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Integrative Analysis of Genetic and Epigenetic Alterations in the CBX7 Gene Reveals Its Tumor-Suppressive Function by Regulating the Cell Cycle in Human Breast Cancer

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Authors' contributions
HC, JPL, and JHM conceived and designed the overall study; JPL and JHM acquired funding; CY, HC, and JHM performed the bioinformatics analyses and conducted statistical tests; HC, JPL, and JHM wrote the manuscript. All authors have read and edited the manuscript.

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Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this article.

ABSTRACT

CBX7 is a member of the chromobox gene family, which plays an important role in epigenetic transcriptional regulation. In this study, we found that compared to normal mammary tissues, mRNA levels of *CBX7* are consistently significantly downregulated in breast cancers (BCs) across different datasets. Integrative multiomics analysis revealed the genetic and epigenetic mechanisms for the loss of *CBX7* expression in BCs. Lower expression levels of *CBX7* are significantly associated with shorter overall, disease-free, and distant metastasis-free survival of patients with BC. These prognostic impacts of *CBX7* are independent of estrogen receptor status and PAM50 molecular subtypes. Coexpression analysis identified 207 genes consistently coexpressed with *CBX7* (157 negatively and 50 positively). Gene Ontology, KEGG, and Reactome enrichment analysis revealed that cell cycle-, DNA replication-, and mitosis-related pathways are significantly overrepresented within the set of *CBX7* negatively coexpressed genes, suggesting that *CBX7* functions as a suppressor of the cell cycle. Moreover, transcription factor enrichment analysis detected the E2F family of transcription factors significantly associated with *CBX7* negatively coexpressed genes, consistent with E2F function regulating the cell cycle. Furthermore, we found that loss of *CBX7* expression significantly increases genomic instability and tumor mutation burden. Our findings indicate that *CBX7* acts as a tumor suppressor in BC through its potential role in the negative regulation of cell proliferation and the maintenance of genome integrity.

Keywords: Breast cancer, *CBX7*, genetic alteration, epigenetic alteration, prognosis, gene co-expression.

INTRODUCTION

Breast cancer (BC) incidence continues to rise globally, with over 2 million new cases diagnosed yearly [1-3]. In the last two decades, the advent of biotechnologies, especially next-generation sequencing, has extensively cataloged the multiomics landscape of BCs [4-7], which has deepened insights into its heterogeneity and expanded our understanding of the disease. Despite the excellent progress made in the treatment and management of patients with BC, therapeutic resistance and distant metastasis, which inevitably lead to patient death, continue to be a daily challenge [1-3]. Therefore, the need to discover novel therapeutic targets remains one of the holy grails of BC research.

The epigenetic modifications, such as DNA methylation, histone modifications, and noncoding RNAs, lead to altered gene expression independent of changes in the primary genomic sequence, which contributes to cancer development and progression [8-10]. Cancer cells have been discovered to harbor epigenetic abnormalities [9, 11] in addition to genetic alterations. The chromobox (CBX) protein family, containing a chromodomain that binds to H3K27me3, plays an essential role in the epigenetic regulation of transcription of genes [12, 13], including both tumor suppressors and oncogenes. Recent studies have revealed the distinct

functions among CBX family members in cancer. For example, CBX3 was found to be upregulated, whereas *CBX7* was downregulated in many types of human cancer [14-22]. Some studies have shown that CBX3 transcriptionally suppresses the p21 gene to promote cell proliferation [23-25], while CBX7 transcriptionally suppresses CCNE1 expression to inhibit cell proliferation [26]. These studies indicate that some CBX genes function as oncogenes and others act as tumor suppressor genes. Moreover, the expression of many CBX genes has a prognostic impact on human cancer [14-22].

Although it has shown that the expression of *CBX7* is significantly reduced in BC [19, 27, 28], the mechanisms for the downregulation of *CBX7* expression and its functional role in BC remain largely unknown. In this study, we further investigated the *CBX7* gene using integrative multiomics analysis. Our results showed a loss of *CBX7* expression through genetic and epigenetic alteration in BC. We also discovered that *CBX7* is negatively coexpressed with important cell cycle-related genes, including CCNB1, CCNB2, and CCNE1. Therefore, we concluded that *CBX7* acts as a tumor suppressor in BC through its potential role in the negative regulation of cell proliferation.

MATERIALS AND METHODS

Datasets and Online Analytic Tools Used in this Study

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) data are publicly available and downloaded from cBioPortal (https://cbioportal.org/) [29, 30]. The online analytic tools included the following: TNMplot (https://tnmplot.com/analysis/) [31]; SMART (http://bioinfo-zs.com/smartapp/) [32]; bc-GenExMiner v4.8 (http://bcgenex.ico.unicancer.fr/BC-GEM/GEM-Accueil.php?js=1) [33]; WebGestalt (http://webgestalt.org/) [34].

Statistical Analysis

The difference in expression of *CBX7* between BCs and normal breast tissues was examined using TNMplot. Genetic alternation frequency was assessed in METABRIC [5, 6] and TCGA-BRCA [4, 7] using cBioPortal. Epigenetic alterations were analyzed using SMART. The association of *CBX7* expression with genetic and epigenetic alterations was assessed using the Mann–Whitney *U* test and Spearman's correlation in SPSS (IBM SPSS Statistics 24), respectively.

The associations of *CBX7* expression with overall (OS), disease-free (DFS), and distant metastasis-free (DMFS) survival were examined using GenExMiner. The patients were optimally divided into two groups based on *CBX7* expression levels in both pooled microarray and RNA-seq datasets in GenExMiner.

The list of *CBX7* coexpressed genes was identified in the METABRIC and TCGA-BRCA datasets using cBioPortal. Functional enrichment analyses, including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Reactome pathway, and transcriptional factor network of *CBX7* coexpressed genes were conducted using WebGestalt.

Differences in aneuploidy score, MSI sensor score, fraction of genome altered, mutation count, and tumor mutation burden (TMB) were assessed by the Wilcoxon test in the TCGA-BRCA dataset using cBioPortal.

The figure panels were downloaded from analytic tools or generated using SPSS (IBM SPSS Statistics 24). A two-tailed p-value or FDR <0.05 was considered statistically significant.

RESULTS

Loss of *CBX7* Expression through Genetic and Epigenetic Alteration in Breast Cancer

We used TNMplot to assess *CBX7* expression change in BCs. In both microarray and RNA-seq datasets, we found that *CBX7* mRNA expression levels were diminished more than twofold in BC compared to normal breast tissues (microarray: fold change = 0.43, p = 4.74E-54; RNA-seq: fold change = 0.27, p = 4.92E-156) (**Figure 1A, B**). To identify the mechanism for reduced expression of *CBX7*, we examined the copy number alterations (CNA) in *CBX7* using METABRIC and TCGA-BRCA datasets and found that a single copy of *CBX7* was frequently deleted in BCs (METABRIC: 32.3%; TCGA-BRCA: 45.0%) (**Figure 2A**), which leads to a significant reduction of its expression ($p < 0.0001$) (**Figure 2B**). Surprisingly, we observed that *CBX7* mRNA expression levels in tumors with a gain of *CBX7* were significantly lower than those without copy number alteration ($p < 0.0001$) **(Figure 2A**). This result led to exploring the possibility of epigenetic alterations in *CBX7* using methylation profile data, where different probes were used in the different locations of *CBX7* (**Figure 3A**). Methylation levels of *CBX7* in all probes were significantly increased in BCs compared to normal breast tissue ($p < 0.0001$) (**Figure 3B,** four left panels) using SMART. Moreover, high methylation levels of *CBX7* significantly negatively correlated with its mRNA expression levels ($p < 0.01$) (**Figure 3C,** four left panels). Moreover, combined methylation levels (average methylation levels of four probes) were significantly increased in BCs compared to normal breast tissue ($p < 1.0E-15$) (**Figure 3B,** last panel) and significantly negatively correlated with its mRNA expression levels (p = 1.8E-12) (**Figure 3C,** last panel). All these findings suggest that the mechanism for reduced expression of *CBX7* is through genetic and epigenetic alteration.

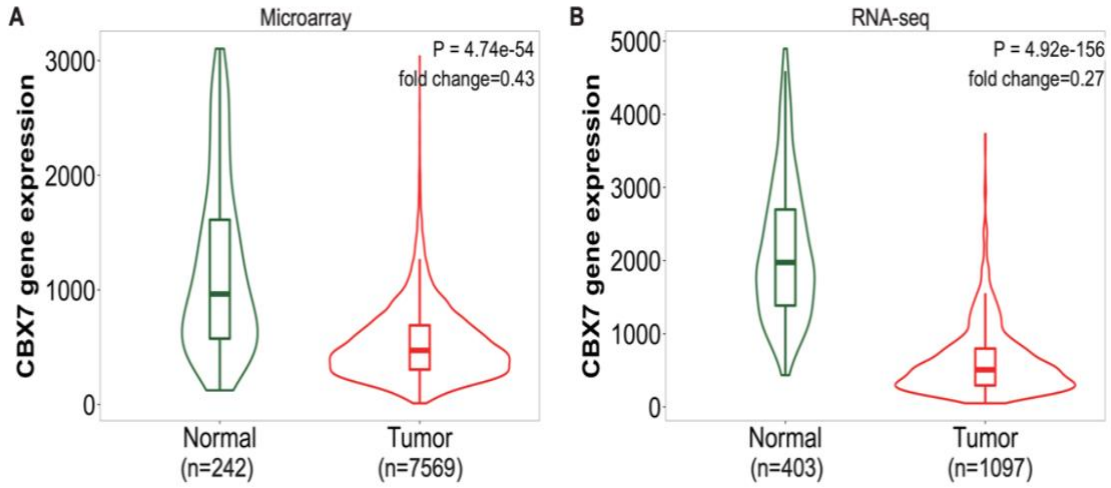

Figure 1. Loss of *CBX7* expression in breast cancers.
The violin plots were generated in **(A)** microarray and **(B)** RNA-seq datasets using TNMplot. The p values were obtained by the Mann–Whitney *U* test.

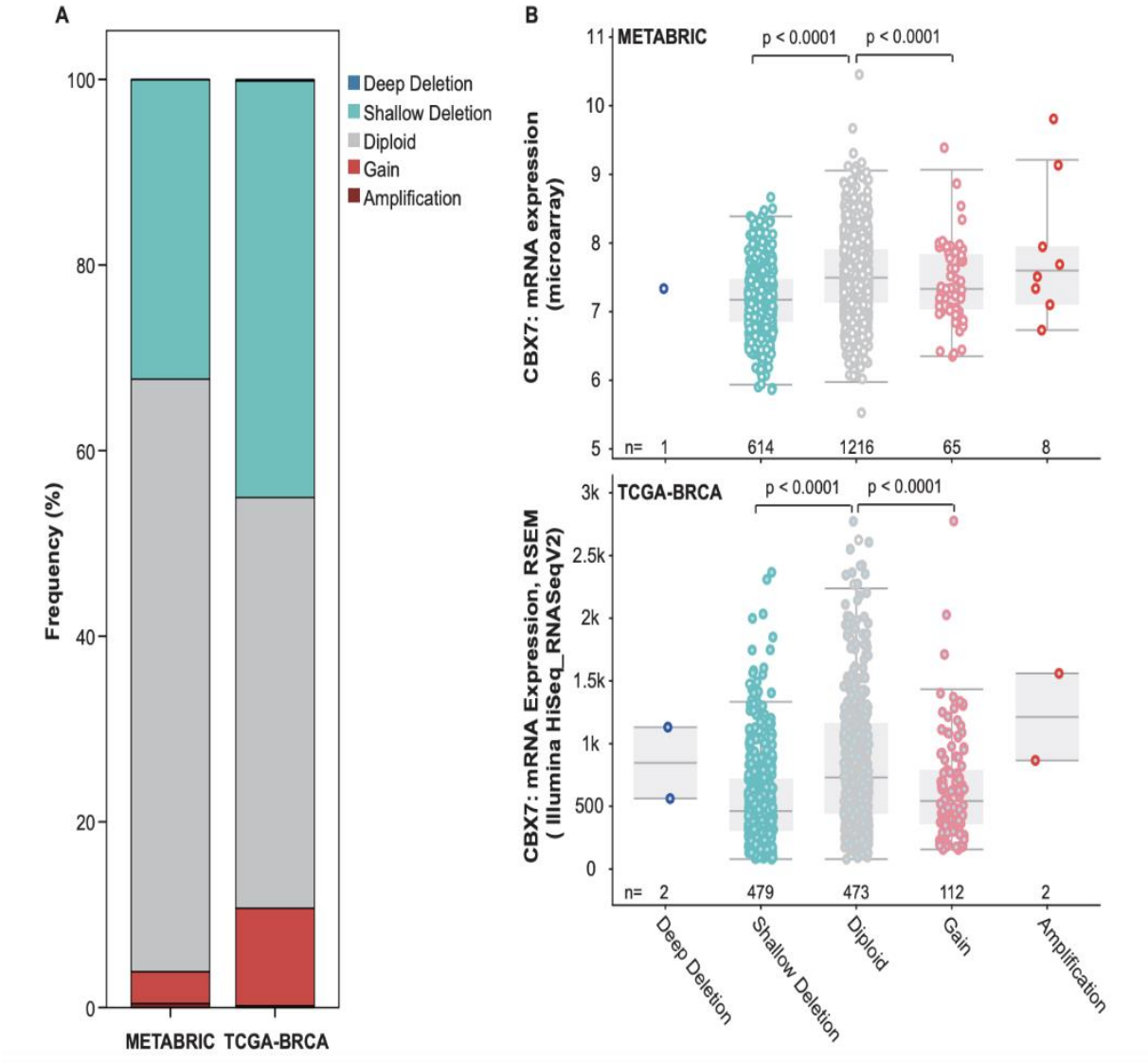

Figure 2. Genetic alterations in *CBX7* gene in breast cancers.
(A) Frequency of genetic alterations. (**B**) Correlation between genetic alterations and gene expression in METABRIC and TCGA-BRCA datasets. The p values were obtained from the Mann–Whitney *U* test.

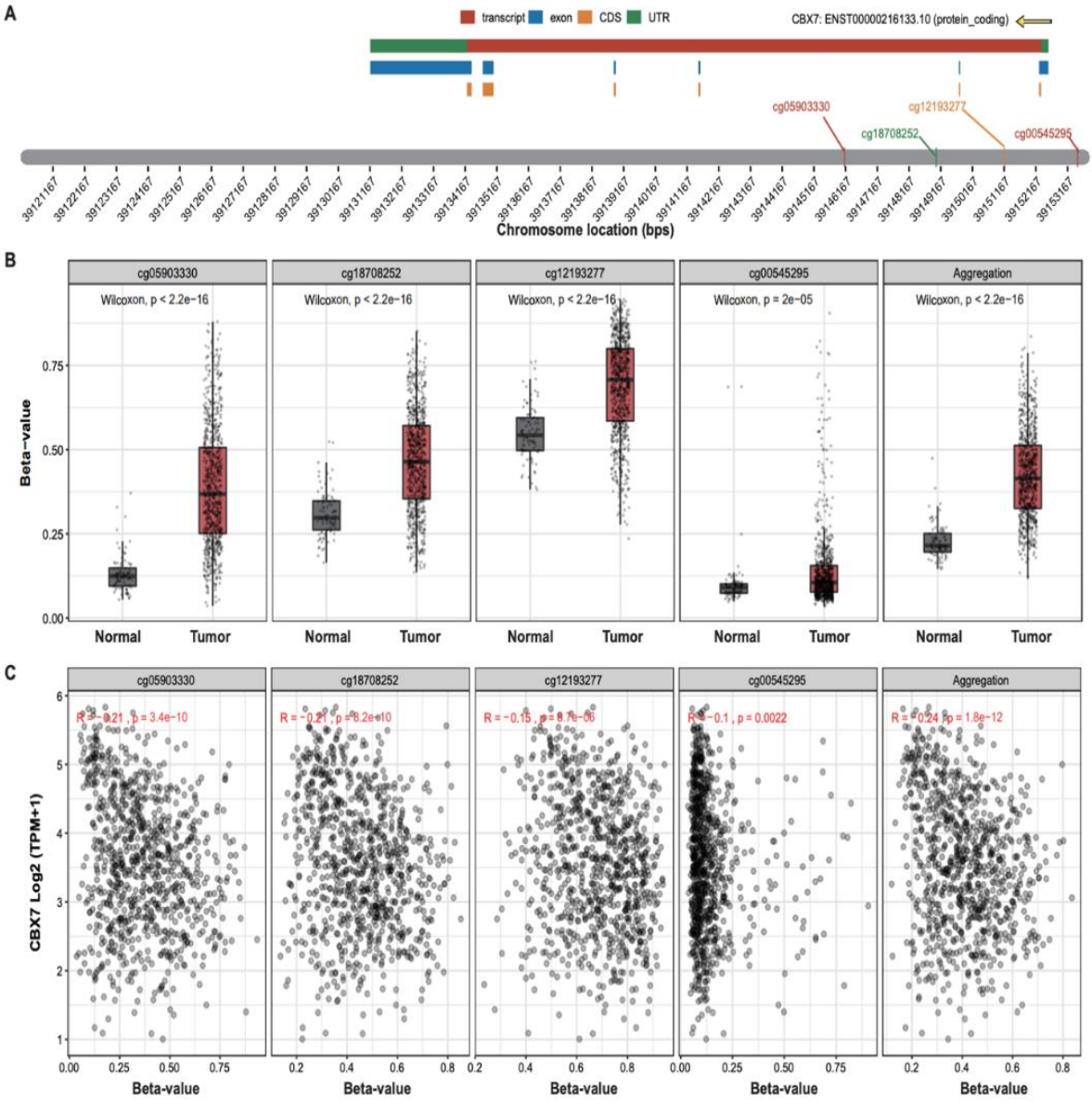

Figure 3. Epigenetic alterations in *CBX7* gene in breast cancers.

(A) Chromosome location of the probes used to detect the methylation of the *CBX7* gene in Illumina HumanMethylation27K or HumanMethylation450 BeadChip. (**B**) Comparison of the *CBX7* gene methylation level between normal breast and breast cancer tissues. The p values were obtained from the Wilcoxon test. (**C**) Correlation between the *CBX7* gene methylation and gene expression. The p values were obtained from Spearman correlation analysis.

Prognostic Value of *CBX7* Expression in Breast Cancer

The bc-GenExMiner was used to evaluate the prognostic value of *CBX7* mRNA expression in BC. We found that patients with BC with higher levels of *CBX7* had significantly longer overall survival (OS) (HR = 0.61, 95% CI of HR: 0.55–0.68, p < 0.0001) (**Figure 4A, Supplementary Figure 1A**), disease-free survival (DFS) (HR = 0.65, 95% CI of HR: 0.60–0.71, p < 0.0001) (**Figure 4B, Supplementary Figure 1B**), and DMFS (HR = 0.60, 95% CI of HR: 0.54–0.66, p < 0.0001) (**Figure 4C**). Since estrogen receptor (ER) status and the intrinsic molecular subtypes defined by PAM50 in BC are important prognostic factors, we next addressed whether the prognostic value of CBX7 is independent of these well-known clinical factors. Therefore, we conducted a subset analysis of *CBX7* in ER+ and ER- tumors or in different molecular subtype tumors. Higher levels of *CBX7* expression were significantly associated with longer OS, DFS, and DMFS in both ER+ and ER- patients (**Figure 5, Supplementary Figure 2**). As shown in **Figure 5** and **Supplementary Figure 2**, *CBX7* mRNA expression levels were significantly associated with OS, DFS, and DMFS in luminal B, HER2, and basal BC subtypes, significantly associated with OS in luminal A subtype, and significantly associated with DFS and DMFS in the normal-like subtype. These findings suggest that *CBX7* expression levels provide an additional prognostic value to these known clinical factors.

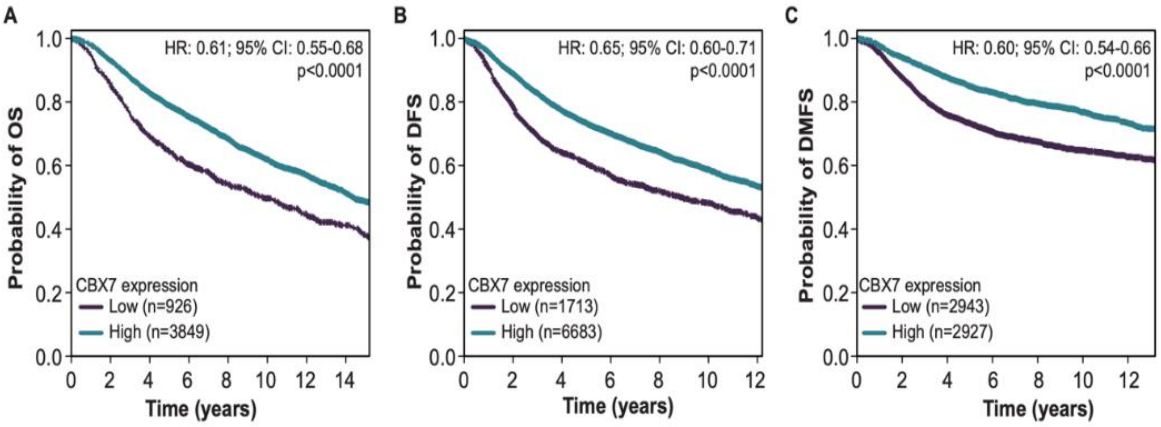

Figure 4. Association of *CBX7* gene expression with prognosis in breast cancers.
(A) Overall survival (OS) analysis. **(B)** Disease-free survival (DFS) analysis. **(C)** Distant metastasis-free survival (DMFS) analysis. Kaplan-Meier curves. The p values were obtained from the log-rank test.

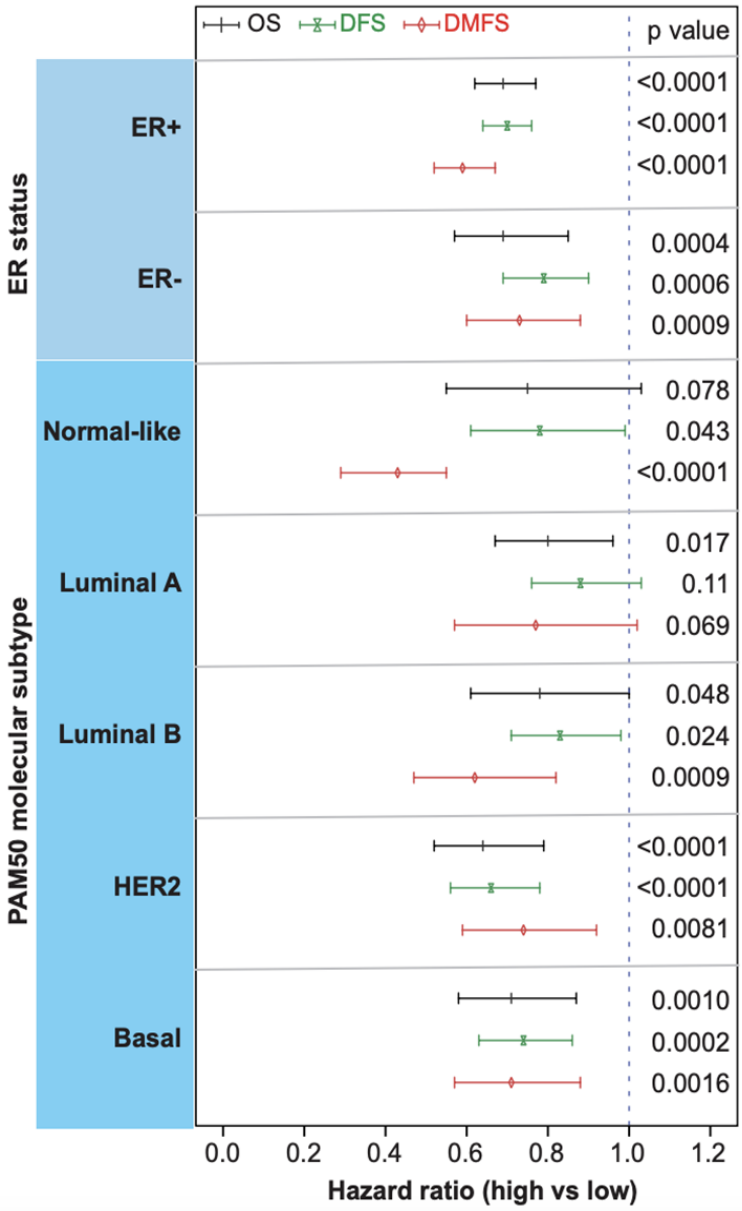

Figure 5. Association of *CBX7* gene expression with overall, disease-free, and distant metastasis-free survival analysis regarding ER status and PAM50 molecular intrinsic subtypes of breast cancer.
The p values were obtained by the log-rank test.

Potential Mechanisms for the Contribution of *CBX7* Loss to Breast Cancer Development

Gene coexpression analysis was used to explore the potential mechanisms for the contribution of *CBX7* loss to BC development. We identified 207 genes consistently coexpressed with *CBX7* in both METABRIC and TCGA-BRCA datasets ($|R| \geq 0.4$ and FDR < 0.05) (**Supplementary Table 1**). *CBX7* was negatively coexpressed with 157 genes, whereas it was positively coexpressed with 50 genes (**Supplementary Table 1**). The set of *CBX7* negatively coexpressed genes contains many well-known cell cycle genes, such as *CCNB1*, *CCNB2*, *CCNA2*, and *CCNE1* (**Figure 6A**). Furthermore, functional enrichment analyses of *CBX7* negatively and positively coexpressed genes were conducted separately. Although we did not find any biological processes and pathways significantly associated with *CBX7* positively coexpressed genes, GO analysis revealed that cell cycle-, DNA replication-, and mitosis-related biological processes (**Figure 6B**) and cyclin-dependent protein kinase activity (**Supplementary Figure 3**) were significantly overrepresented within the set of *CBX7* negatively coexpressed genes (FDR < 0.05). Besides, KEGG analysis showed that cell cycle- and DNA replication-related pathways were also enriched with *CBX7* negatively coexpressed genes (FDR < 0.05) (**Figure 6C**). These observations were further confirmed by the Reactome pathway analysis (**Supplementary Figure 4**). To further discover which transcription factors cooperating with CBX7 to regulate these genes, we conducted transcription factor enrichment analysis and identified the E2F family of transcription factors significantly associated with *CBX7* negatively coexpressed genes (**Supplementary Figure 5**). Finally, we found that loss of *CBX7* expression significantly increases aneuploidy score (p < 1.0E-10) (**Figure 7A**), fraction genome altered (p < 1.0E-10) (**Figure 7B**), MSI sensor score (p < 1.0E-10) (**Figure 7C**), mutation count (p < 1.0E-10) (**Figure 7D**), and tumor mutation burden (TMB) (p < 1.0E-10) (**Figure 7E**). These findings suggest that *CBX7* is a tumor suppressor by suppressing the cell cycle and maintaining genome integrity.

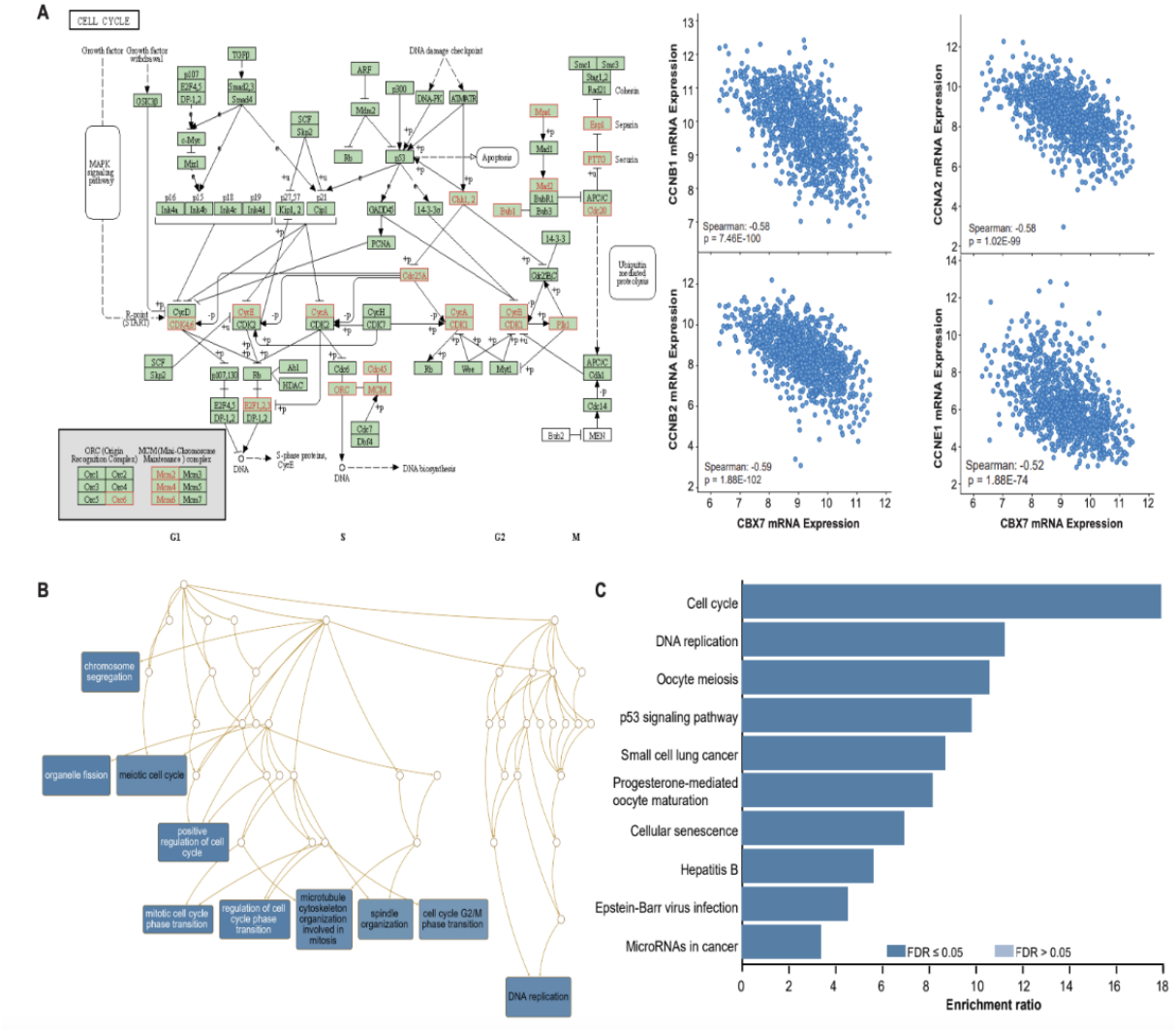

Figure 6. Functional analysis of *CBX7* negatively coexpressed genes.

(A) Negative correlation between expression of *CBX7* and cell cycle genes. The genes highlighted in red in the cell cycle pathway indicate they are significantly or negatively coexpressed with *CBX7* (left panel). The dot plots show some representative genes (right panels). The p values were obtained from Spearman correlation analysis. (**B**) The biological processes enriched within *CBX7* negatively coexpressed genes. (**C**) The KEGG pathways enriched within *CBX7* negatively coexpressed genes.

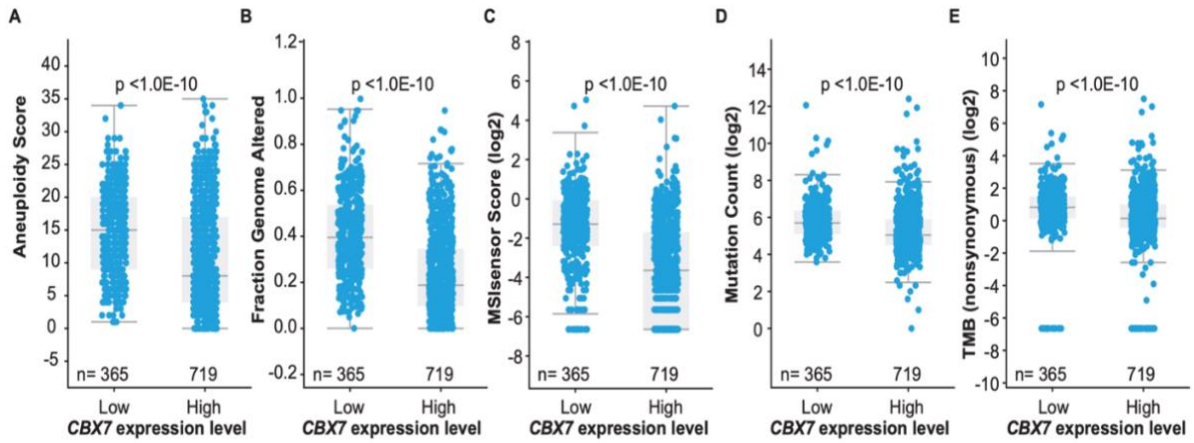

Figure 7. Loss of *CBX7* expression increases genome instability.
(A) Aneuploidy score. (**B**) Fraction of genome altered. (**C**) MSI sensor score. (**D**) Mutation count. (**E**) Tumor mutation burden (nonsynonymous). The p values were obtained from the Wilcoxon test.

DISCUSSION

We used an integrative multiomics approach to systematically assess the potential role and function of *CBX7* in BC. Our results showed that low expression of *CBX7* in BCs is due to genetic and epigenetic alteration. The *CBX7* locus is frequently deleted, and frequent promoter hypermethylation of CBX7 is detected in BCs. These genetic and epigenetic changes lead to the downregulation of *CBX7* in BCs. Moreover, loss of *CBX7* expression has been reported in primary human tumors of the blood [14], brain [22], head and neck [16], lung [20], skin [17], stomach [21], and ovary [15]. Moreover, the knockout of the *Cbx7* gene in mice resulted in the spontaneous development of lung and liver tumors [26]. These findings indicate that *CBX7* is a general tumor suppressor gene in human cancer.

We demonstrated that patients with BC with higher expression levels of *CBX7* had significantly longer OS, DFS, and DMFS independent of ER status and PAM50 molecular subtype, suggesting that *CBX7* independently adds clinical value to stratify patients to inform treatment and care. These results are consistent with the fact that *CBX7* regulates the sensitivity to cancer treatments [35]. For example, low expression of *CBX7* makes cancer cells sensitive to triciribine and rapamycin treatment but resistant to GSK1487371 and ICRF-193 treatment [35]. Therefore, *CBX7* may serve as a biomarker for selecting patients for specific therapies. Significant association with DMFS suggests that *CBX7* participates in the regulation of cancer metastasis. *CBX7* has been shown to regulate the epithelial-mesenchymal transition by sustaining the expression of the E-cadherin gene [36]. Moreover, a recent study showed that *CBX7* inhibits metastasis in basal-like BC by regulating the TWIST1/EPHA2 pathway [37]. Therefore, CBX7 plays an essential role in cancer progression and impacts the prognosis of cancer patients.

Our gene coexpression analysis revealed that *CBX7* negatively coexpressed with many cell cycle and proliferation genes, including CCNE1, consistent with findings in the *Cbx7* knockout model where knockout of the *Cbx7* gene led to upregulated expression of multiple cell cycle components [26]. We discovered the possibility that CBX7 cooperates with the E2F family of transcription factors to regulate these genes through transcription factor enrichment analysis, consistent with the E2F function regulating the cell cycle [38]. Interestingly, a recent study revealed a distinct role of two isoforms of CBX7 ($p36^{CBX7}$ and $p22^{CBX7}$) in cell proliferation [39]. In particular, $p22^{CBX7}$ potentially interacts with cell cycle regulators [40]. We found that low expression of *CBX7* increases genome instability, suggesting that its loss may lead to misregulation of the cell cycle and subsequently cause chromosomal instability. Taking it all together, we concluded that *CBX7* plays a tumor-suppressive role by regulating the cell cycle and maintaining genome integrity.

Overall, the findings in this study increase our understanding of the function and role of CBX7 in carcinogenesis, which needs further investigation by experimental approaches.

CONCLUSION

Integrative omics analysis of the *CBX7* gene reveals its tumor-suppressive function through regulating the cell cycle and maintaining genome integrity, and *CBX7* expression is a prognostic factor for patients with BC.

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