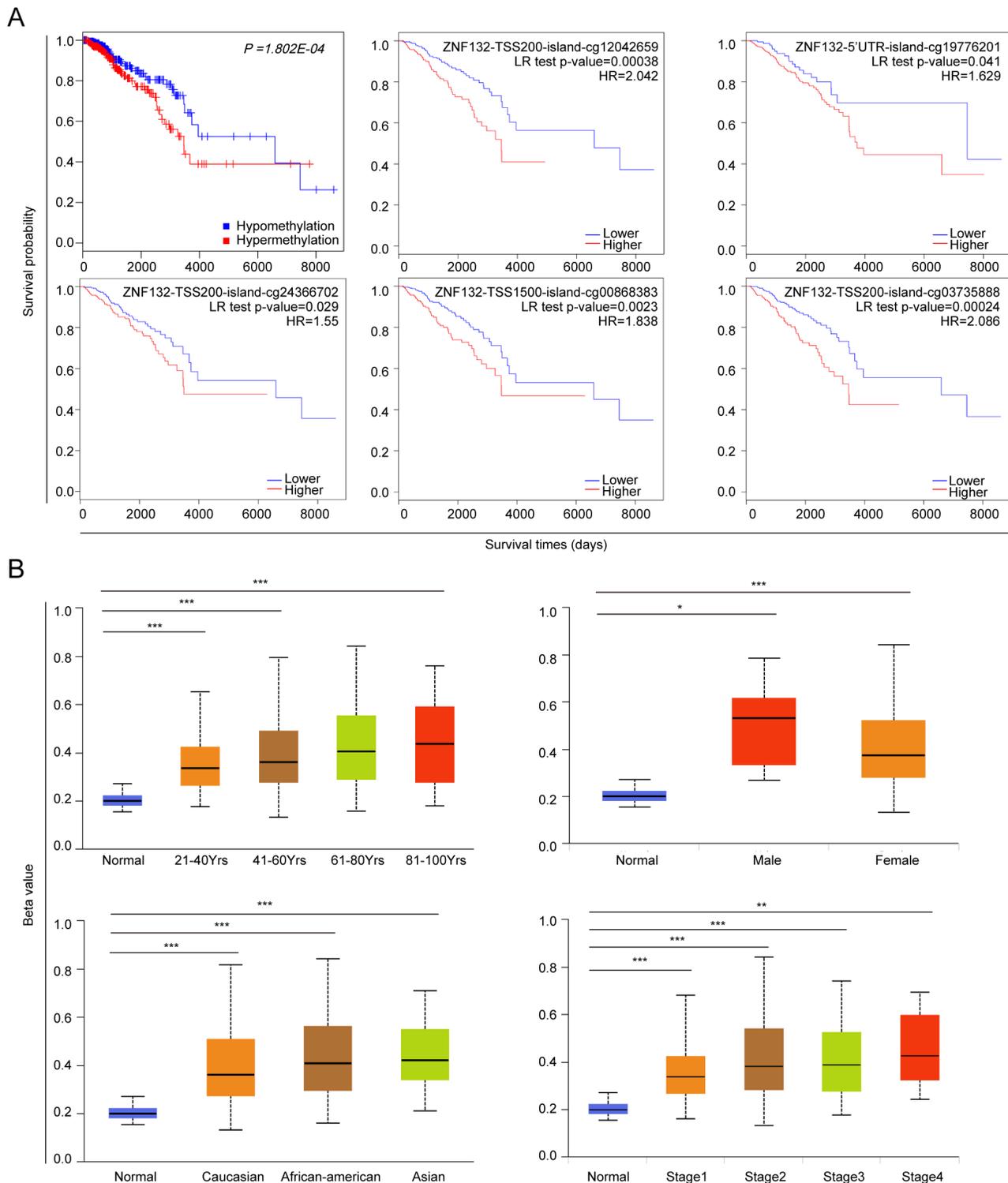


Figure 6

The effect of ZNF132 methylation on prognosis and clinicopathological features. (A) The high methylation level of ZNF132 was negatively correlated with overall survival in BC patients. (B) UALCAN

was used to evaluate the impact of aberrant methylation on the clinicopathological features of BC patients.