Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Down-Regulation of lncRNA CKMT2-AS1 Predicts Poor Prognosis and Promotes Breast Cancer Progression

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Purpose: This research aimed to ascertain the clinical implications of lncRNA CKMT2-AS1 and its underlying molecular mechanism in patients with breast cancer.

Patients and Methods: CKMT2-AS1 expression were assessed in breast cancer. The prognostic implication of CKMT2-AS1 was evaluated using Cox regression analysis. Functional assays were conducted to explore the effects of CKMT2-AS1 on the cellular activities associated with breast cancer. Mechanistic investigations included dual-luciferase reporter assays, bioinformatics, and Western blot analyses to elucidate the molecular pathways involving CKMT2-AS1.

Results: The analysis revealed that CKMT2-AS1 was significantly reduced in breast cancer tissue specimens and cell lines ($P<0.05$). Its expression was correlated with advanced stages of tumors ($P=0.031$) and the presence of lymph node metastasis ($P=0.044$). The upregulation of CKMT2-AS1 led to an obvious decline in cell proliferation, migration, and invasion, while also enhancing apoptosis in breast cancer cells ($P<0.05$). Furthermore, mechanistic studies indicated that CKMT2-AS1 directly interacts with miR-106b-5p, a microRNA that is elevated in breast cancer, thereby influencing the expression of its target gene MECP2, which is recognized as a tumor suppressor.

Conclusion: CKMT2-AS1 exerts its tumor-suppressing function in breast cancer via sequestering miR-106b-5p and modulating MECP2 expression. Its decreased levels are linked to the unfavorable prognosis, positioning CKMT2-AS1 as a prospective indicator for prognosticating breast cancer.

Keywords: CKMT2-AS1, breast cancer, cell activity, prognosis, miR-106b-5p, MECP2

Introduction

Breast cancer is among the most prevalent types of cancer globally, constituting a predominant cause of tumor-associated mortality among female populations.[1,2] The onset of breast cancer is shaped by an interplay of genetic and environmental risk factors. Genetic factors encompass mutations in susceptibility genes and single nucleotide polymorphisms.[3,4] Environmental factors include increasing age, a history of breast pathology, obesity, physical inactivity, alcohol consumption, and reproductive factors.[5] Early diagnosis and intervention have been shown to substantially enhance overall survival rates.[6] However, even with advancements in early detection and treatment, the molecular mechanisms that drive its progression and spread are not fully understood. This disease is heterogeneous and is categorized into specific subtypes according to hormone receptor status and molecular characteristics, with each subtype displaying distinct clinical behaviors and responses to therapy.[7,8] Factors such as tumor recurrence, resistance to drugs, and the dissemination of cancerous cells lead to a poor prognosis, emphasizing the necessity for innovative biomarkers and therapeutic strategies to better patient outcomes.[9]

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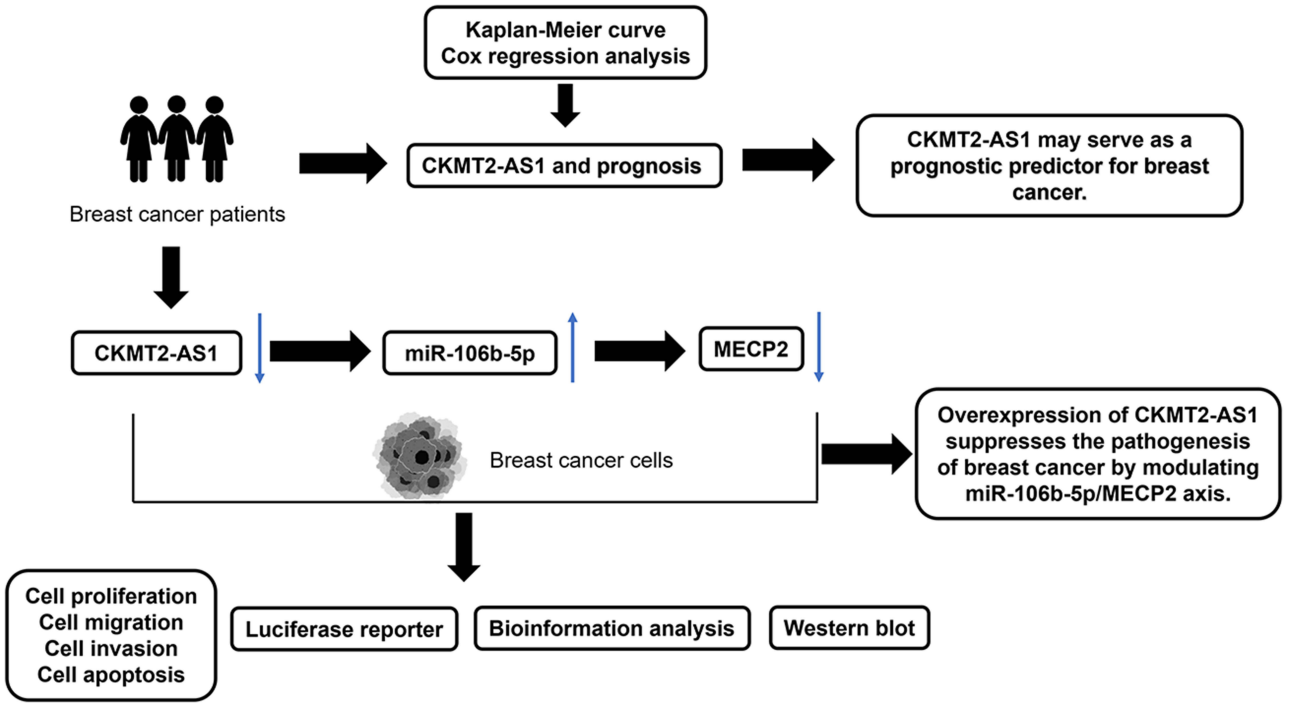
Graphical Abstract



Long non-coding RNAs (lncRNAs) have been recognized as significant modulators of gene expression and various cellular activities in the context of cancer. These transcripts, which exceed 200 nucleotides in length and do not possess protein-coding capabilities, play a role in tumor development by engaging with DNA, RNA, or proteins, thereby affecting chromatin restructuring, transcriptional control, and modifications that occur post-transcription.[10] Growing evidence implicates lncRNAs in breast cancer progression, where they act as oncogenes or tumor suppressors by regulating proliferation, apoptosis, metastasis, and therapy resistance.[11,12] For instance, LINC01535, an oncogenic lncRNA, promotes breast cancer progression by sponging tumor-suppressive miR-214-3p, correlating with advanced TNM stage and poor prognosis.[13] Conversely, lncRNA MIR4435-2HG drove triple-negative breast cancer aggressiveness by remodeling the tumor microenvironment (TME) and activating the JNK/c-Jun and p38 MAPK pathways, thereby enhancing epithelial-mesenchymal transition (EMT) and chemoresistance.[14] These findings highlighted the dual roles of lncRNAs as both oncogenic drivers and tumor suppressors, with their dysregulation serving as potential biomarkers and therapeutic targets. As an intriguing lncRNA, Creatine Kinase Mitochondrial 2 antisense RNA 1 (CKMT2-AS1) is transcribed from the antisense strand of CKMT2 gene locus. Its emerging role in cancers is complex and context-dependent. For instance, in colorectal cancer, CKMT2-AS1 acts as a tumor suppressor by inhibiting the AKT/mTOR signaling pathway.[15,16] In papillary renal cell carcinoma, CKMT2-AS1 was predicted to be a potential biomarker for the prognosis and immunotherapy response.[17] Critically, in breast cancer, the functional role and clinical significance of CKMT2-AS1 are almost entirely unknown. A recent bioinformatics by Pan et al based on The Cancer Genome Atlas (TCGA) demonstrated CKMT2-AS1 as one of several cuproptosis-related lncRNAs with prognostic potential in cases of breast cancer.[18] However, this study remained purely predictive; it did not provide any experimental validation of CKMT2-AS1's expression, function, or mechanism in breast cancer. This significant knowledge gap, coupled with its prognostic prediction from independent databases, makes it both necessary and compelling to systematically investigate CKMT2-AS1. Therefore, our study is designed to move beyond bioinformatic prediction to definitively establish its

clinical relevance and underlying molecular mechanism in breast cancer, thereby addressing a critical unmet need in the field.

In the present research, we examined the relative expression status of CKMT2-AS1 in breast cancer, and evaluated its prognostic significance in the tumor. Moreover, the influences of CKMT2-AS1 on cellular events were also explored. Through integrated approaches, including clinical correlation studies, functional assays, and mechanistic validations, we aimed to elucidate the CKMT2-AS1/miR-106b-5p/MECP2 axis and its clinical relevance. Our findings may provide insights into lncRNA-mediated regulatory networks and pave the way for novel therapeutic strategies in breast cancer.

Materials and Methods

Participants and Sample Collections

A total of 103 breast cancer tissues and their paired adjacent non-cancerous tissues were obtained from patients who were subjected to surgical resection in The Second Hospital of Shanxi Medical University. All tissue samples were diagnosed by two professional pathologists, and no patients underwent anti-cancer therapies, such as chemotherapy, or radiotherapy pre-operation. The fresh tissues were rapidly frozen in liquid nitrogen and preserved at −80°C till subsequent experiments.

Additionally, at the same period, 21 healthy individuals, 18 patients with breast benign diseases, and 25 breast cancer patients were included at the same hospital. Serum samples were obtained from these participants to further verify the expression status of CKMT2-AS1 in breast cancer.

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of The Second Hospital of Shanxi Medical University (Approval No: 2024YX460). Informed consent was obtained from all individual participants included in the study.

The clinical characteristics and 5-year progression-free survival time were collected and statistically analyzed in the enrolled breast cancer patients.

Cell Lines Culture and Transfection

Human breast cancer cell lines, including MCF-7, BT-474, MDA-MB-231, and ZR-75-1, as well as the human breast epithelial cell line MCF-10A, were acquired from the Chinese Academy of Sciences located in Shanghai, China. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), sourced from Wisent, which was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at a temperature of 37°C in a humidified incubator that provided an atmosphere composed of 5% carbon dioxide to ensure optimal growth conditions. The overexpression vector for CKMT2-AS1 (designated as oe-CKMT2-AS1), along with the miR-106b-5p mimic and inhibitor, were constructed and acquired from General Biosystems, located in Anhui, China. Additionally, corresponding negative control vectors (NC) were also included in this study. The transfection of all these vectors was carried out utilizing Lipofectamine 3000 (Invitrogen, Shanghai, China). The transfection procedures were performed in accordance with the detailed instructions provided by the manufacturer to ensure optimal results.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Method

TRIzol reagent (Roche, Indianapolis, IN, USA) was employed for the extraction of total RNA from the samples. Following the RNA extraction, reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), in strict accordance with the manufacturer's instructions. The expression of miRNAs and mRNAs was determined via qRT-PCR detection with SYBR Green PCR Kit (Qiagen, Duesseldorf, Germany). GAPDH and RNU6B served as the normalization controls for lncRNA, mRNA, and miRNA, respectively. To quantify the relative expression levels of CKMT2-AS1, miR-106b-5p, and MECP2, the $2^{-\Delta\Delta Ct}$ method was applied.

The primer sequences of these genes were as follows:

CKMT2-AS1-forward, 5'- AACCTACCACTATAATCCA-3';

CKMT2-AS1-reverse, 5'-ATTCTGTCCACTGTATCT-3';

MECP2-forward, 5'-GCCGAGAGCTATGGACAGCA-3';
MECP2-reverse, 5'-CCAACCTCAGACAGGTTTCCAG-3';
MiR-106b-5p-forward, 5'-TGCGGCAACACCAGTCGATGG-3';
MiR-106b-5p-reverse, 5'-CCAGTGCAGGGTCCGAGGT-3';
GAPDH-forward, 5;-GTGCTCAACCAGTTGGCACC-3';
GAPDH-reverse, 5'- AGCCTCGCTCCACCTGACTT-3';
RNU6B-forward, 5'-CTCGCTTCGGCAGCACA-3';
RNU6B-reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Cell Proliferation Assay

In this study, we utilized BT-474 and MDA-MB-231 cell lines to assess the impact of various treatments on cell growth. The procedure began with seeding 5,000 cells per well in a 96-well plate and incubating them overnight to allow attachment. Following this, different treatments were added, including oe-CKMT2-AS1, co-transfection with miR-106b-5p mimic, oe-NC, and mimic-NC. The Cell Counting Kit-8 (CCK-8) reagent was then added to each well, and the plates were incubated at 37°C for 4 hours, depending on the experimental design. The CCK-8 reagent reacts with viable cells to produce a colored product, and the absorbance at 450 nm was measured using a microplate reader.

Cell Migration and Invasion Assay

Transwell assay was utilized to evaluate the migration and invasion of breast cancer cells BT-474 and MDA-MB-231. For migration, cells were placed in the upper chamber of Transwell inserts without Matrigel, and after 24 hours, cells that migrated to the lower chamber were stained and counted. Invasion was assessed similarly, but the inserts were Matrigel-coated to mimic the extracellular matrix. Following incubation, invasive cells on the lower side were stained, and the number was quantified. Each experiment was done at least three times.

Cell Apoptosis Assay

To assess the apoptosis rate in BT-474 and MDA-MB-231 cell lines, an annexin V-FITC/PI Apoptosis detection kit (KeyGen, Nanjing, China) was utilized. The cells were transfected with oe-NC, oe-CKMT2-AS1+mimic-NC, or oe-CKMT2-AS1+miR-106b-5p mimic for a duration of 48 hours, followed by harvesting and staining with Annexin V-FITC and propidium iodide (PI). Flow cytometry (BD FACSCanto™ II) was employed to quantify the apoptotic cells, identifying early apoptosis with Annexin V+/PI− and late apoptosis with Annexin V+/PI+. The analysis of the data was performed using FlowJo V10 software. To ensure reproducibility, experiments were conducted in triplicate.

Luciferase Reporter Assay

In this study, the 3'-untranslated regions (3'-UTRs) of CKMT2-AS1 and MECP2 were cloned into the psiCHECK-2 vector (Promega, Madison, MI), placing them downstream of the Renilla luciferase gene. Following this preparation, the cells were co-transfected with the reporter construct alongside either a mimic or an inhibitor of miR-106b-5p, or a negative control (NC) mimic/inhibitor. After allowing a period of 48 hours for the transfection, the luciferase activity within the cells was evaluated using the Dual-Glo® Luciferase Assay System, sourced from Promega, based in Madison, WI, USA. To ensure accurate comparisons, the firefly luciferase activity was normalized against the Renilla luciferase signal, allowing for a clear assessment of the interactions being tested.

Bioinformatics Analysis

Publicly available data from the TCGA and ENCORI databases were downloaded and analyzed for initial hypothesis generation and to provide supporting evidence from independent cohorts. All key conclusions are drawn from our own experimental validation detailed below. To explore the functional roles of miR-106b-5p and its target genes, a thorough bioinformatics analysis was carried out. Initially, a Gene Ontology (GO) enrichment analysis was executed to classify the targets into biological processes (BP), cellular components (CC), and molecular functions (MF). Subsequently, the Kyoto Encylopedia of Genes and Genomes (KEGG) pathway analysis was utilized to pinpoint significantly enriched signaling

pathways, including those related to cancer and viral infections. For constructing protein-protein interaction (PPI) networks, the STRING database was employed.

Western Blot Analysis

To evaluate the expression levels of MECP3 protein following various treatments, breast cancer cell lines, specifically BT-474 and MDA-MB-231, were subjected to lysis in a radioimmunoprecipitation assay (RIPA) buffer that included protease inhibitors. For the subsequent analysis, a total protein amount of 20 micrograms from each sample was utilized. The proteins were then separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, after which they were transferred to polyvinylidene fluoride (PVDF) membranes to prepare for antibody binding. After the transfer process, the membranes underwent a blocking step using 5% non-fat milk to minimize nonspecific binding. The primary antibodies utilized in this experiment included anti-MECP2 (1:1000, 10861-1-AP, Proteintech) and anti-GAPDH (1:5000, 60,004-1-Ig, Proteintech), which served as the loading control. The membranes were incubated overnight at 4°C to ensure optimal binding of the antibodies. Following this incubation, the membranes were treated with a horseradish peroxidase (HRP)-conjugated secondary antibody. To visualize the protein bands, enhanced chemiluminescence (ECL) was employed, and the intensity of the bands was quantified using the software ImageJ.

Statistics

Continuous variables were represented as the mean along with the standard deviation (SD). To compare these continuous variables across different groups, the independent samples *t*-test was utilized for comparisons involving two groups, while one-way ANOVA was employed when analyzing differences among three or more groups. On the other hand, categorical variables were summarized as frequencies and percentages (n %), and the Chi-square test was used to evaluate the relationships or differences within these categorical data. For the survival analysis component of the study, the Kaplan-Meier method was implemented via Log rank test. The prognostic value of CKMT2-AS1 in breast cancer was evaluated via univariate and multivariate Cox regression. A P-value<0.05 was recognized to be statistically significant. Analyses were conducted using SPSS 23.0 or GraphPad Prism 9.0.

Results

Relative Expression Pattern of CKMT2-AS1 in Breast Cancer Patients

Relative expression abundances of CKMT2-AS1 were comprehensively analyzed across different samples and conditions related to breast cancer using qRT-PCR assay. A significant decline was found in CKMT2-AS1 expression in breast cancer tissue samples against paired non-cancerous tissue samples (P<0.001, Figure 1A). Additionally, there was a progressive decrease in expression from Stage I–II to III–IV, with the lowest expression observed in Stage III–IV (n=28), suggesting an important role of CKMT2-AS1 in the formation of breast cancer. The serum analysis revealed a significant decrease in CKMT2-AS1 expression in breast cancer (n=25) and patients with benign conditions (n=18) compared with healthy individuals (n=21) (P<0.001, Figure 1B).

A marked decrease in the expression levels was detected between the breast cancer cell line and normal breast cell line. The tested breast cancer cell lines (MCF-7, BT-474, MDA-MB-231 and ZR-75-1) exhibited a significant decline in expression relative to MCF-10A (P<0.001, Figure 1C). To assess the generality of this observation, we analyzed data from the ENCORI database, which confirmed that the expression of CKMT2-AS1 was reduced in breast cancer samples against non-cancerous specimens (P<0.001, Figure 1D). Additionally, based on TCGA samples, in breast invasion carcinoma (BRCA), the expression of CKMT2-AS1 was reduced in tumor tissue specimens against non-cancerous tissues (P<0.001, Figure 1E). When stratified by tumor stage (Figure 1F), the expression decreases as the stage advances.

Relationship of CKMT2-AS1 with Clinicopathological Factors of Breast Cancer Patients

The research examined the relationship between the expression of CKMT2-AS1 and several clinicopathological factors in a cohort of 103 patients diagnosed with breast cancer. Utilizing the mean expression level of CKMT2-AS1, the

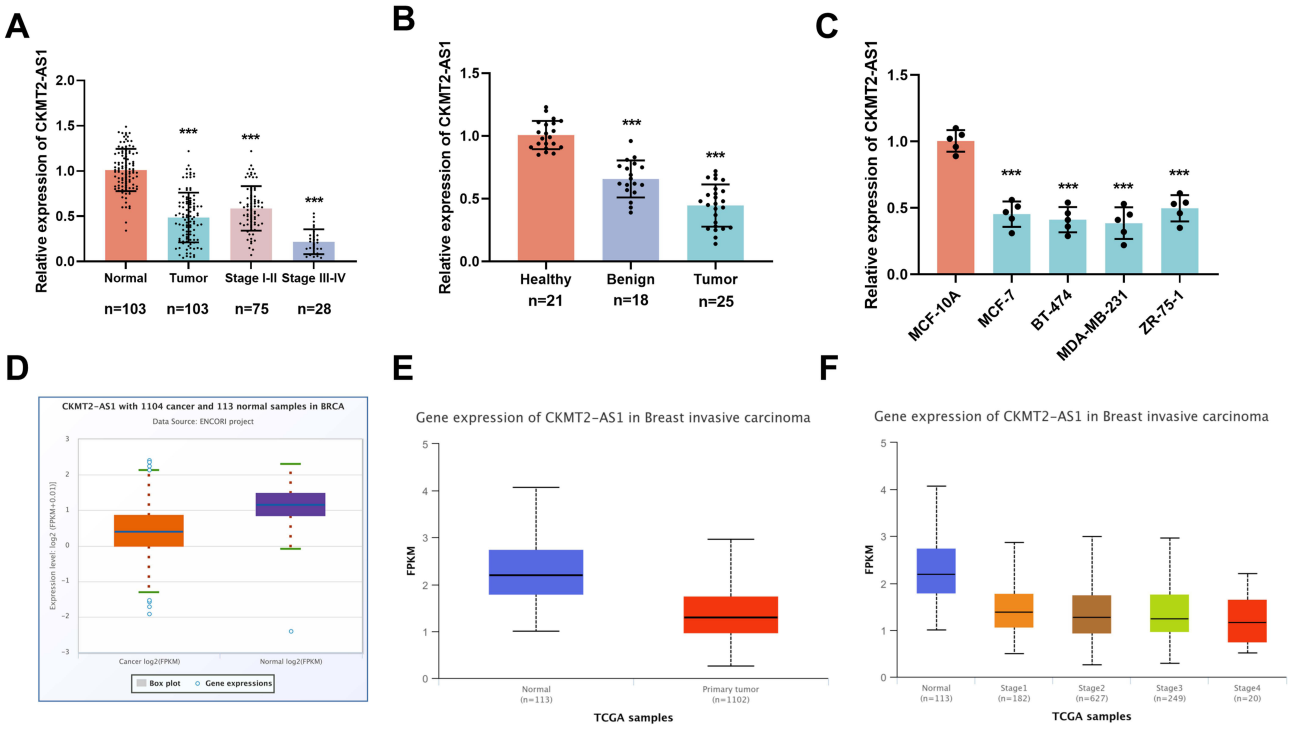


Figure 1 Expression status of CKMT2-AS1 in breast cancer. (**A**). Relative expression levels of CKMT2-AS1 in breast cancer tissues (n=103) and tumor tissues stratified by stage, along with adjacent normal tissues (n=103) (***P<0.001 vs normal). (**B**). Relative expression levels of CKMT2-AS1 in serum samples from healthy individuals, patients with benign breast conditions, and breast cancer patients (***P<0.001 vs healthy). (**C**). Relative expression amounts of CKMT2-AS1 in human breast cancer cell lines and a normal breast epithelial cell line (***P<0.001 vs MCF-10A). (**D**). Box plot showing the expression levels of CKMT2-AS1 in 1104 breast cancer samples and 113 normal samples from ENCORI database. (**E**). Box plot showing the gene expression of CKMT2-AS1 in breast invasive carcinoma samples from The Cancer Genome Atlas (TCGA) database. (**F**). Box plot showing the gene expression of CKMT2-AS1 in breast invasive carcinoma samples from TCGA, stratified by tumor stage.

patients were categorized into two distinct groups: one with low CKMT2-AS1 expression (n=52) and another with high CKMT2-AS1 expression (n=51). Findings indicated a significant association between CKMT2-AS1 expression and both the TNM stage (P=0.031) and the status of lymph node metastasis (LNM) (P=0.044). Specifically, patients with advanced TNM stages (III–IV) had a higher proportion of low CKMT2-AS1 expression compared to those with early stages (I–II). Similarly, a lower CKMT2-AS1 expression was observed in patients with positive LNM compared to those with negative LNM. However, CKMT2-AS1 expression did not show significant associations with age (P=0.250), progesterone receptor (PR) status (P=0.202), estrogen receptor (ER) status (P=0.765), human epidermal growth factor receptor 2 (HER2) status (P=0.719), tumor size (P=0.089), or histological type (P=0.095) (Table 1).

Table 1 Associations of CKMT2-AS1 Expression and Clinicopathological Features of Breast Cancer Patients

Factors	Cases (n=103)	CKMT2-AS1 Expression		*P* value
		Low (n=52)	High (n=51)	
Age (years)				
≤60	38	22	16	0.250
>60	65	30	35	

(*Continued*)

Table 1 (Continued).

Factors	Cases (n=103)	CKMT2-AS1 Expression		*P* value
		Low (n=52)	High (n=51)	
PR status				
Negative	48	21	27	0.202
Positive	55	31	24	
ER status				
Negative	53	26	27	0.765
Positive	50	26	24	
HER2 status				
Negative	71	35	36	0.719
Positive	32	17	15	
Tumor size (cm)				
≤2	58	25	33	0.089
>2	45	27	18	
Histological type				
Well-moderate	73	33	40	0.095
Poor	30	19	11	
TNM Stage				
I–II	75	33	42	**0.031**
III–IV	28	19	9	
LNM				
Negative	78	35	43	**0.044**
Positive	25	17	8	

Note: Bold text means significant difference (*P*<0.05).
Abbreviations: PR, progesterone reporter; ER, estrogen reporter; HER2, human epidermal growth factor reporter 2; TNM, tumor node metastasis; LNM, lymph node metastasis.

Clinical Implication of CKMT2-AS1 Expression in Breast Cancer Patients

To appraise the association of CKMT2-AS1 with 5-year progression-free survival of breast cancer, Kaplan-Meier method was conducted. As listed in Figure 2, patients with high CKMT2-AS1 expression (n=51) exhibited better progression-free survival than those expressing low CKMT2-AS1 expression (n=52) (Log rank test $P<0.001$). Cox regression analysis was conducted to evaluate the prognostic significance of CKMT2-AS1 expression for progression-free survival in breast cancer patients. Univariate analysis showed that CKMT2-AS1 expression was significantly associated with progression-free survival (HR=0.176, 95% CI=0.072–0.429, $P<0.001$), along with TNM stage (HR=7.316, 95% CI=3.516–15.222, $P<0.001$) and LNM (HR=7.086, 95% CI=3.483–14.417, $P<0.001$). In the multivariate analysis, the expression of CKMT2-AS1 was identified as an independent prognostic indicator for progression-free survival (HR=0.264, 95% CI=0.106–0.658, $P=0.004$), as well as TNM stage (HR=3.115, 95% CI=1.165–8.328, $P=0.024$) and LNM (HR=2.682, 95% CI=1.038–6.930, $P=0.042$) (Table 2).

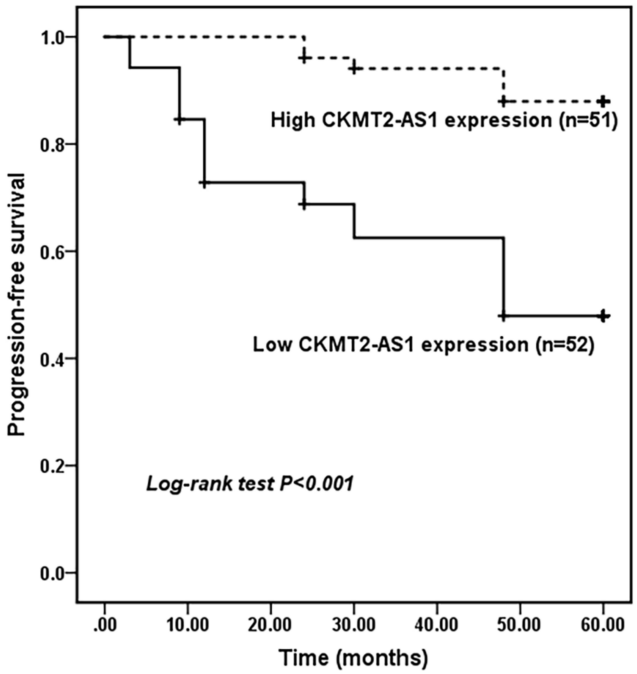


Figure 2 Kaplan-Meier survival analysis of CKMT2-AS1 expression was performed in breast cancer patients via Log rank test. Patients with low CKMT2-AS1 exhibited a poorer overall survival of breast cancer (*P*<0.001).

Impacts of CKMT2-AS1 Expression on Cellular Behaviors

In the BT-474 and MDA-MB-231 cell lines, the relative expression levels of CKMT2-AS1 were elevated in the oe-CKMT2-AS1 group against the oe-NC group (*P*<0.05, Figure 3A), indicating effective overexpression of CKMT2-AS1 in these models. Furthermore, in both BT-474 and MDA-MB-231 cell lines, heightened CKMT2-AS1 expression significantly decreased cell proliferation, migration, and invasion, while enhancing apoptosis relative to controls (*P*<0.001, Figure 3B–F).

Table 2 Cox Regression Analysis of Clinicopathological Features for Progression-Free Survival in Breast Cancer Patients

Factors	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age (years)	0.660(0.329–1.321)	0.240	/	/
PR status	1.330(0.657–2.693)	0.429	/	/
ER status	1.320(0.656–2.654)	0.436	/	/
HER2 status	1.531(0.756–3.100)	0.237	/	/
Tumor size (cm)	1.863(0.926–3.746)	0.081	/	/
Histological type	1.768(0.873–3.581)	0.114	/	/
TNM stage	7.316(3.516–15.222)	<0.001	3.115(1.165–8.328)	0.024
LNM	7.086(3.483–14.417)	<0.001	2.682(1.038–6.930)	0.042
CKMT2-AS1	0.176(0.072–0.429)	<0.001	0.264(0.106–0.658)	0.004

Note: *P*<0.05 means significant difference.
Abbreviations: PR, progesterone reporter; ER, estrogen reporter; HER2, human epidermal growth factor reporter 2; TNM, tumor node metastasis; LNM, lymph node metastasis; HR, hazard ratio; CI, confidence interval.

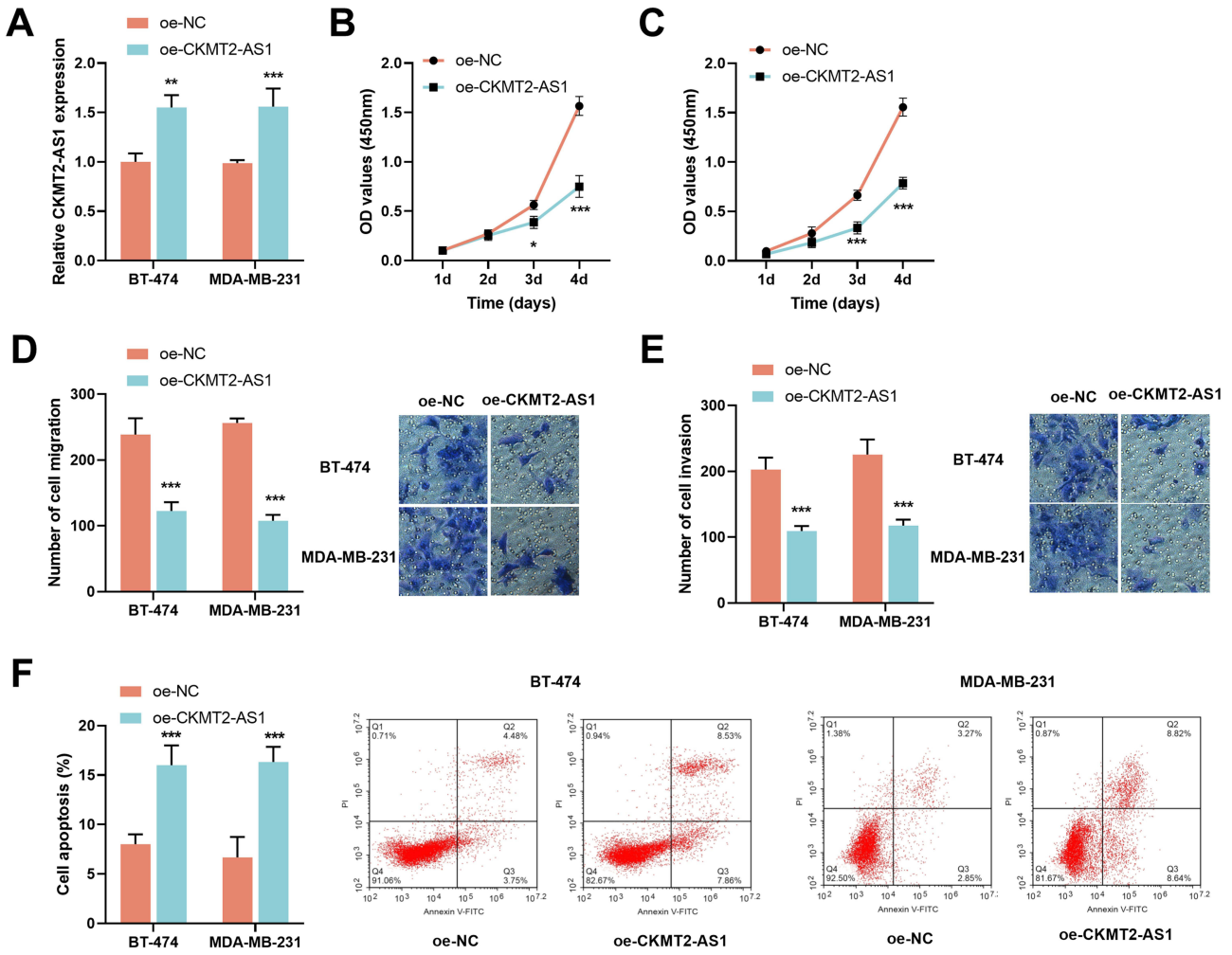


Figure 3 Impact of CKMT2-AS1 overexpression on cellular behaviors in breast cancer. (**A**). The relative expression levels of CKMT2-AS1 in breast cancer cell lines were examined following transfection with oe-CKMT2-AS1. (**B** and **C**). Cell proliferation ability was measured post-transfection with oe-CKMT2-AS1 in BT-474 and MDA-MB -231 cells. (**D**). In the oe-CKMT2-AS1 group, cell migration number significantly decreased in breast cancer cells compared to the oe-NC group. (**E**). High CKMT2-AS1 expression inhibited cell invasion ability in breast cancer cells. (**F**). An increased apoptosis rate was found in breast cancer cells following transfection with oe-CKMT2-AS1. (***P*<0.01; ****P*<0.001 vs oe-NC).

CKMT2-AS1 Directly Targeted miR-106b-5p

To delve deeper into the molecular mechanism by which CKMT2-AS1 influences breast cancer, the complementary binding sequence of CKMT2-AS1 and miR-106b-5p was predicted through the ENCORI database, as illustrated in Figure 4A, establishing a theoretical foundation for their potential interaction. In both BT-474 (Figure 4B) and MDA-MB -231 (Figure 4C) cell lines, co-transfection of the miR-106b-5p mimic with CKMT2-AS1-wt (wild-type) resulted in a significant reduction in luciferase activity compared to the mimic NC (P<0.001). Conversely, co-transfecting the miR-106b-5p inhibitor with CKMT2-AS1-wt caused a notable increase in luciferase activity (P<0.001). Notably, no such changes were detected in the CKMT2-AS1-mut (mutant) group. In a cohort of 103 tumor samples, miR-106b-5p displayed an obviously higher relative expression when compared to 103 normal samples (P < 0.001, Figure 4D). A distinct negative correlation was also observed between CKMT2-AS1 expression and miR-106b-5p expression (r=−0.6026, P<0.001, Figure 4E). In breast cancer cell lines (MCF-7, BT-474, MDA-MB-231, ZR-75-1) compared to MCF-10A, relative miR-106b-5p expression was elevated (P<0.001, Figure 4F), aligning with the results observed at the tissue level. Additionally, analysis of data from 1085 cancer samples and 104 normal samples within the BRCA framework provided by the ENCORI project demonstrated that miR-106b-5p levels were higher in cancer samples than in normal counterparts (Figure 4G).

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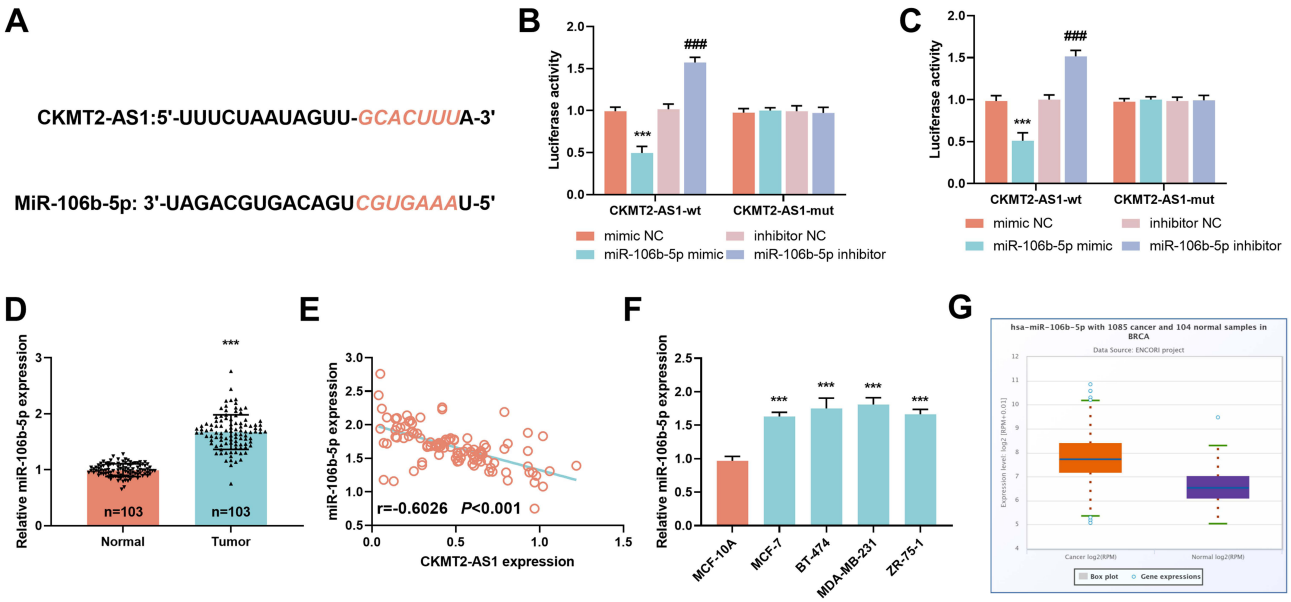

Figure 4 CKMT2-AS1 directly targeted miR-106b-5p. (**A**). The predicted binding sites were listed between CKMT2-AS1 and miR-106b-5p via the ENCORI database. (**B** and **C**). The luciferase activities were compared in BT-474 and MDA-MB-231 cells. (**D**). The relative expression levels of miR-106b-5p were examined in breast cancer tissues. (**E**). CKMT2-AS1 expression was negatively correlated with miR-106b-5p expression. (**F**). The relative expression of miR-106b-5p was measured in breast cell lines. (**G**). Based on the ENCORI project, the expression levels of miR-106b-5p were analyzed. (**B**–**C**): *** $P<0.001$ vs mimic-NC and $^{\#\#\#}P<0.001$ vs inhibitor NC; (**D**) *** $P<0.001$ vs normal; (**F**) *** $P<0.001$ vs MCF-10A.

CKMT2-AS1 Modulated Cellular Events by Targeting miR-106b-5p

In the BT-474 and MDA-MB-231 cell lines, an elevation in CKMT2-AS1 expression resulted in a marked decrease in the relative levels of miR-106b-5p against the oe-NC group ($P<0.001$). The co-transfection of miR-106b-5p mimic negated this effect ($P<0.001$), demonstrating that CKMT2-AS1 functioned as a negative regulator of miR-106b-5p expression (Figure 5A). In the BT-474 (Figure 5B) and MDA-MB-231 (Figure 5C) cell lines, the enhancement of CKMT2-AS1 notably suppressed cell proliferation relative to oe-NC ($P<0.001$). Co-transfection with the miR-106b-5p mimic partially mitigated the suppressive effect on proliferation caused by CKMT2-AS1 upregulation ($P<0.001$), implying that miR-106b-5p has the potential to diminish the antiproliferative actions of CKMT2-AS1. The oe-CKMT2-AS1 group exhibited a significant reduction in the number of migrating and invading cells compared to the oe-NC in both cell lines ($P<0.001$). The introduction of the miR-106b-5p mimic was found to enhance cell migration and invasion ($P<0.001$, Figure 5D and E), indicating that CKMT2-AS1 suppresses these processes through the regulation of miR-106b-5p. Furthermore, the rate of cell apoptosis was elevated in the oe-CKMT2-AS1 group against the oe-NC across both cell lines ($P<0.01$). Co-transfection with the miR-106b-5p mimic caused a reduction in the apoptosis rate ($P<0.05$) (Figure 5F).

Bioinformatics Analysis of miR-106b-5p Potential Targets

The Venn diagram revealed the overlap of predicted miR-106b-5p targets from three databases: TargetScan, miRTarBase, and ENCORI. There are 117 targets in the intersection identified by all three databases (Figure 6A). The GO analysis was divided into three ontologies: BP, CC, and MF. The most significantly enriched terms included BP such as glial cell differentiation, cytokinesis and regulation of mesenchymal cell proliferation, CC like PML body, trans-Golgi network, and clathrin-coated vesicle, and MF such as RNA polymerase II transcription cofactor activity, DNA-binding transcription activator activity, and regulatory RNA binding (Figure 6B). The pathways included Kaposi sarcoma-associated herpesvirus infection, Pancreatic cancer, EGFR tyrosine kinase inhibitor resistance, and several others related to cancer and viral infections (Figure 6C).

The network diagram visualized the relationships between miR-106b-5p targets (high confidence=0.700). Nodes represented different targets, and edges represented their interactions. The size of the nodes corresponded to their degree of connectivity, highlighting key proteins in the network (Figure 6D).

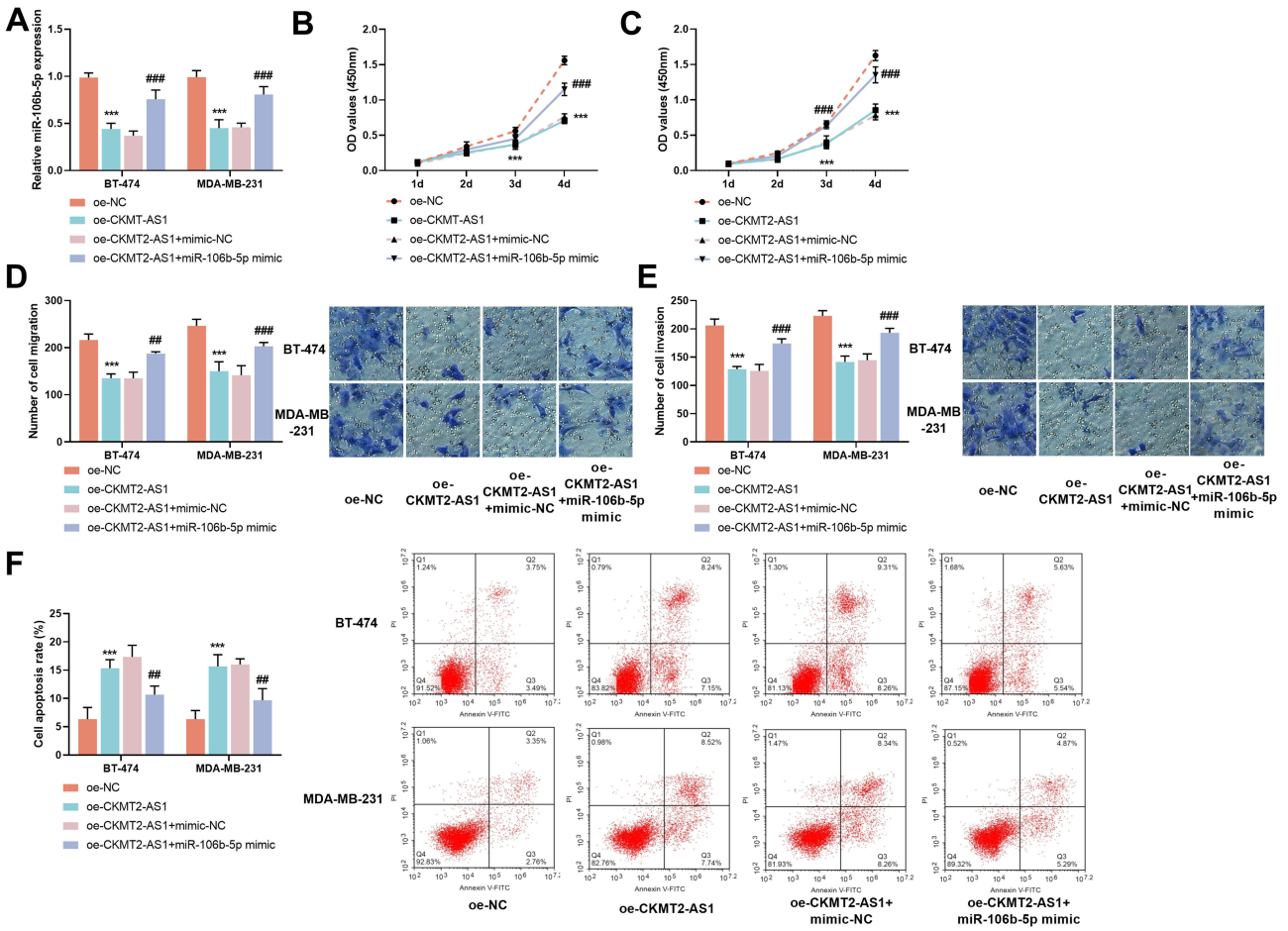

Figure 5 CKMT2-AS1 regulated cellular behaviors via sponging miR-106b-5p. (**A**). The relative expression of miR-106b-5p was examined in breast cancer cell lines under different transfection conditions. (**B** and **C**). Cell proliferation was examined in BT-474 and MDA-MB-231 for different treatment groups. (**D** and **E**). Migrating and invasive cell numbers in BT-474 and MDA-MB-231 cells were analyzed for different treatment groups. (**F**). Cell apoptosis rate in BT-474 and MDA-MB-231 cells was distinctly increased following transfection with oe-CKMT2-AS1, the effects of which were counteracted by the co-transfection with miR-106b-5p mimic. (***P<0.001 vs oe-NC; ##P<0.05 and ###P<0.001 vs oe-CKMT2-AS1+mimic-NC).

MECP2 Was a Target of miR-106b-5p

Figure 7A illustrated the complementary binding regions between miR-106b-5p and the 3'-untranslated region (3'-UTR) of MECP2 mRNA, suggesting a potential target interaction. In the BT-474 and MDA-MB-231 cell lines, a notable decline in luciferase activity was observed when the miR-106b-5p mimic was co-transfected with the MECP2-wt 3'-UTR reporter construct, in contrast to the mimic NC group. Conversely, the luciferase activity remained largely unaffected when co-transfected with the MECP2-mut 3'-UTR reporter construct (P<0.001, Figure 7B and C). Additionally, MECP2 expression was markedly reduced in breast cancer tissues against non-cancerous tissue specimens (P<0.001, Figure 7D). Furthermore, a significant negative correlation was identified between the expression levels of miR-106b-5p and MECP2 mRNA (r=−0.7343, P<0.001, Figure 7E), reinforcing the regulatory relationship. In breast cancer cell lines, a significant decline of MECP2 mRNA expression was found compared to normal breast epithelial cell lines (P<0.001, Figure 7F). In Figure 7G, a box plot was presented comparing MECP2 expression levels in 1104 breast cancer samples and 113 normal samples from the ENCORI project, indicating that MECP2 expression was decreased in cancer samples compared to normal samples (P<0.001).

CKMT2-AS1 Modulated MECP2 Expression by Sponging miR-106b-5p

To delve deeper into the interactions between CKMT2-AS1, miR-106b-5p, and MECP2, transfections were performed with oe-NC, oe-CKMT2-AS1, oe-CKMT2-AS1+mimic-NC, or oe-CKMT2-AS1+miR-106b-5p mimic in BT-474 and MDA-MB -231 cells. In both cell lines, the expression levels of CKMT2-AS1 were found to significantly elevate MECP2 mRNA levels against the oe-NC group (P<0.001). Additionally, the introduction of the miR-106b-5p mimic caused a notable decline in

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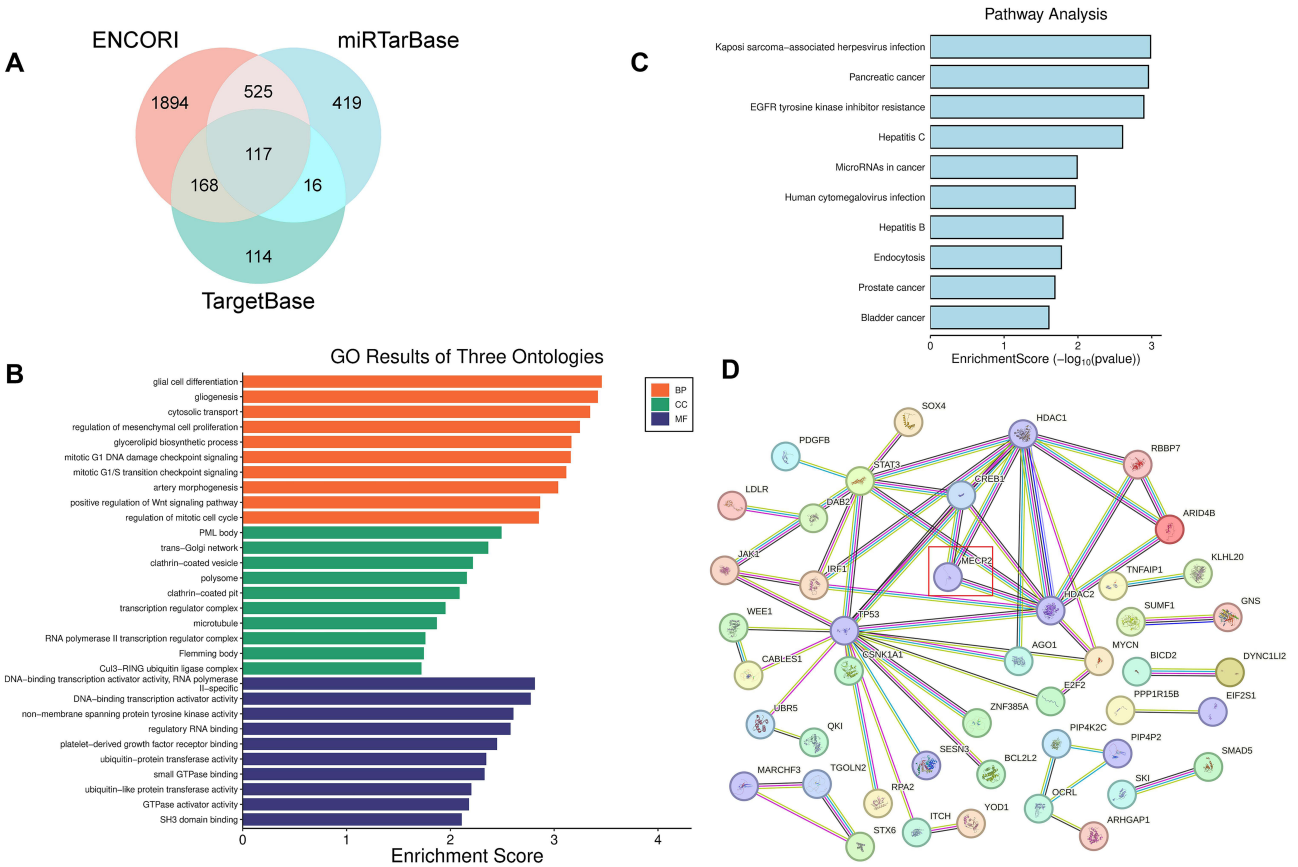

Figure 6 Bioinformatics analysis of miR-106b-5p targets. (**A**). The overlap of predicted miR-106b-5p targets from three databases: TargetScan, miRTarbase, and ENCORI, was listed using a Venn diagram. (**B**). Gene ontology (GO) enrichment analysis was performed. (**C**). KEGG pathway enrichment associated with miR-106b-5p targets was conducted. (**D**). The protein-protein interaction (PPI) networks of miR-106b-5p were established. The gene marked with a red box was selected for the subsequent experiment.

MECP2 mRNA expression, which was stimulated by the overexpression of CKMT2-AS1 ($P<0.05$, Figure 7H and I). In the BT-474 and MDA-MB-231 lines, oe-CKMT2-AS1 triggered a significant upsurge in the relative expression of MECP2 protein compared to oe-NC ($P<0.05$). Nonetheless, co-transfecting with the miR-106b-5p mimic resulted in a reduction of MECP2 protein levels ($P<0.05$, Figure 7J and K).

Discussion

This research investigates the role of CKMT2-AS1 as a tumor inhibitory in breast cancer through miR-106b-5p/MECP2 pathway, thus providing new insights into lncRNA-mediated processes involved in cancer development. Our study identifies the lncRNA CKMT2-AS1 as a novel tumor suppressor in breast cancer, the downregulation of which portends a poor prognosis. These results align with prior studies identifying lncRNAs as critical modulators of breast cancer aggressiveness and patient outcomes.[19,20] Importantly, the reduction of CKMT2-AS1 parallels the oncogenic function of miR-106b-5p, identified as a direct target of CKMT2-AS1 in our study. This finding aligns with previous research that shows an increase in miR-106b-5p levels in more aggressive subtypes of breast cancer, which correlates with poorer survival outcomes,[21–23] further emphasizing the clinical significance of our results.

Mechanistically, CKMT2-AS1 functions as a molecular sponge for miR-106b-5p, thereby upregulating MECP2, a transcriptional regulator implicated in chromatin remodeling and tumor suppression.[24,25] Our luciferase assays and rescue experiments confirmed this regulatory axis, with CKMT2-AS1 overexpression reversing miR-106b-5p-mediated suppression of MECP2. This is important as MECP2 functions a role in inhibiting EMT and metastasis in breast cancer.[26] Additionally, the inverse relationship was found between miR-106b-5p and MECP2 in clinical samples, suggesting that the CKMT2-AS1/miR-106b-5p/MECP2 pathway may represent a potential therapeutic target. The dual role of CKMT2-AS1 as both a regulator of miR-106b-5p and an upstream modulator of MECP2 highlights its potential as a central player in breast cancer pathogenesis.

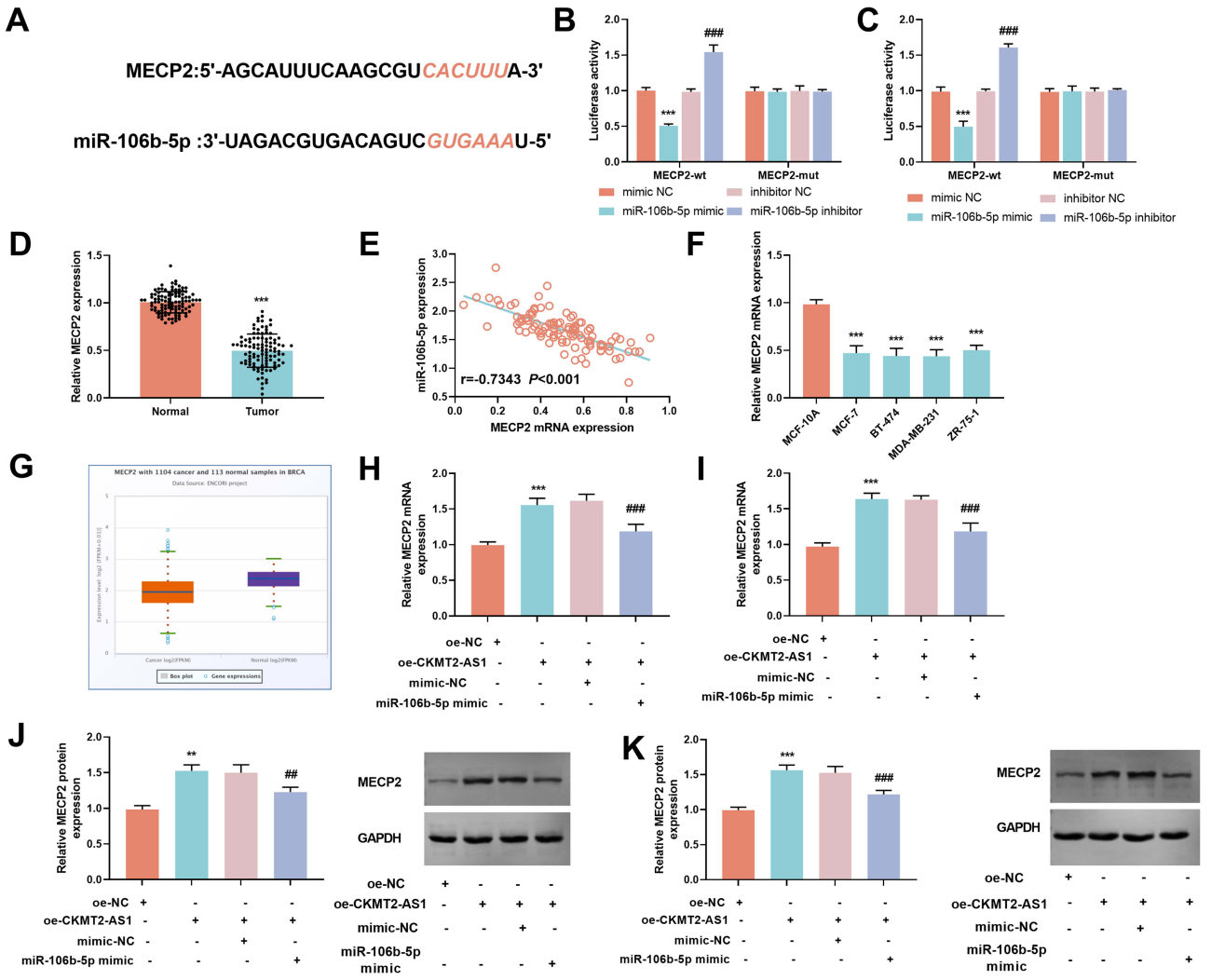

Figure 7 MECP2 is a target of miR-106b-5p and its regulation in breast cancer. (**A**). The targeting binding sequences of MECP2 and miR-106b-5p were predicted using the ENCORI database. (**B** and **C**). The relative luciferase activity was examined in BT-474 and MDA-MB-231 cells. (***P<0.001 vs mimic NC, $^{###}P$<0.001 vs inhibitor-NC) (**D**). The relative MECP2 mRNA expression was increased in breast cancer tissues (n=103) relative to adjacent normal tissues (n=103). (***P<0.001 vs normal) (**E**) A negative correlation was observed between MECP2 mRNA expression and miR-106b-5p expression (r=−0.7343, P<0.001). (**F**). The relative expression of MECP2 mRNA was measured in breast cancer cell lines and normal breast epithelial cell lines. (***P<0.001 vs MCF-10A) (**G**). The expression levels of MECP2 in 1104 cancer samples and 113 normal samples were analyzed in BRCA (breast invasive carcinoma). (**H** and **I**). The relative MECP2 mRNA expression was examined in BT-474 and MDA-MB-231 cells. (**J** and **K**). Relative MECP2 protein expression was measured in BT-474 and MDA-MB-231 cells. (**P<0.01 and ***P<0.001 vs oe-NC, $^{##}P$<0.01 and $^{###}P$<0.001 vs oe-CKMT2-AS1+mimic-NC).

The selection of CKMT2-AS1 for this study was based on its emerging but under-explored role in breast cancer. While numerous lncRNAs, such as HOTAIR, MALAT1, and GAS5, have been extensively documented in breast cancer pathogenesis,[27–29] CKMT2-AS1 represents a novel and distinct entity. Its importance is highlighted by several key findings from our study. Firstly, its significant downregulation in tissues and serum, coupled with its strong association with advanced stage and metastasis, provides immediate clinical relevance that rivals that of many established lncRNAs. Second, we have defined a clear and functionally validated mechanistic axis: CKMT2-AS1 exerts its tumor-suppressive effects not in isolation but through direct sequestration of miR-106b-5p and subsequent modulation of MECP2. This places it within a specific and therapeutically targetable pathway. Finally, its role as a ceRNA is particularly crucial given the oncogenic nature of miR-106b-5p, which itself is associated with poor prognosis and therapy resistance. Therefore, CKMT2-AS1 is a central regulator of a novel pathway, offering a dual potential as a robust prognostic biomarker and a candidate for RNA-based therapeutic strategies.

Furthermore, our study highlights CKMT2-AS1's dual potential as a prognostic biomarker and therapeutic target. The correlation of decreased CKMT2-AS1 expression with inferior progression-free survival, independent of traditional clinicopathological factors, positions it as a robust prognostic indicator. Moreover, the ability of CKMT2-AS1 to

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counteract miR-106b-5p's oncogenic effects suggests that lncRNA-based therapies could be explored to restore MECP2 activity in aggressive breast cancer subtypes. This approach may be especially relevant for triple-negative breast cancer, which lacks targeted therapies and often exhibits dysregulated lncRNA and miRNA networks.[14] The potential for CKMT2-AS1 to serve as a non-invasive biomarker is further supported by its detectable downregulation in serum samples from breast cancer patients, offering a less invasive diagnostic tool compared to tissue biopsies.

The broader implications of our findings extend beyond breast cancer. The CKMT2-AS1/miR-106b-5p/MECP2 axis may represent a conserved regulatory mechanism applicable to other malignancies where miR-106b-5p is dysregulated, such as colorectal or cervical cancers.[30,31] While the competing endogenous RNA (ceRNA) mechanism is a recognized paradigm, our study provides robust functional evidence for its operation in this specific instance. The use of rescue experiments to reverse the phenotypic effects of CKMT2-AS1 by restoring miR-106b-5p activity is a critical strength, offering direct proof of the functional dependency within this axis beyond mere correlative observation. Furthermore, the success of RNA-based therapies in other diseases suggests that targeting this axis could pave the way for innovative treatments in oncology. However, the development of such therapies will require overcoming technical hurdles, such as ensuring stable delivery and minimizing off-target effects. Collaborative efforts between basic researchers and clinicians will be crucial to translate these findings into tangible benefits for patients.

Although our research offers strong support for the CKMT2-AS1/miR-106b-5p/MECP2 axis, several limitations warrant mention. First, the sample size may limit the generalizability of our clinical correlations. Second, our study was conducted entirely in vitro; thus, the tumor suppressive effects of CKMT2-AS1 await validation in in vivo models. Future studies employing animal xenograft models are essential to assess the therapeutic efficacy of CKMT2-AS1 restoration or miR-106b-5p inhibition in suppressing tumor growth and metastasis, including their potential side effects and delivery challenges in a more physiologically relevant context. Finally, the precise mechanisms by which MECP2 suppresses tumorigenesis remain to be fully elucidated. Additionally, the interplay between CKMT2-AS1 and other miRNAs or signaling pathways in breast cancer progression warrants further investigation.

Conclusion

In conclusion, our study provides compelling evidence that establishes CKMT2-AS1 as a novel tumor-suppressive lncRNA in breast cancer. Our findings collectively define the CKMT2-AS1/miR-106b-5p/MECP2 axis as a crucial regulatory pathway in breast cancer pathogenesis. This work not only enhances our understanding of lncRNA-mediated gene regulation but also positions CKMT2-AS1 as a promising prognostic biomarker and a candidate for therapeutic development. Future studies will focus on validating this axis in vivo and exploring the potential of RNA-based therapies targeting this network.

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Disclosure

The author(s) report no conflicts of interest in this work.

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