Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

ALKBH5 Promotes Breast Cancer Stemness Through Regulating Wnt/β-Catenin Signaling

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Background: Breast cancer is the most prevalent disease and the fourth cause of cancer death among female globally. The N6-methyladenylate methylation (m^{6}A) demethylase alpha-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) decreases modification of RNA, while its role in regulating breast cancer development remains unclear.

Methods: ALKBH5-silenced breast cancer cell-line MCF-7 was constructed to investigate its functional impact. Cell proliferation, migration and invasion ability were evaluated by CCK8 and transwell assays under ALKBH5 inhibition. Spheroid formation and in vitro extreme limiting dilution analysis (ELDA) were performed to elucidate the effect of ALKBH5 deficiency on stemness of MCF-7 cells. The m^{6}A modification level of *CTNNB1* and the interaction of ALKBH5 and *CTNNB1* were investigated by Methylated RNA immunoprecipitation (MeRIP) and RIP assay respectively.

Results: Silencing ALKBH5 significantly suppressed MCF-7 cell proliferation, migration, and invasion abilities. Moreover, ALKBH5 depletion also diminished the stemness of breast cancer cells in vitro. Further investigation illustrated that ALKBH5 may regulate Wnt/β-catenin signaling via an m^{6}A-dependant manner. Clinical data analysis demonstrated a strong positive relationship between ALKBH5 and β-catenin expression.

Conclusion: This study establishes a link between ALKBH5 and cancer stemness in breast cancer, providing insights into the functional role of demethylase ALKBH5 in breast cancer progression.

Keywords: N6-methyladenosine, (m6A), breast cancer, ALKBH5, stemness, CTNNB1

Introduction

Breast cancer is the second leading cause of tumor-related mortality among women worldwide. Early detection and screening are crucial for reducing morbidity and mortality, and improving survival rate.[1,2] However, due to the complexity and heterogeneity of breast invasive carcinoma (BRCA), it is challenging to select the appropriate treatment options and conduct accurate clinical prognostic analysis.[3–5] A deeper understanding of the underlying molecular mechanisms driving breast cancer development is essential to address these challenges.[6]

RNA N6-methyladenosine (m^{6}A) modification is the most abundant mRNA modification, playing a critical role in regulating gene expression.[7,8] The m^{6}A RNA modification is a dynamic and reversible process coordinated by methyltransferase (m^{6}A writers), m^{6}A-binding proteins (m^{6}A readers), and m^{6}A demethylase (m^{6}A erasers).[9–12] The m^{6}A modification determines the fate of mRNA, including splicing, translation, or degradation.[13–15] Accumulating evidence demonstrated that alteration of m^{6}A levels participated in tumor pathogenesis and progression by regulating expression of tumor-related genes.[16,17]

In breast cancer, it was found that m^{6}A writer METTL3 induced m^{6}A modification of *LATS1* mRNA, which was further recognized by m^{6}A reader YTHDF2, promoting both tumorigenesis and glycolysis.[18] IGF2BP1, as an m^{6}A reader, could induce breast cancer metastasis by enhancing *CPT1A* mRNA stability in an m^{6}A-dependent

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manner.[19] Fat mass and obesity associated protein (FTO) and a-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5) were two major m^{6}A erasers, reversing the RNA methylation process.[20–22] Niu et al demonstrated that FTO promoted proliferation, colony formation and metastasis of breast cancer cell in vitro and in vivo through demethylation of m^{6}A marks from BNIP3.[23] In addition, several studies have also indicated the role of FTO in facilitating chemoresistance and epithelial–mesenchymal transition (EMT) in breast tumor cells.[24–26] However, the contribution of ALKBH5 to breast cancer remain poorly understood. It was found that ALKBH5-mediated m^{6}A demethylation not only induced glycolysis and resistance to HER2-targeted therapy, but also contributed to cancer stem cells (CSCs) characteristics and doxorubicin-resistance in breast cancer.[27,28] There are yet few studies on the involvement of ALKBH5 in breast cancer progression.

This study aimed to investigate the potential role of ALKBH5 in breast tumor development and progression. It is expected to provide new clues and insights into breast cancer management.

Methods

Cell Culture

Breast cancer cell-line MCF-7 was obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Lentiviral Transfection

We performed cell infection experiments using lentiviral vectors encoding shRNA sequences targeting the gene of *ALKBH5* (shALKBH5 sequence: GATCCTGGAAATGGACAAAGA). Breast cancer cells were seeded in a 6-well plate and infected with the lentiviral particles at a multiplicity of infection (MOI) of 20. After 48 h of infection, the cells were subjected to 4 µg/mL puromycin selection to establish stable *ALKBH5*-knockdown cell line.

Western Blot Analysis

Total protein was extracted from MCF-7 breast cancer cell line using RIPA buffer supplemented with protease inhibitors. Protein concentrations were determined using a BCA protein assay kit (ThermoFisher Scientific). Equal amounts of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% non-fat milk and then incubated with primary antibodies targeting ALKBH5 (Novus, NBP1-82188), β-catenin (Abcam, ab32572), AFP (Abcam, ab284388), CK19 (ABclonal, A19040), C-Myc (SANTA CRUZ, sc-40), Cyclin D1 (Cell Signaling Technology, 2922), GAPDH (ABclonal, A19056), N6-methyladenosine (ABclonal, A22411) overnight at 4°C. Following incubation with secondary antibodies in room temperature for 1 h, protein bands were visualized using the LI-COR Odyssey imaging system.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent following the manufacturer's instructions. Complementary DNA was synthesized using a reverse transcription kit (Promega). Quantitative PCR was carried out with SYBR® Green Master Mix (Roche) and a Roche LightCycler 96. All primers used in this study are listed in Additional file Table S1.

Methylated RNA Immunoprecipitation-qPCR (MeRIP-qPCR)

The MeRIP assay was performed with the RNA Immunoprecipitation Kit. MCF-7 cells were used to extract RNA. RNA samples were then immunoprecipitated with m^{6}A antibody or IgG. The isolated m^{6}A-RIP RNA was quantified by qRT-PCR.

RNA Immunoprecipitation (RIP)

RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit. Cells were lysed, and the RNA-binding proteins were immunoprecipitated using specific antibody against ALKBH5. RNA was extracted from immunoprecipitated complexes and subjected to qRT-PCR.

M^{6}A Dot Blot Analysis

The levels of N6-methyladenosine modification in RNA were determined using a dot blot assay. Briefly, RNA samples were spotted onto a nitrocellulose membrane and UV-crosslinked (254nm). The membrane was then blocked and probed with an m^{6}A-specific antibody overnight at 4°C. The membrane was incubated with fluorescent secondary antibody and the dot blots were visualized by Odyssey® CLx Infrared Imaging System. Total input RNA was spotted by staining with 0.02% Methylene blue.

Cell Viability Assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at a density of 1000 cells per well. CCK-8 solution was added to each well and incubated for 1 hours at 37°C. Absorbance at 450 nm was measured and the cell viability was normalized to day 0.

Colony Formation Assay and Sphere Formation Assay

Cells were plated at a density of 1000 cells per well in a 6-well plate and allowed to grow for 7–10 days. At the end of the incubation period, colonies were fixed with 10% formalin and stained with crystal violet. Sphere formation assay was performed using ultra-low attachment 6-well plates and the cells were incubated for 7–14 days to allow for spheroid formation. Spheroid formation was assessed using an inverted microscope, and the number was measured.

Migration and Invasion Assay

For the migration assay, a total of 1x10^5 cells were seeded in the upper chamber of a 24-well Transwell plate with a pore size of 8 μm. The lower chamber contained complete growth medium with 10% FBS as a chemoattractant. After 48 hours of incubation, the cells that had migrated through the membrane were fixed with 10% paraformaldehyde, stained with crystal violet, and then photographed under a light microscope. For the invasion assay, a similar setup was used as in the migration assay, but the Transwell membrane was pre-coated with Matrigel (BD Biosciences) to mimic the extracellular matrix. After 48 hours of incubation, the invaded cells were fixed, stained, and photographed as described for the migration assay.

Extreme Limiting Dilution Assay (ELDA)

To assess the stemness of breast cancer cells, ELDA was performed. MCF-7 cells were sorted into ultra-low attachment 96-well plates at a range of dilutions, starting from 500 cells down to 1 cell per well. The plates were incubated for 1–2 weeks to allow for the formation of mammospheres. The frequency of tumorigenic cells was calculated using the ELDA web tool.

Statistical Analysis

Data were analyzed using GraphPad Prism 9.0 software. Results were presented as means ± standard deviation of at least three independent experiments. Statistical significance was determined using two-tailed *T*-test, with $p<0.05$ considered significant.

Results

ALKBH5 Promotes Breast Cancer Cell Proliferation, Colony Formation, Migration and Invasion Abilities

To investigate the role of demethylase ALKBH5 in breast cancer cell, we generated stable ALKBH5-knockdown (shALKBH5) and control (shNC) MCF-7 cell line. The transfection efficiency was verified by qRT-PCR and Western blot (Figure 1A). Next, we performed CCK8 and colony formation assay to evaluate cell growth under ALKBH5 knockdown and found that depletion of ALKBH5 significantly inhibited MCF-7 cell proliferation and colony formation abilities (Figure 1B and C). Transwell assay further demonstrated that ALKBH5 silencing dramatically reduced cell migration and invasion abilities (Figure 1D). These functional experiments collectively revealed silencing ALKBH5

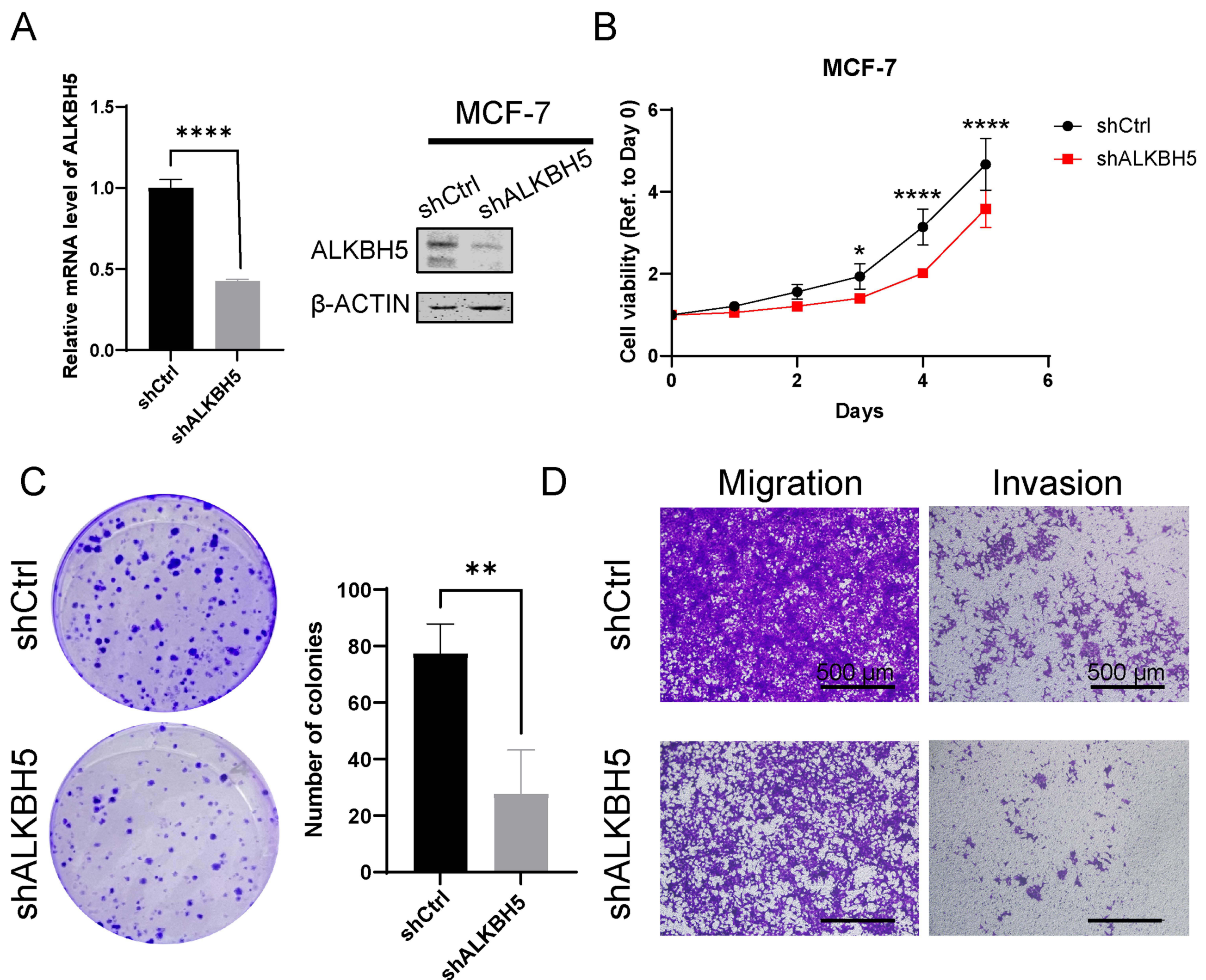

Figure 1 ALKBH5 promotes MCF-7 cells proliferation, migration, and invasion. (**A**) The knockdown efficacy of shALKBH5 in MCF-7 cells was verified by qRT-PCR and Western blot. (**B**). Cell growth was measured using CCK8 assay. (**C**). Representative images of colony formation assay and the number of colonies was counted in *ALKBH5*-knockdown cells. (**D**). Transwell assay was conducted to determine cell migration and invasion abilities after silencing ALKBH5. Statistical significance was determined using two-tailed *T*-test, with p<0.05 considered significant. *P<0.05, **P<0.01, ****P<0.0001.

dampened malignant behaviors of MCF-7 cells, including proliferation, colony formation, migration and invasion abilities in vitro.

Depleting ALKBH5 Decreases CSCs Characteristics of MCF-7 Cells

Methyltransferase WTAP was proved to be affected by NRP1 to enhance stem cell properties and induce radioresistance of breast cancer cells.[29] METTL3 regulated m^{6}A level of *SOX2* mRNA to promote the stemness and malignant progression of MCF-7 cell.[30] YTHDF1 could target *E2F8* and modulate its mRNA stability, leading to breast cancer cell growth, DNA damage and chemoresistance.[31] Given the established roles of m^{6}A regulators in stem cell properties and breast cancer progression, we investigated whether ALKBH5 was involved in breast cancer stemness. The correlation of m^{6}A modification enzymes (*ALKBH5, FTO, WTAP, METTL14, YTHDF2*, and *IGF2BP1*) and stemness-related genes (*EPCAM, KRT19, CD44, NANOG, MYC* and *AFP*) was analyzed by the Breast Cancer Gene-Expression Miner v5.1 database (bc-GenExMiner v5.1) (Figure 2 and Supplementary Figure 1).[32] The result showed *ALKBH5* but not other enzymes was positively correlated with most stemness marker genes, indicating ALKBH5 may contribute to CSCs feature regulation. Sphere formation assay is commonly used to determine cell-line stemness.[33] As shown in Figure 3A

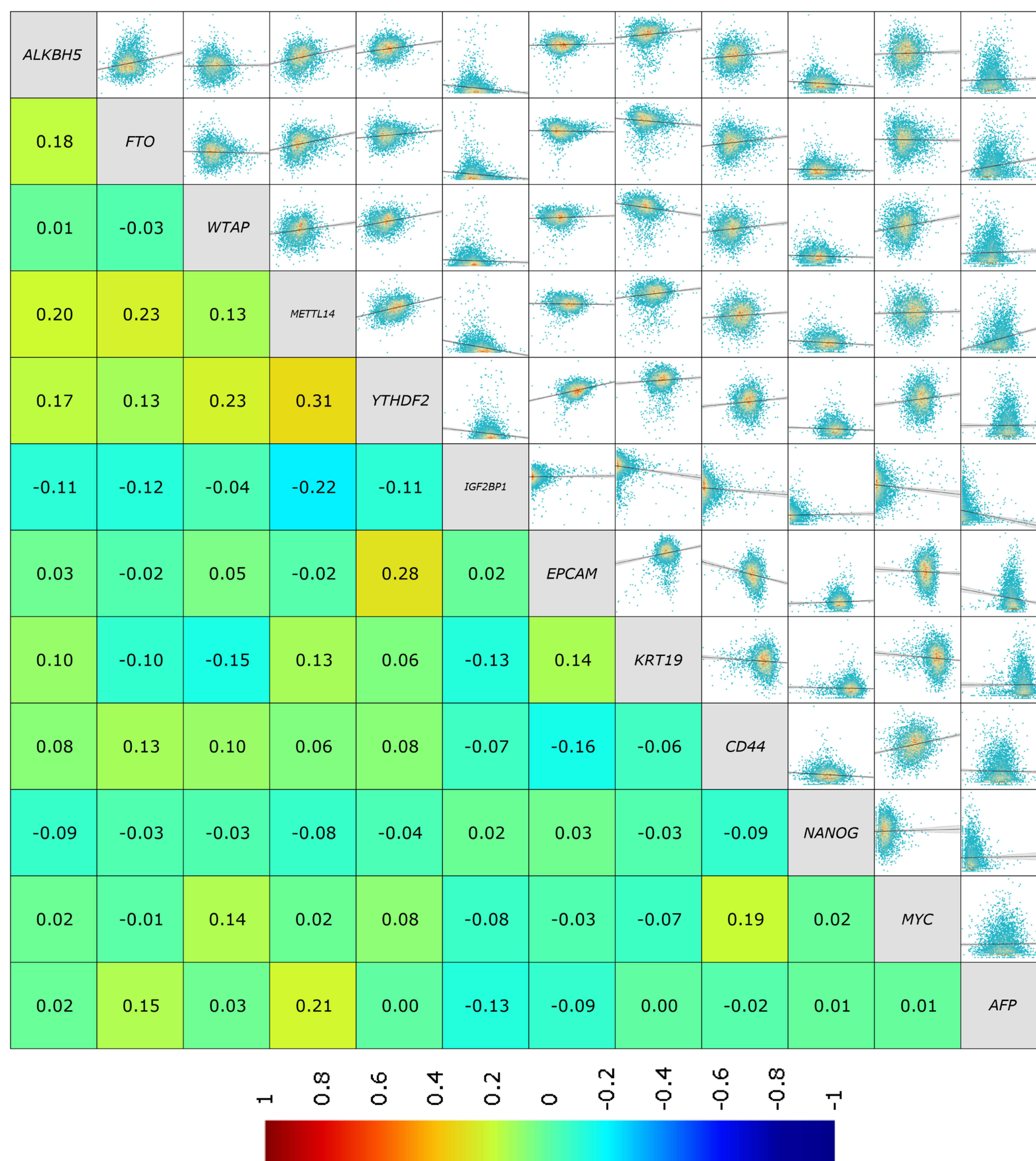

Figure 2 Correlation between m6A modification enzymes and stemness-associated genes. The relationship between m6A modification enzymes gene and stemness-associated genes was analyzed by the Breast Cancer Gene-Expression Miner v5.1 database (bc-GenExMiner v5.1).

and B, knockdown of ALKBH5 suppressed sphere formation in MCF-7 cells (Figure 3A and B). Furthermore, in vitro limiting dilution assays verified significant decrease of stem cell frequency in shALKBH5 MCF-7 cells compared with counterpart control cells (p=0.0381) (Figure 3C and D). Stem cell frequency was estimated using public ELDA software (http://bioinf.wehi.edu.au/software/elda/).[34] We also assessed the mRNA level of stemness marker genes following ALKBH5 knockdown using qRT-PCR (Figure 4E and Supplementary Figure 2). AFP and CK19 represented the degree of differentiation. Western blot assay suggested ALKBH5 silence notably decreased the protein level of AFP and CK19,

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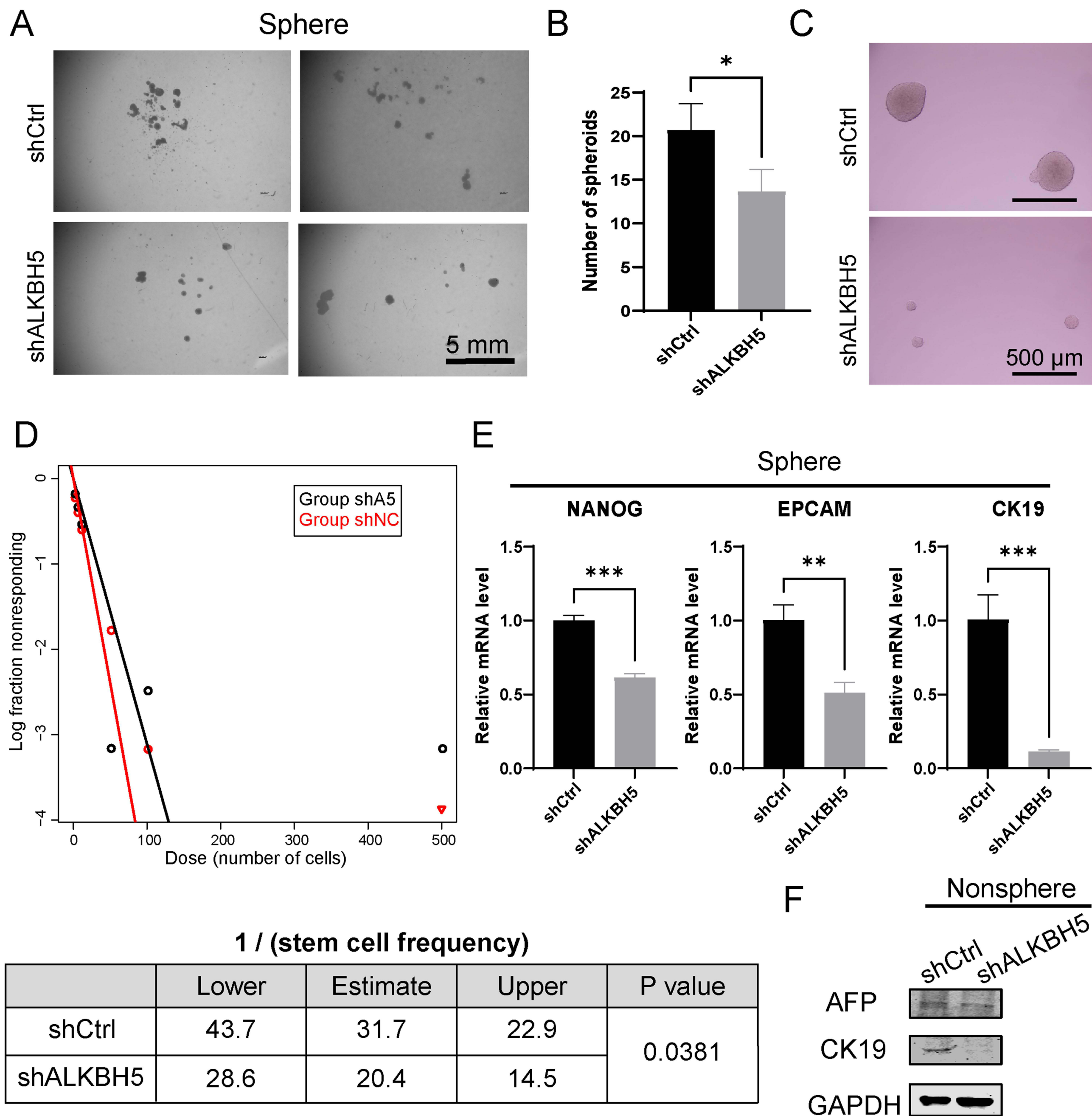

	Lower	Estimate	Upper	P value
shCtrl	43.7	31.7	22.9	0.0381
shALKBH5	28.6	20.4	14.5	

Figure 3 Silencing ALKBH5 inhibits sphere formation capability of MCF-7 cells. (**A**) Mammosphere-forming ability of the stable cell lines was measured in ultra-low attachment plates. (**B**) The number of spheroids formed was quantified. (**C**) Representative images of spheroids formed in ELDA assay. (**D**) ELDA expressing median values from *ALKBH5*-knockdown MCF-7 cells (Control, black curves; *ALKBH5*-knockdown, red curves). The table shows the stem cell frequency and p value calculated by ELDA. (**E**) The mRNA level of stemness-related genes (*NANOG, EPCAM, CK19*) was measured by qRT-PCR in *ALKBH5*-knockdown MCF-7 spheroids. (**F**) The protein levels of AFP and CK19 in nonsphere cell line. Statistical significance was determined using two-tailed *T*-test, with p<0.05 considered significant. *P<0.05, **P<0.01, ***P<0.001.

further confirmed the impact of ALKBH5 on CSCs characteristics of MCF-7 cells (Figure 4F). Taken together, our results strongly suggested that ALKBH5 plays a critical role in enhancing stemness in breast tumor cell.

ALKBH5 Regulated m^{6}A Modification of *CTNNB1*

M^{6}A dot blot assay was employed to examine m^{6}A level of cells. The result showed that ALKBH5 knockdown significantly increased the global RNA m^{6}A modification levels (Figure 4A). Wnt/β-catenin signaling is known to be involved in maintaining stemness and cell proliferation.[35–38] Interestingly, among ALKBH5-associated stemness genes

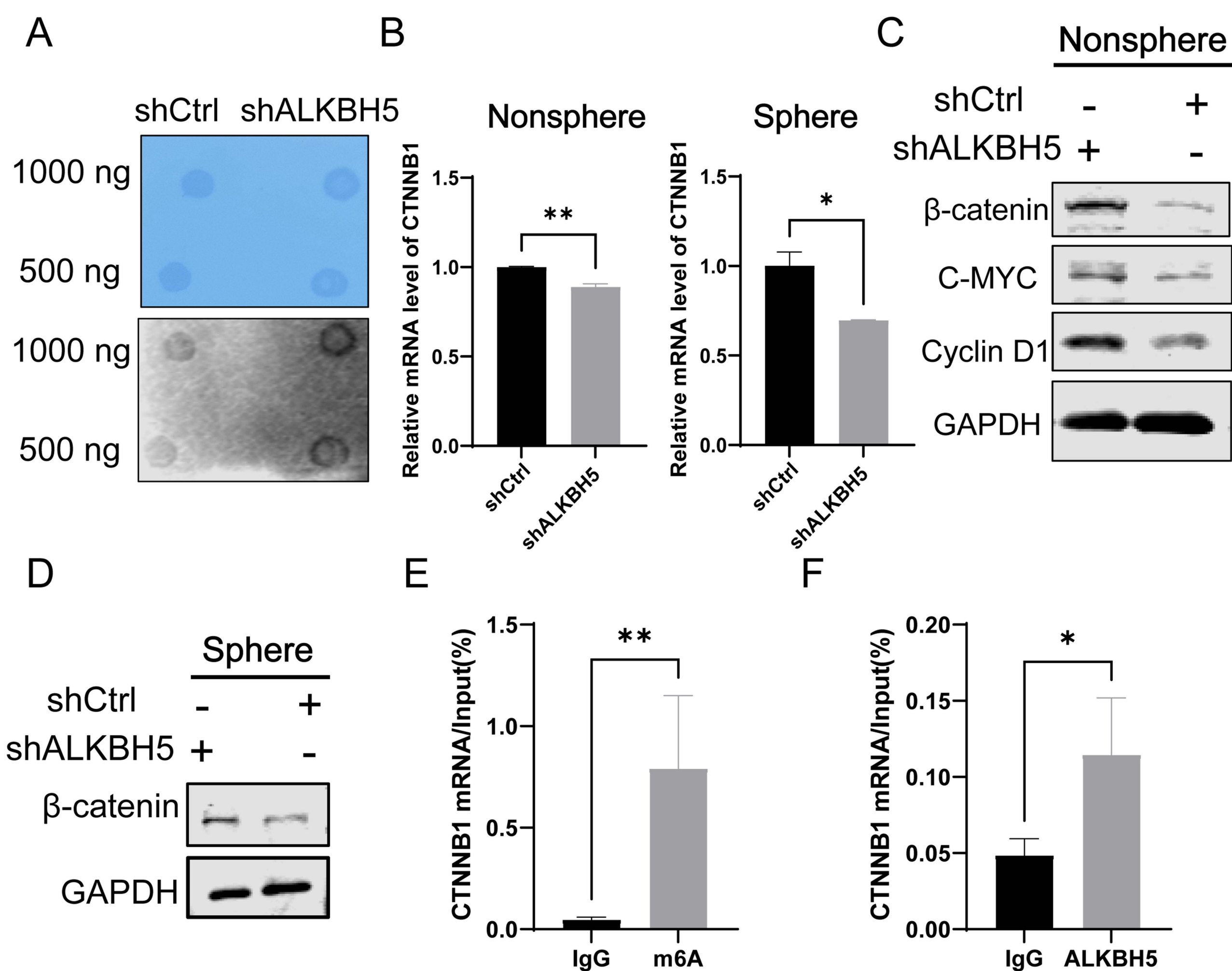

Figure 4 ALKBH5 regulates β-catenin expression via an m^{6}A-dependent manner. (**A**) Dot blot analyses of m^{6}A levels in total RNA. (**B**) The mRNA level of *CTNNB1* in adherent MCF-7 cells and spheroids under ALKBH5 inhibition. (**C**) The protein expression level of β-catenin, C-MYC, and Cyclin D1 in *ALKBH5*-silenced MCF-7 cells. (**D**) The protein level of β-catenin in MCF-7 forming sphere after suppressing ALKBH5. (**E**) Enrichment of m^{6}A in *CTNNB1* mRNA by Me-RIP qPCR assay. (**F**) RIP-qPCR confirmed ALKBH5 binding to *CTNNB1* mRNA. Statistical significance was determined using two-tailed *T*-test, with p<0.05 considered significant. *P<0.05, **P<0.01.

analyzed by public dataset, *CD44* and *MYC* were canonical Wnt target genes (Figure 2).[39,40] This prompted us to investigate the expression of *CTNNB1* in MCF-7 sphere and nonsphere cells by qRT-PCR. A consistent decline of *CTNNB1* mRNA was observed after ALKBH5 loss, indicating Wnt/β-catenin pathway may be potential target of ALKBH5 in breast tumor (Figure 4B). Western blot analysis further demonstrated this signaling was markedly suppressed in ALKBH5-silenced MCF-7 cells, as evidenced by reduced levels of β-catenin and its downstream target, C-MYC and Cyclin D1, in adherent cells (Figure 4C).[41] Moreover, this downregulation of β-catenin protein was also observed in ALKBH5-silenced MCF-7 spheroid cells (Figure 4D). According to the above results, we preliminarily speculated that *CTNNB1* might be a direct target of ALKBH5. To substantiate this conjecture, the m^{6}A abundance of *CTNNB1* was measured by methylated RNA immune-precipitation qPCR (Me-RIP qPCR), which revealed that *CTNNB1* mRNA was affected by m^{6}A modification (Figure 4E). In addition, the direct binding of ALKBH5 and *CTNNB1* mRNA was observed by RIP-qPCR using anti-ALKBH5 antibody (Figure 4F). The m^{6}A sites of *CTNNB1* were predicted using two databases, RMBase v3.0 (https://rna.sysu.edu.cn/rmbase3/) and RM2Target (http://rm2target.canceromics.org/).[42–46] A total of 98 and 4 potential m^{6}A sites were identified by these two tools, respectively (Figure 5A and B). Collectively, these initial analyses have suggested that ALKBH5 depletion may regulate Wnt/β-catenin signaling pathway via an m^{6}A-dependent manner, contributing to impaired cell proliferation, migration, invasion, and stemness characteristics. *CTNNB1* appeared to be a potential target of ALKBH5, warranting further exploration to elucidate this interaction.

A

Details for m6A sites in gene CTNNB1 (ENSG00000168036.18) of hg38

Gene	CTNNB1	Gene ID	ENSG00000168036.18
Gene Type	protein_coding,retained_intron,nonsense_mediated_decay,processed_transcript		
Mod Num	98		

Examples of m^{6}A sites in gene CTNNB1				
Mod ID	Mod Site Loc	Mod Strand	Region	Sequence
m6A_site_584498	chr3:41235772-41235773	+	exon	AGGTTGTACCGGAGCCCTTCACATCCTAGCTCGGGATGTTC
m6A_site_584508	chr3:41236685-41236686	+	exon	TGACCAGCTCTCTCTTCAGAACAGAGCCAATGGCTTGGAA
m6A_site_584529	chr3:41240064-41240065	+	utr	AAGTTATAGTGAATACTGCTACAGCAATTTCTAATTTTTAA
m6A_site_584488	chr3:41233552-41233553	+	exon	GGATGGAAGGTCTCCTTGGGACTCTTGTTCAGCTTCTGGGT

B

RM2Target ID	WERs Name	WERs Type	Target Gene	Modification	Organism	Cell Line/Tissue
RM2Target_632503	ALKBH5	Eraser	CTNNB1	m6A	Homo sapiens	NOMO1
RM2Target_632502	ALKBH5	Eraser	CTNNB1	m6A	Homo sapiens	HEK293T
RM2Target_632505	ALKBH5	Eraser	CTNNB1	m6A	Homo sapiens	THP1
RM2Target_632504	ALKBH5	Eraser	CTNNB1	m6A	Homo sapiens	MOLM13

Figure 5 The predicted m^{6}A modification sites. **(A** and **B**) 98 and 4 predicted m^{6}A sites generated by RMBase v3.0 (**A**) and RM2Target (**B**) respectively.

The Positive Correlation of β-Catenin and ALKBH5 in Clinical Data

To discuss the clinical significance of ALKBH5 in breast cancer, we first utilized the Cancer Proteogenomic Data Analysis Site database (cProSite) (https://cprosite.ccr.cancer.gov) to explore the relative protein abundance of ALKBH5 across various cancers compared to their corresponding normal tissues (Figure 6A). Specifically, relative ALKBH5 expression was the most significantly elevated in breast tumor, followed by lung adenocarcinoma (Figure 6B). This implied a potential pro-carcinoma function of ALKBH5 in breast cancer. Kaplan–Meier survival analysis of overall survival (OS) and disease-free survival (DFS) was generated with Breast Cancer Gene-Expression Miner v5.1 platform (bc-GenExMiner v5.1). Contrary to our expectations, ALKBH5 was not significantly correlated with overall survival (OS) of breast cancer patients. Moreover, higher expression of ALKBH5 even predicted a better disease-free survival (DFS), suggesting the involvement of other mechanisms regulated by ALKBH5 and highlighting its complex role in breast cancer (Figure 6C). Moreover, we analyzed the correlation between ALKBH5 expression and prognosis across different subtypes, including ER, PR, nodal status, and molecular subtype HER2-E classification. Consistent with prior observations, ALKBH5 expression did not demonstrate a significant prognostic impact (Supplementary Figure 3). We further analyze the association between ALKBH5 and β-catenin expression. The result revealed that patients with high *ALKBH5* mRNA levels tended to have high *CTNNB1* expression (Figure 6D). This positive correlation was also observed by the Gene Expression Profiling Interactive Analysis (GEPIA) database (Figure 6E).[47] Additionally, according to

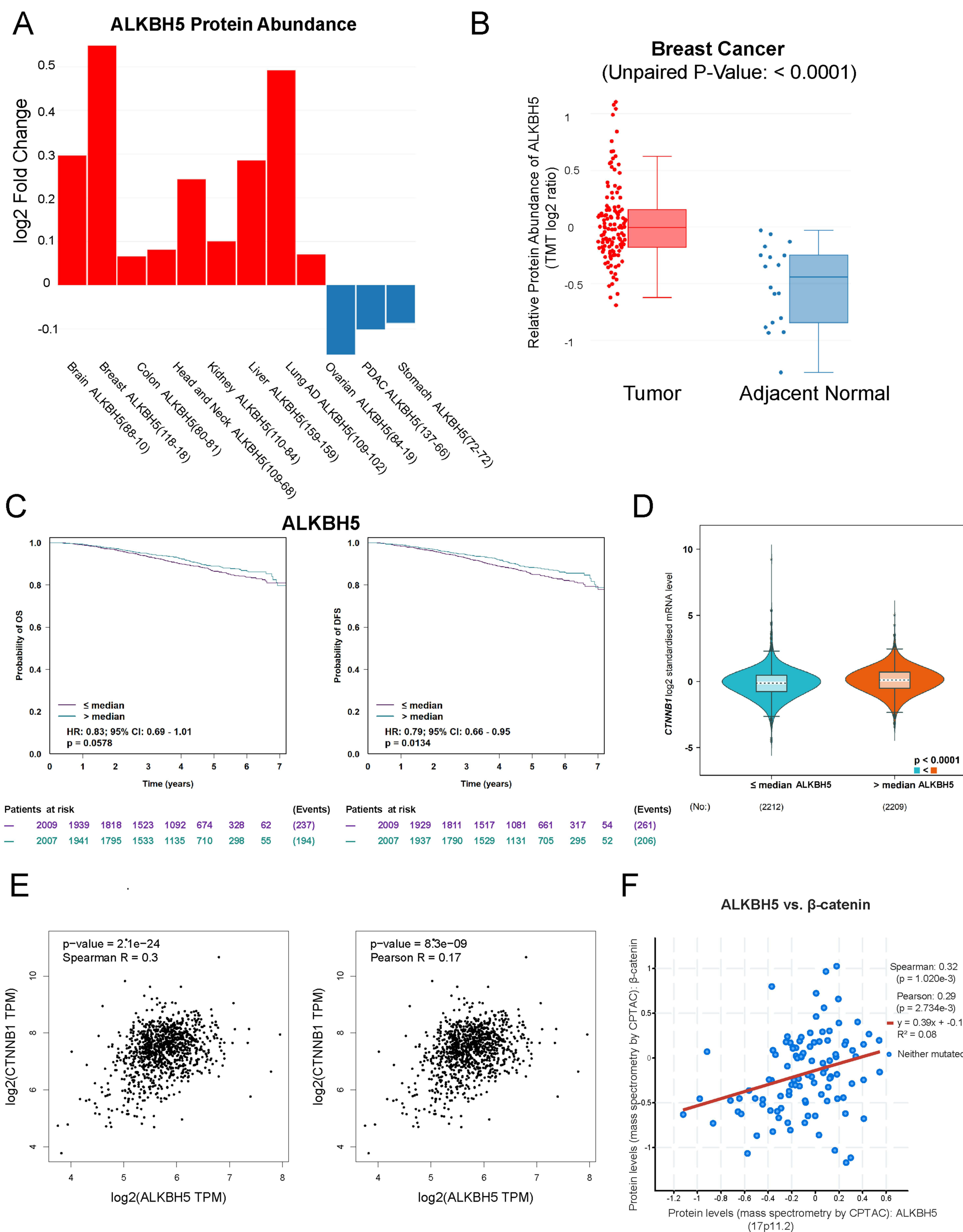

Figure 6 ALKBH5 is positively correlated with CTNNB1 expression in breast cancer. (**A** and **B**) Using cProSite database to analyse the relative ALKBH5 protein abundance in various tumor types (**A**) and the relative protein expression level of ALKBH5 in breast tumor and adjacent normal tissue (**B**). (**C**) Kaplan-Meier survival analysis of overall survival (OS) and disease-free survival (DFS) by Breast Cancer Gene-Expression Miner v5.1 platform. (**D**) Patients with high ALKBH5 expression demonstrated high *CTNNB1* mRNA levels. (**E**) Gene Expression Profiling Interactive Analysis (GEPIA) database showed the positive correlation of *ALKBH5* and *CTNNB1* mRNA. (**F**) The protein level of ALKBH5 was significantly positively associated with β-catenin protein level performed with cBioportal database.

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analysis from cBioportal (http://www.cbioportal.org/),[48] the protein levels of ALKBH5 correlated positively with β-catenin expression (Figure 6F). Altogether, although ALKBH5 may not be an ideal prognostic factor on its own, its significant association with β-catenin was evident in multiple clinical datasets. These findings suggest that combining ALKBH5 and β-catenin expression levels may offer a more robust prognostic marker for breast cancer.

Conclusion

The involvement of m^{6}A epigenetic modifications in breast cancer has been extensively studied in recent years. Previous research has found that high expression of METTL14, WTAP and FTO, but not ALKBH5 was correlated with good metastasis relapse-free survival in breast cancer. Induction of m^{6}A by overexpression of METTL14 or knockdown of ALKBH5 inhibited cell viability, colony formation, and migration.[49] Zhang et al proved that knockdown of ALKBH5 not only decreased expression of NANOG, which is a pluripotency factor promoting CSCs phenotype, but also confirmed ALKBH5 was required for tumorigenesis and lung metastasis in breast cancer within the hypoxia condition.[50,51] These researches highlighted the significant role of ALKBH5 in breast tumor development and progression. In our study, we suggested ALKBH5 mediated demethylation of *CTNNB1* mRNA, thus affecting Wnt/β-catenin signaling pathway and stemness associated genes including *NANOG*. Whether *NANOG* was regulated directly by ALKBH5 in this study warranted further verification. In addition to Wnt/β-catenin pathway, it is important to note that other stemness-related pathways, such as Hippo, Hedgehog may also be involved.[52–54] Further research is needed to explore the potential interplay of ALKBH5 with these pathways in the context of breast cancer stemness.

While our study focuses on ALKBH5, it is essential to acknowledge the complex interplay between different m^{6}A regulators in breast cancer. Previous studies highlight that other m^{6}A erasers like FTO also contribute to tumorigenesis and metastasis.[25] Further exploration of which m^{6}A reader recognizing m^{6}A modification on *CTNNB1* induced by ALKBH5 in MCF-7 cells was still needed. Understanding how ALKBH5 interacts with other regulators would provide a more comprehensive picture of the m^{6}A landscape in breast cancer and its implications for targeted treatment. Despite we revealed that ALKBH5 could promote stemness of breast cancer cell in vitro, several avenues like the role of ALKBH5 in different breast cancer subtypes and its potential as a biomarker for patient stratification are worthy of future studies. Additionally, in vivo models would be invaluable for assessing the therapeutic potential of targeting ALKBH5 in breast cancer treatment.

In conclusion, we found depletion of ALKBH5 significantly inhibited the proliferation, colony formation, and migration, invasion abilities of MCF-7 cells in vitro. We also observed that the CSCs characteristics in MCF-7 cells were suppressed under ALKBH5 knockdown. These findings establish a crucial link between ALKBH5 and breast cancer progression, particularly through its impact on cancer stemness and the Wnt/β-catenin signaling pathway. These insights pave the way for potential therapeutic interventions aimed at modulating ALKBH5 activity in breast cancer management.

Data Sharing Statement

All data analyzed during this study are included in this published article and its supplementary information files.

Ethical Approval

No ethical approval is required in this study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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