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Distinct 5-methylcytosine profiles of LncRNA in breast cancer brain metastasis

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Abstract

Background Recent studies have identified a complex relationship between methylation patterns and the development of various cancers. Breast cancer (BC) is the second leading cause of cancer mortality among women. Approximately 5–20% of BC patients are at risk of BC brain metastases (BCBM). Although 5-methylcytosine (m5C) has been identified as an important regulatory modifier, its distribution in BCBM is not well understood. This study aimed to investigate the distribution of m5C in BCBM.

Materials and methods Samples from BCBM (231-BR cells) and BC (MDA-MB-231 cells) groups were subjected to a comprehensive analysis of the m5C methylation in long non-coding RNA (lncRNA) using methylated RNA immunoprecipitation next-generation sequencing (MeRIP-seq). The expression levels of methylated genes in BC and adjacent tissues were verified through quantitative real-time polymerase chain reaction (RT-qPCR). Enrichment pathway analyses were through Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) to predict the potential functions of m5C in BCBM.

Results The MeRIP-seq analysis identified 23,934 m5C peaks in BCBM and 21,236 m5C in BC. A total of 9,480 annotated genes (BCBM) and 8,481 annotated genes (BC) were mapped. Notably, 1,819 methylation sites in lncRNA were upregulated in BCBM, whereas 2,415 methylation sites were upregulated in BC. Significant m5C hypermethylated lncRNAs included ENST00000477316, ENST00000478098 and uc002gtt.1, whereas hypomethylated lncRNAs included ENST00000600912, ENST00000493668, ENST00000544651 and ENST00000464989. These results were verified by qPCR and MeRIP-qPCR in BC and BCBM. Considering the strong association between m5C RNA methylation regulators and lncRNA, we examined the expression levels of 13 m5C RNA methylation regulators and observed significant differences between BC tissues and adjacent normal tissues. In addition, the interaction between regulators of altered expression and the differentially expressed genes in vitro was analyzed. The GO and KEGG

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pathways analyses revealed that genes significantly associated with m5C sites in lncRNA were linked to the BCBM signaling pathways.

Conclusion This uncovered significant variations in the levels and distribution of m5C in BCBM compared to BC. The findings provide a new theoretical understanding of the mechanisms of BCBM.

Keywords Breast Cancer Brain Metastases (BCBM), 5-methylcytosine (m5C), Long non-coding RNA (lncRNA), Methylated RNA Immunoprecipitation next-generation sequencing (MeRIP-seq), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), Methylation regulators

Introduction

RNA post-transcriptional modifications are increasingly being studied in recent years [1, 2]. These modifications, which exceed 100 in number, include pseudouridine, N6-methyladenosine (m6A) [3], N1-methyladenosine (m1A) [4], and 5-methylcytosine (m5C) [5]. The important role of m6A modification for RNA has been documented by several studies, including its involvement in the development of various cancers, such as colon cancer [3], hepatocellular carcinoma [6], and glioblastoma [7, 8]. Another noteworthy post-transcriptional modification of RNA, m5C, has been identified in stable and abundant tRNAs, as well as in various RNAs and mRNAs [9].

The widespread m5C modification in cells underscores its crucial role in the regulation of gene expression and RNA stability [5]. Catalyzed by RNA methyltransferase or DNA methyltransferase 2 (DNMT2) [10], m5C can be demethylated by tet methylcytosine dioxygenase [11]. m5C-modified mRNA is recognized by ALYREF, which facilitates its nuclear export. In contrast, Y-box binding protein 1 (YBX1) directly interacts with m5C-methylated mRNA, contributing to its stabilization [12]. Recent studies have highlighted the importance of m5C methylation and its related modifying enzymes in tumor initiation and progression. For instance, NSUN2-mediated m5C methylation could promote tumorigenesis and progression of bladder cancer [5], and the m5C methyltransferase NSUN1 alters chromatin structure by interacting with BRD4, influencing the responsiveness of leukemia tumor cells to the chemotherapeutic agent 5-azacytidine [13].

Studies show that m5C modification is essential for the stability and efficiency of tRNA translation, which is critical in the processing, construction, and translation of rRNA [14]. Moreover, m5C modification also occurs in noncoding RNAs (ncRNAs) such as long non-coding RNAs (lncRNAs) and mRNA [15]. Comprehensive analysis of the m5C profiles of mammalian transcriptomes have revealed that the m5C modification contributes to the export of mRNA and post-transcription regulation [16–18]. Studies show that the m5C-modified H19 lncRNA can specifically interact with the G3BP1 protein, causing accumulation of the oncoprotein Myc and promoting the occurrence and development of liver cancer [19].

LncRNAs, with lengths exceeding 200 nucleotides and lacking protein-coding functions [20–22], have been associated with several epigenetic mechanisms, such as gene silencing, histone modification, regulation of transcription, interference in transcriptional processes, and nuclear transport; which are associated with the progression of various human diseases [23]. Several lncRNAs have been linked to the formation and progression of tumors [23]. For instance, the specific sequence of LINC00942 recruits the methyltransferase METTL14, stabilizing the downstream targets of LNC942 and enhancing the initiation and progression of breast cancer (BC) [24]. Currently, the distribution and functional significance of m5C modification of lncRNA in breast cancer brain metastases (BCBM) is not fully understood.

BC is the second most common cause of cancer-related deaths among women, with the BCBM incidence ranging from 10–20% [25, 26]. BCBM was found to be correlated with unfavorable prognosis, negatively affecting cognitive and sensory functions and severely limiting the patient's quality of life [25, 27]. Treatment modalities for BCBM comprise surgical intervention, whole-brain radiotherapy (WBRT), stereotactic radiation therapy (SRS), and chemotherapy. These face challenges due to the blood-brain barrier (BBB), leading to unsatisfactory longevity expectations for BCBM patients and limiting penetration and chemoresistance [28]. Recent studies indicates that methylation could be a crucial therapeutic target for BCBM, given its role in the development and progression of the condition [29].

This study adds new insights into the effect of m5C methylation of lncRNAs on BCBM and BC. By using m5C-specific in-depth bioinformatics analyses, we demonstrated that m5C modification was higher in BCBM than in BC, and this phenomenon varied across all chromosomes.

Materials and methods

Preparation of RNA sequencing samples and cell culture

Patricia S Steeg gifted BCBM (231-BR) and BC (MDA-MB-231) cells [30, 31] (National Cancer Institute, NIH, Bethesda, MD, USA), and all subsequent experiments

were performed with a comparison between the two groups. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Corporation, MO, USA) enriched with 10% (v/v) Fetal Bovine Serum (FBS, Sangon Biotech, Shanghai, China) at 37℃ in a 5% CO_2 incubator. Total RNA was extracted from cultured cells utilizing TRIzol reagent (Thermo Fisher, MA, USA) by the manufacturer's guidelines. The concentration and purity of the RNA were evaluated using a NanoDrop 2000 ultraviolet-visible spectrophotometer (Thermo Fisher, MA, USA), where OD260/OD280 ratios ranging from 1.8 to 2.1 were deemed acceptable.

RNA MeRIP-seq library construction & sequencing

MeRIP-Seq was conducted using Cloudseq Biotech Inc (Shanghai, China) following a slightly modified version of an established protocol [32]. Fragmented RNA was incubated with an anti-m5C antibody for 2 h at 4 °C in IPP buffer. The mixture was then subjected to immunoprecipitation using protein-A beads (Thermo Fisher, MA, USA) at 4 °C for an additional 2 h. The RNA was subsequently eluted using a free m5C adenosine analog. RNA MeRIP-seq was performed with three biological replicates per group. The eluted RNA was extracted using Trizol reagent (Thermo Fisher, MA, USA). Purified RNA was used for library construction with RNA Library Kit (New England Biolabs, Inc, USA), and paired-end 150 bp sequencing was performed on an Illumina Hiseq sequencer (Fig. 1).

Analysis of sequencing data

Quality control was conducted on paired-end reads (Q30 > 80%, Supplementary Table S1), followed by the trimming of 3' adapters, and low-quality reads were filtered out using Cutadapt software (v1.9.3). The clean reads derived from the input library were accurately mapped to the reference genome using the STAR software [33], subsequently leveraging the DCC software to identify circRNAs [34]. The clean reads were mapped to the reference genome using Hisat2 software (v2.0.4) [35]. Methylated genes in each sample were identified using MACS software [36]. The portions that overlap with the exons of known lncRNAs were selected using the proprietary program of Shanghai CloudSeq Biotechnology Company. The threshold for MACS identification was set as $P <= 0.00001$ and Fold enrichment $>= 2$. Subsequently, the diffReps software [37] was utilized to identify differentially methylated sites and conduct corresponding annotations. The dataset can be accessed at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE246721.

Analysis of pathway enrichment

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses used DAVID to interpret the functional enrichment and annotation of differentially methylated genes. *P*-values < 0.05 were regarded as significant for both analyses.

Quantitative real-time PCR (RT-qPCR) analysis

Total RNA from all samples was isolated using Trizol reagent (Thermo Fisher, MA, USA). The first complementary DNA (cDNA) strand was synthesized using reverse transcription kits (Beyotime, Beijing, China). cDNA underwent RT-qPCR utilizing a SYBR Green qPCR Master Kit (Toroivd, Shanghai, China) along with specific primers (sequences provided in Supplementary Table S2) under the reaction condition of 95℃ for 5s and 60 ℃ for 30s for 35 cycles.

Western blotting

Protein was extracted using RIPA buffer (Applygen, Beijing, China). The BCA protein quantification kit was then used to assess the protein concentration in the sample (Beyotime, Beijing, China). The protein samples were separated through SDS-PAGE gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk at room temperature for 1 h to prevent nonspecific binding and then incubated overnight at 4 °C with primary antibodies targeting NSUN2 (1:1000, #52901S, Cell Signaling Technology, USA) or β-actin (1:1000, #sc-47778, Santa, USA). Once the primary antibody incubation was finished, the PVDF membrane was rinsed three times using TBST buffer. An HRP-conjugated secondary antibody was then applied at room temperature. Finally, the PVDF membrane was treated with an enhanced chemiluminescence reagent (Beyotime, Beijing, China). The protein signal was captured and analyzed using a chemiluminescent imaging system (Tanon, Shanghai, China).

Knockdown of NSUN2 in BCBM cells via lentiviral infection

For NSUN2 gene knockdown, the BCBM cells were transfected with Lv-shNSUN2 (Genechem, Shanghai, China) or the negative control Lv-NC at a multiplicity of infection (MOI) of 10 for 12 h, following the manufacturer's protocol. The successfully infected cells were then selected by treating them with 2 μg/mL puromycin (Beyotime, Beijing, China) for 48 h.

Statistical analysis

Data analysis was conducted using GraphPad Prism v5.0 software. Student's t-test was utilized to compare statistical significance, and $P < 0.05$ was considered statistically significant. All experiments were conducted in triplicate.

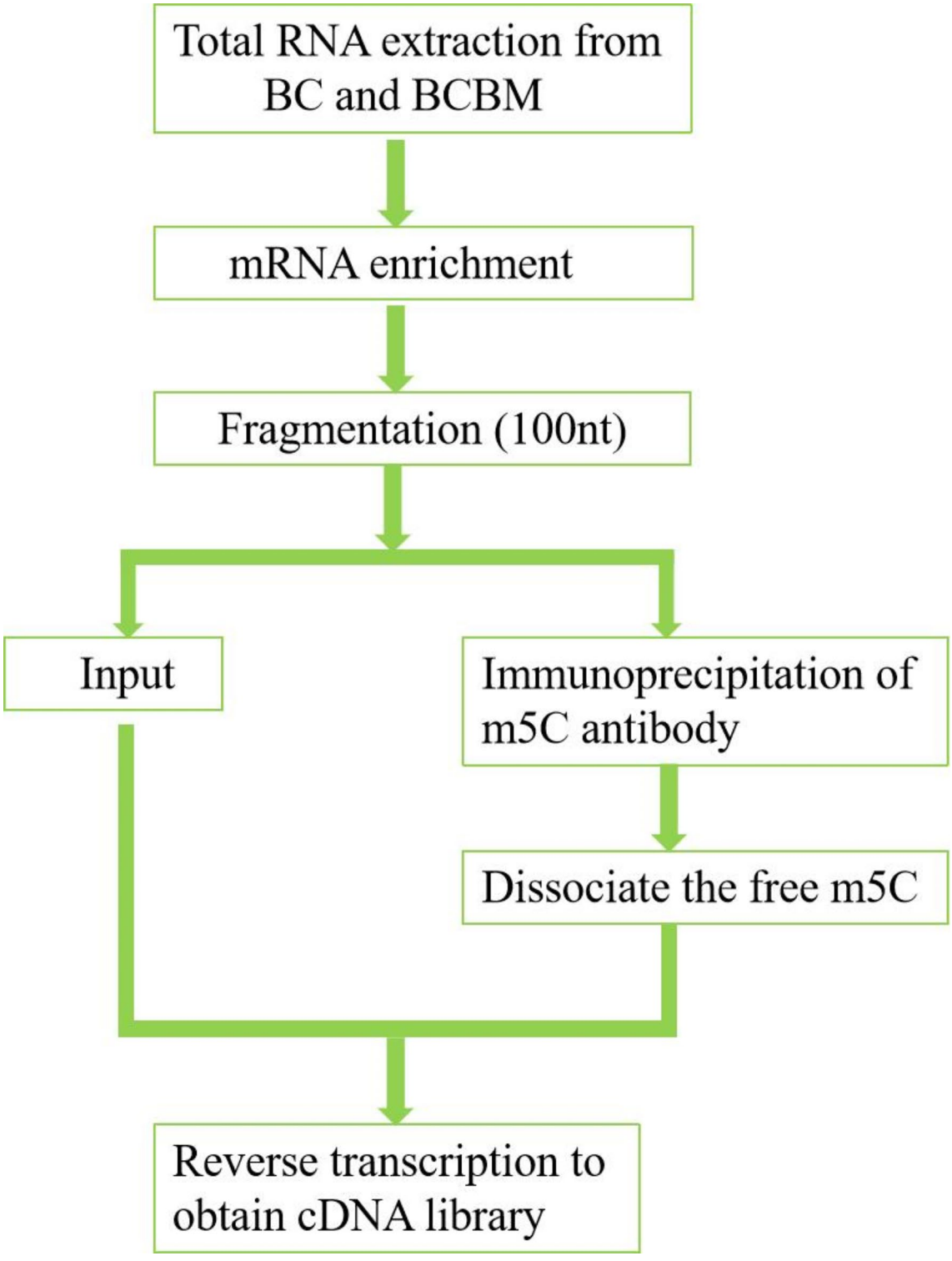

Fig. 1 RNA MeRIP-seq library construction and sequencing

Results

General features of m5C methylation in BC and BCBM

Transcriptome-wide m5C methylation was assessed in BC and BCBM cells through RNA MeRIP-seq of LncRNA. BCBM cells exhibited 23,934 m5C peaks compared to 21,236 in BC cells. The BCBM and BC cells shared 7030 methylation sites, 16,904 were exclusive to BCBM, while 14,206 were exclusive to BC (Fig. 2A). A total of 9,480 annotated genes were mapped in BCBM, and 8,481 in BC, with 6,246 genes shared between the

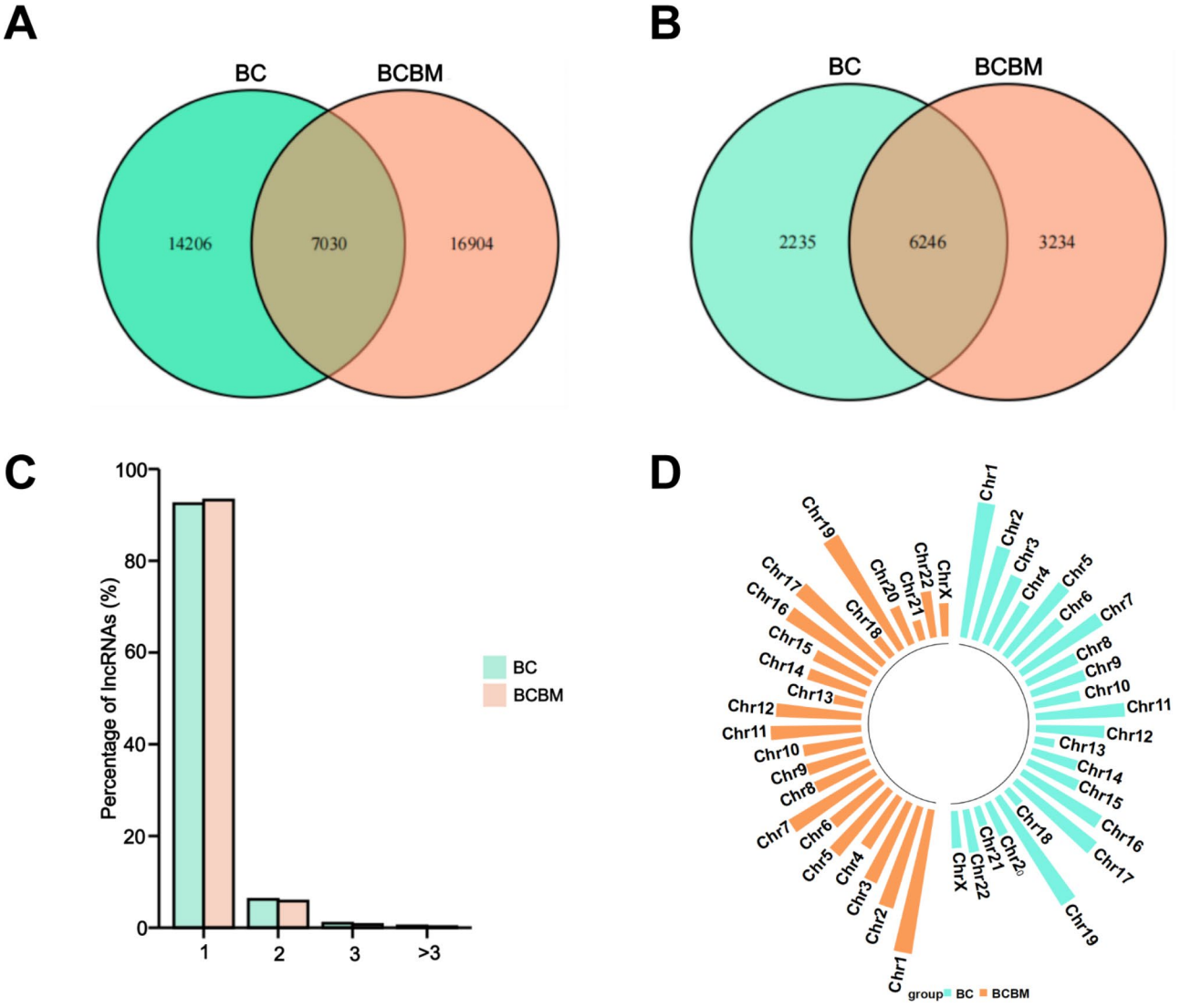

Fig. 2 RNA MeRIP-seq was performed to determine the transcriptome-wide m5C methylation and the overall characteristics of lncRNA in BCBM. (**A**) A Venn diagram illustrating the m5C methylation sites in lncRNA from BCBM and BC. (**B**) A Venn diagram displaying the m5C genes in both BCBM and BC. (**C**) The proportion of lncRNAs with varying m5C methylation peaks between BCBM and BC cells, with the majority having only a single m5C peak. (**D**) The histogram showing the distribution of m5C methylation sites in various chromosomes

two (Fig. 2B). Moreover, the results showed that the number of up-methylated transcript sites per gene exclusive to BCBM (5.23 sites/gene) or BC (6.36 sites/gene) was markedly higher than the methylated transcript sites shared between BCBM and BC cells (1.13 sites/gene). This observation highlights the intricate nature of epigenetic regulation in BCBM. The distinct patterns of m5C methylation suggest potential mechanisms by which these cells adapt to their microenvironment and acquire unique functional properties.

Statistical evaluation of peak counts for each LncRNA

Analysis of m5C peaks on lncRNAs showed that the majority had only one methylation peak, with a higher proportion observed in BCBM (93.24%) compared to BC (92.44%). Furthermore, the percentages for two or more methylation peaks on lncRNAs were comparable between BCBM (5.82%) and BC (6.19%). Three methylation peaks (0.71% BCBM vs. 0.99% BC) and more than three methylation peaks (0.235% BCBM vs. 0.39% BC) were detected (Fig. 2C).

Chromosome visualization of m5C in LncRNAs

The distribution analysis of m5C methylation sites across chromosomes in BCBM and BC cells revealed a broad and widespread pattern in all chromosomes. Notably, there were distinct differences in both methylation levels and distribution between these two cell types. Specifically, chromosome 21 exhibited significantly lower

methylation levels compared to the other chromosomes in both BCBM and BC cells, as illustrated in Fig. 2D.

Motif analysis of methylation sites

The sequence of the m5C methylation peak was analyzed to clarify the presence of the m5C motif, which revealed CCAGSCUG (S = C/G) as the most common and reliable motif in BCBM cells (E-value = 2.1e-088) (Fig. 3A). The most consistent motif for m5C peaks in BC cells was identified as GRAGRA (R = G/A) (E-value = 1.2e-119) (Fig. 3B), highlighting distinct motif preferences for m5C peaks in each cell type.

Cluster analysis of differential methylation peaks

Unsupervised hierarchical cluster analysis revealed varying methylation patterns between BCBM and BC cells (Fig. 3C), highlighting both consistency and inter-cellular variability between the two cell types. Differential methylation sites in lncRNAs were identified, with 1819 and 2415 methylation sites in lncRNA upregulated in BCBM and BC cells, respectively. The top ten up and hypomethylated sites in BCBM cells compared to BC cells are shown in Tables 1 and 2.

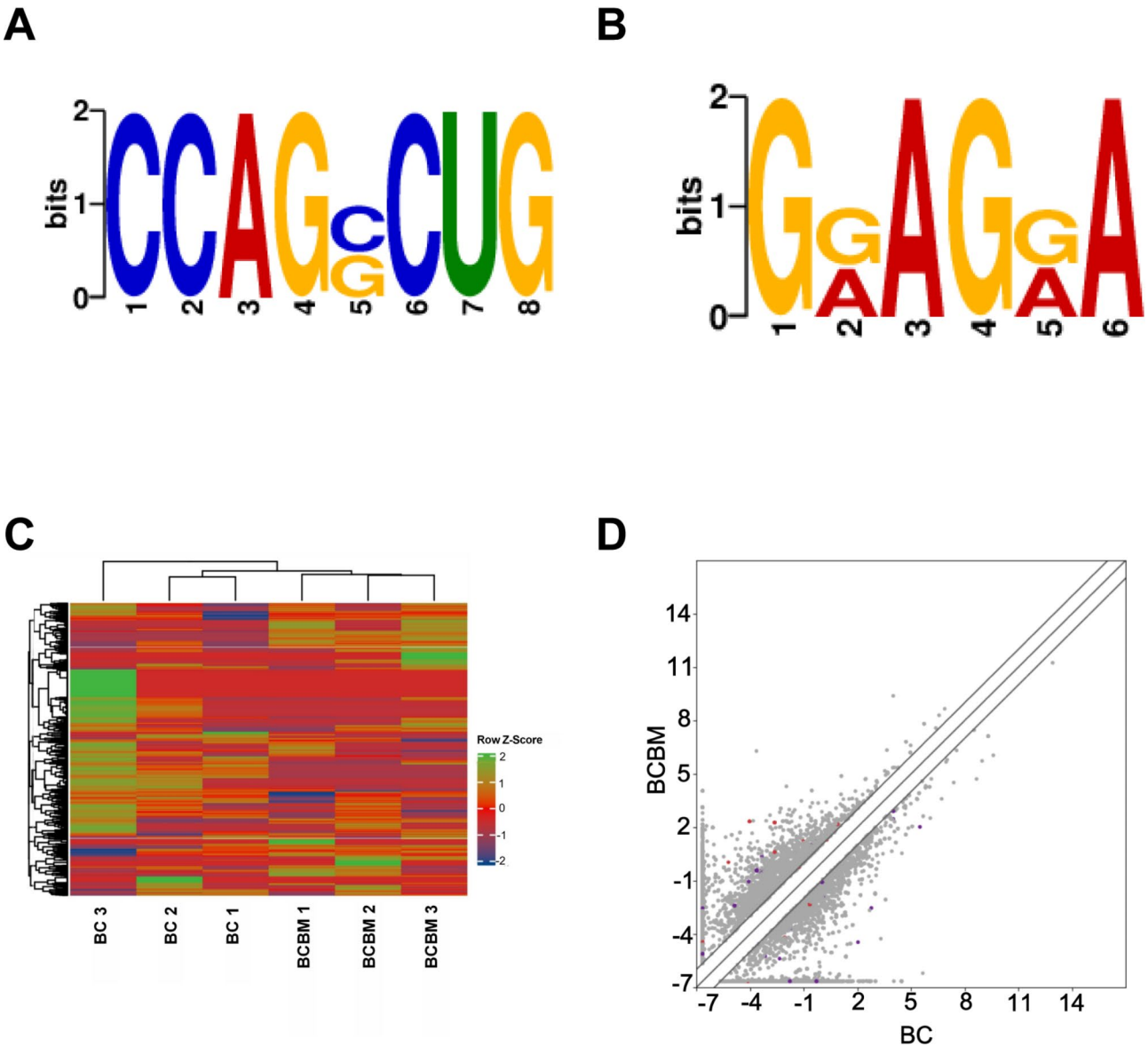

Fig. 3 Motif analysis of methylation sites. (**A**-**B**) The motif of m5C in BCBM and BC. (**C**) Heat map displaying the methylation patterns between BCBM and BC cells. (**D**) Differential expression of lncRNAs between BCBM and BC cells. Up-regulated methylated genes are shown in red, while down-regulated methylated genes are represented by purple

Table 1 Top ten Up-methylated peaks. Up BCBM VS BC

Chrom	txStart	txEnd	transcript_id	GeneName	FoldChange
Chr2	241,496,681	241,496,823	ENST00000477316	ANKMY1	1299.6
Chr1	6,133,792	6,133,907	ENST00000478098	KCNAB2	1276.2
Chr7	43,523,742	43,523,860	ENST00000461842	HECW1	1093.6
Chr14	19,720,481	19,720,589	ENST00000550526	RP11-496I2.5	1035.5
Chr7	150,939,222	150,939,420	ENST00000472103	SMARCD3	1022.2
chr17	18,315,161	18,315,660	uc002gtt.1	AX748015	964.2
chr19	21,751,961	21,752,039	ENST00000594564	RP11-678G14.2	899.1
chr22	16,192,905	16,193,009	NR_122113	DUXAP8	791.9
chr12	56,663,665	56,663,840	ENST00000546464	RP11-977G19.14	759.6
chr22	20,393,533	20,393,604	NR_003563	PI4KAP1	683.2

Table 2 Top ten Down-methylated peaks. Down BCBM VS BC

Chrom	txStart	txEnd	transcript_id	GeneName	FoldChange
Chr19	8,279,249	8,279,413	ENST00000600912	CERS4	959.2
Chr17	73,555,061	73,555,340	ENST00000582860	LLGL2	946
ChrX	9,866,221	9,866,330	ENST00000493668	SHROOM2	703.2
Chr16	15,082,501	15,082,800	ENST00000567634	RP11-680G24.4	675.7
Chr11	67,057,299	67,057,436	NR_030767	ANKRD13D	647.6
Chr1	1,631,701	1,631,701	ENST00000412810	MMP23A	612.9
Chr12	109,629,282	109,629,568	ENST00000544651	ACACB	596.2
Chr2	220,308,521	220,308,781	ENST00000464989	SPEG	484.8
Chr19	46,273,741	46,273,960	ENST00000598272	DMPK	383.5
Chr1	1,019,101	1,019,400	ENST00000487177	C1orf159	318.8

Effect of methylation on transcriptional expression

To investigate the impact of m5C methylation on lncRNA expression, RNA sequencing data (GSE246721) was used to analyze the expression patterns of lncRNA in BCBM cells and their parental BC cells. The results showed that methylation increased the expression level of numerous lncRNAs in BCBM cells (Fig. 3D). In contrast, methylation decreased the expression levels of lncRNAs in BC cells.

GO and KEGG enrichment analysis

The function of differentially methylated lncRNA-associated genes in BCBM and BC cells was determined using GO functional analysis. The main enrichment in biological processes (BP), cell components (CC), and molecular functions (MF) are shown in Fig. 4 (A-F). The analysis of biological processes (BP) revealed that genes with up-methylated m5C in BCBM were mainly involved in RNA splicing through transesterification reactions with bulged adenosine as a nucleophile, along with RNA splicing and regulation of chromosome organization. In contrast, genes with down-methylated m5C in BCBM were primarily associated with tube morphogenesis, protein localization, organelle organization, and macromolecule localization. Regarding cellular components (CC), genes exhibiting up-methylated m5C in BCBM were associated with organelles, nucleoplasm, nuclear lumen, and bounded organelles. In contrast, genes that were down-methylated m5C in BCBM were associated with organelle membranes, membrane-bounded organelles, and intracellular vesicles. For the MF category, the up-regulated methylation sites in BCBM were enriched in transcription coregulator activity, anion antiporter activity, protein binding and nucleoside-triphosphatase regulator activity and small GTPase binding, protein serine/threonine kinase activity, protein binding and phosphotransferase activity alcohol group as acceptor were down-regulated methylated.

The KEGG pathway analysis highlighted pathways enriched in hyper- and hypo-methylated lncRNA (Fig. 5A-B), providing insights into the molecular mechanisms these methylation changes might affect. The KEGG pathway analysis showed that genes associated with methylated lncRNAs were significantly involved in choline metabolism in cancer, oocyte meiosis, and the P53 and MAPK signaling pathways. Conversely, down-methylated genes were linked to regulating the actin cytoskeleton, pathways governing stem cell pluripotency, VEGF, and the Hippo signaling pathway. The dot plot (GeneRatio) shows the ratio of the number of differentially methylated The top 10 pathway items with significant enrichment, based on high fold enrichment, were associated with lncRNA-related genes among the total number of differentially methylated LncRNA-associated genes in BCBM and BC (Fig. 5C-D).

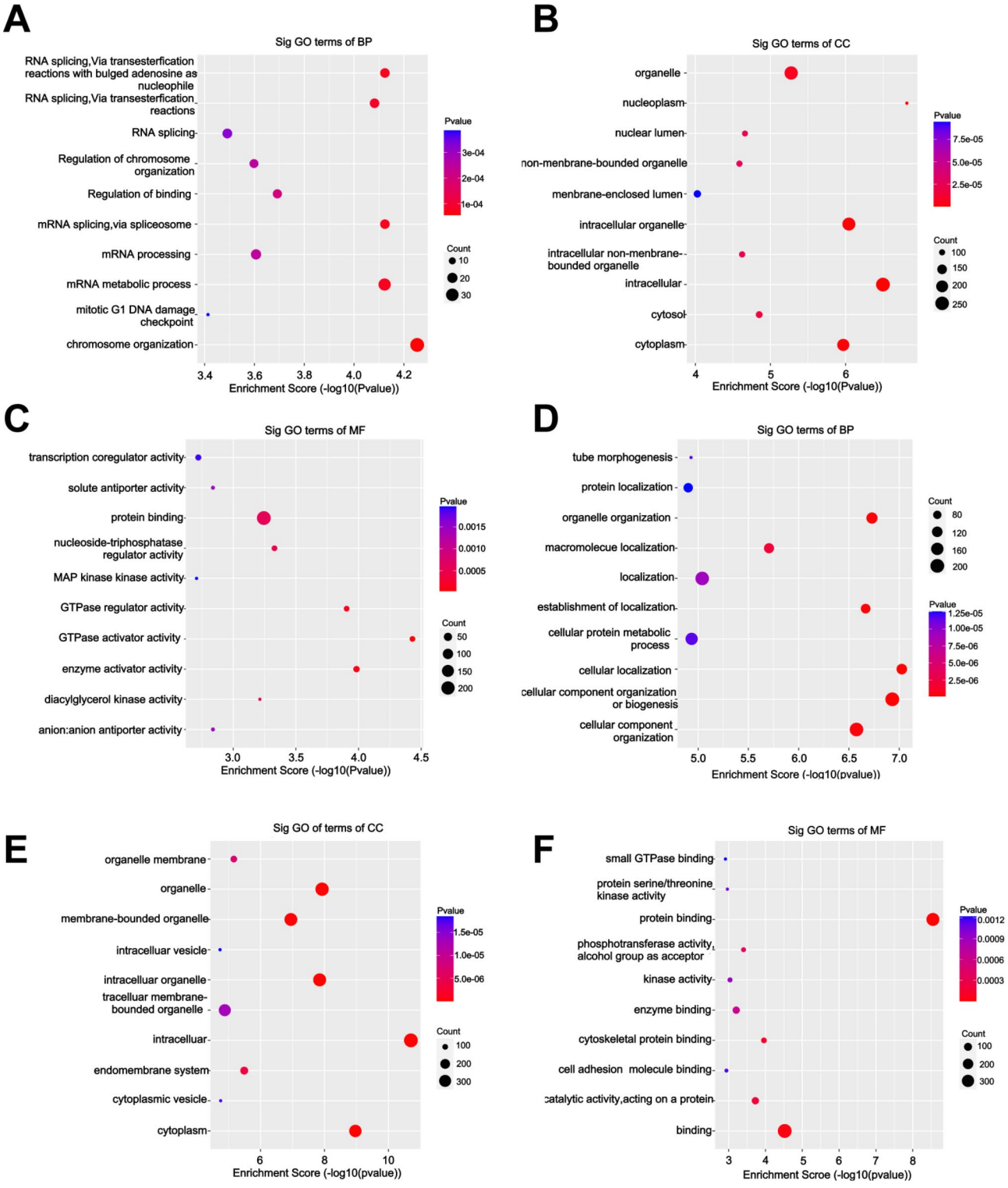

Fig. 4 Gene Ontology (GO) enrichment analysis of the differentially methylated lncRNA-associated genes in BCBM cells. The top 10 GO terms in the (**A**) biological processes (BP), (**B**) cellular components (CC), and (**C**) molecular functions (MF), illustrating the enrichment of the up-methylated m5C genes in BCBM. The top 10 GO terms in the (**D**) BP, (**E**) CC, and (**F**) MF, showing enrichment of the down-methylated m5C genes in BCBM

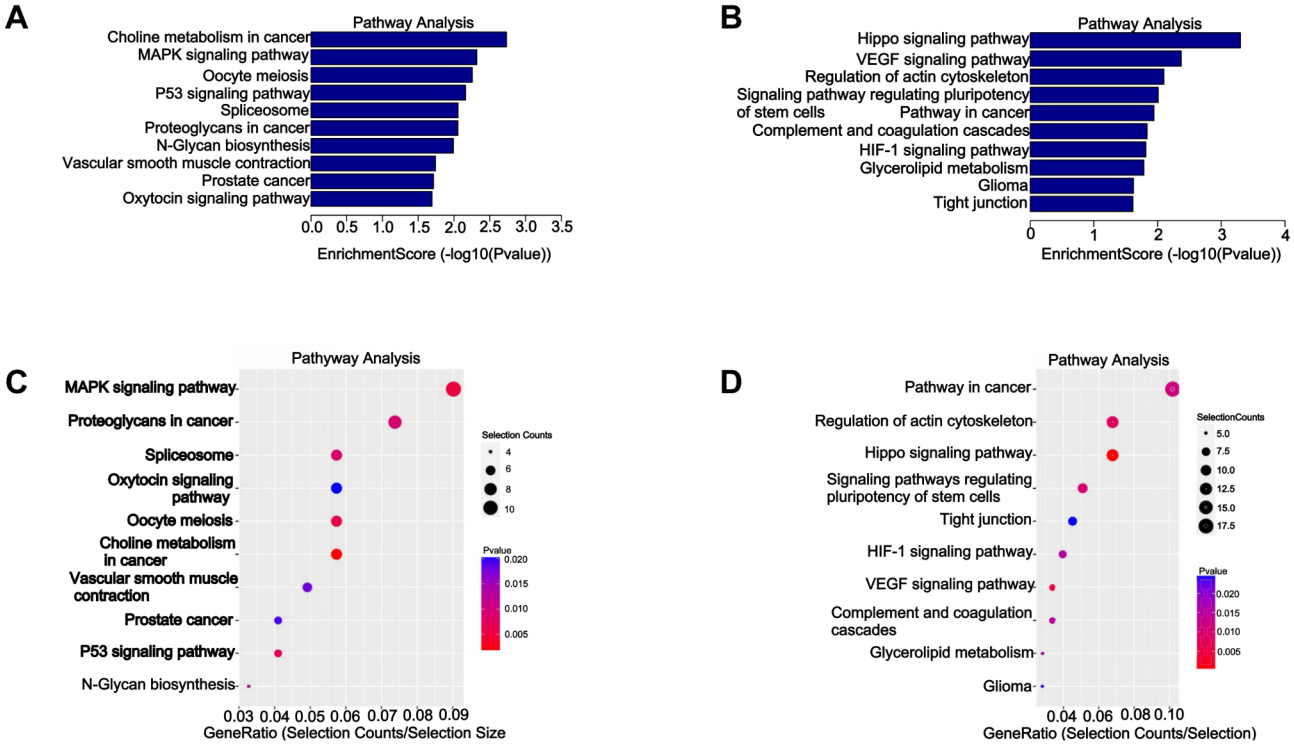

Fig. 5 KEGG pathway analysis for the m5C genes among BCBM lncRNAs. (**A**) The bar chart illustrating the top ten enrichment scores for significant pathways associated with up-methylated m5C genes in BCBM. (**B**) The bar chart showing the top ten enrichment scores of the significant pathways associated with down-methylated m5C genes in BCBM. (**C**) The dot plot indicating the gene ratio values for the ten most significantly enriched pathways associated with the up-methylated m5C genes in BCBM. (**D**) The dot plot showing the gene ratio values of the ten most significantly enriched pathways associated with the down-methylated m5C genes in BCBM

Differential methylation LncRNAs expression in BCBM and BC cells

The top ten up and hypomethylated sites in BCBM cells in comparison with BC cells were validated using qPCR. The results showed that BCBM and BC shared three upregulated lncRNAs ($P<0.05$), ENST00000477316, ENST00000478098 and uc002gtt.1. Furthermore, there are four downregulated lncRNAs which covered ENST00000493668, ENST00000544651, ENST00000600912 and ENST00000464989 ($P<0.05$) (Fig. 6A-G). The findings from MeRIP-qPCR aligned with the results above (Fig. 7A-G). The log2 fold changes of seven methylation genes on lncRNAs were calculated (Fig. 7H). The qPCR results were consistent with RNA MeRIP-seq sequencing data on the relative expression level of these seven methylated genes ($P=0.0105$), indicating that our RNA MeRIP-seq of lncRNA results are reliable (Fig. 7I).

Analytical integration of m5C LncRNA methylation and the expression levels of LncRNA transcripts

Significant differences in global expression patterns of lncRNAs were detected between BCBM and BC cells, with 199 upregulated and 175 downregulated lncRNAs identified in BCBM (Fig. 8A, B). The differences in the expression patterns of lncRNAs in BCBM were displayed in Volcano plots (Fig. 8C; Fold Change > 2, $P<0.05$). The nine-quadrant diagram showed a strong positive correlation between the lncRNA transcriptome and m5C methylation, along with significant variations in this relationship (Fig. 8D; $R=0.991$, $P<0.01$). The Upset diagram showed that two genes were identified in the up-regulated transcriptional group of lncRNAs and the up-regulated methylation group of m5C-modified lncRNAs. In contrast, two genes were identified in both the down-regulated transcriptional group of lncRNAs and the down-regulated methylation group of m5C-modified lncRNAs. Furthermore, three genes were found in the up-regulated transcriptional group of lncRNAs and the down-regulated methylation group of m5C-modified lncRNAs (Fig. 8E).

Validation of differentially methylated LncRNA in BC tissues

Validation of methylation sites in BC and adjacent tissues confirmed differential expression patterns observed in cell experiments. Increased expression of ENST00000477316, ENST00000478098 and uc002gtt.1, and decreased expression of ENST00000493668, ENST00000544651, ENST00000600912 and

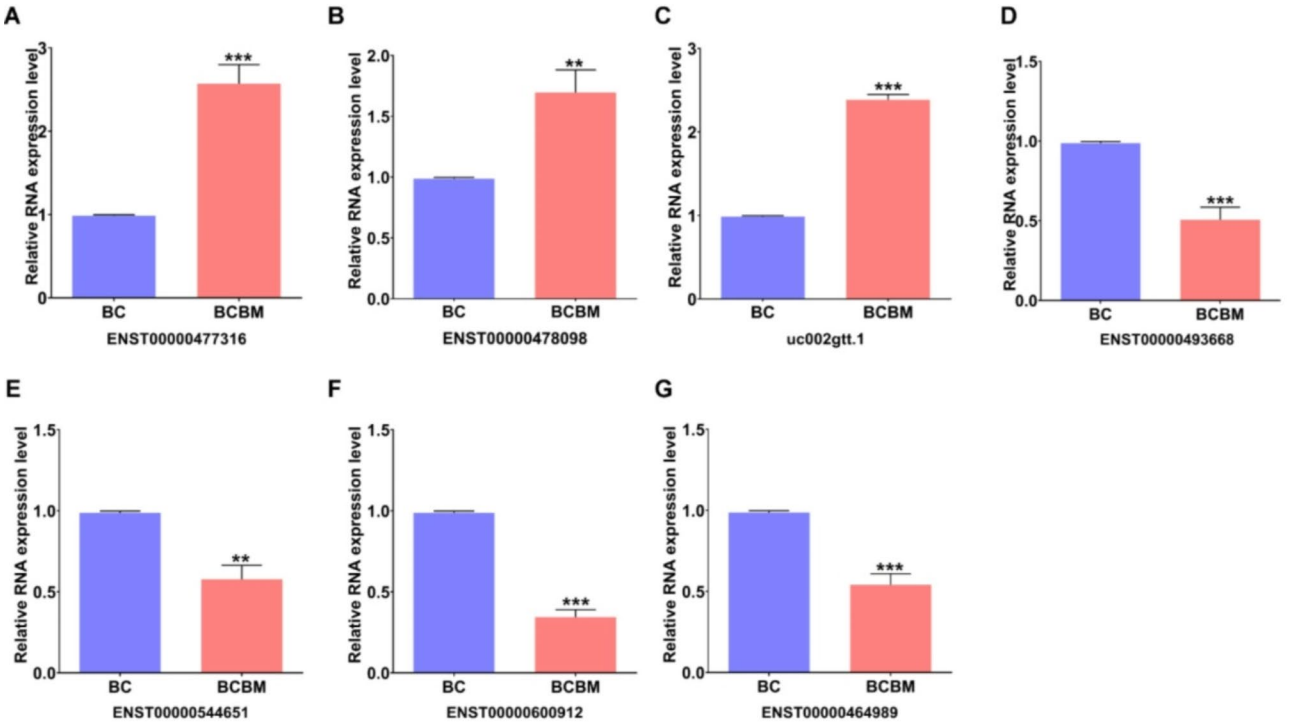

Fig. 6 The expression profile of differential methylation genes in BCBM and BC. (**A**-**G**) The relative expression levels of 7 different methylated genes in BCBM and BC as determined by qPCR ($n = 3$; Data are presented as the mean ± SD; $P < 0.05$)

ENST00000464989 was observed in BC compared to adjacent tissues ($P < 0.05$) (Fig. 9A-G).

Analysis of m5C RNA methylation regulators

RNA methylation regulators for m5C consist of "writers" (methyltransferases), "erasers" (demethylases), and "readers" (binding proteins)[11]. Considering the dynamic regulatory role of m5C in LncRNA methylation, we began by performing a thorough comparison of the expression levels of 13 regulators associated with m5C RNA methylation (Supplementary Table S2) between BC and adjacent tissues (due to the challenge in acquiring specimens of BCBM). The analysis compared the alterations in the expression of m5C RNA methylation regulators between BC and adjacent tissues). Given their elevated expression in BC tissues, NSUN2, NSUN5, YBX1, and ALYREF may play important roles in cancer progression ($P < 0.05$). Methyltransferase DNMT3B, DNMT3A, DNMT1, and TET3 were downregulated ($P < 0.05$), and no difference was observed between BC and adjacent tissues for NSUN3, NSUN4, NSUN5, NSUN6, and TET2 (Fig. 10A).

Further comprehensive analysis of the interplay between regulators of altered expression and differentially expressed genes revealed significant upregulation of NSUN2 expression in BC tissues. Similarly, qPCR and western-blot showed that the expression of NSUN2 was increased in BCBM cells (Fig. 10B-D) implying that NSUN2 is involved in the modulation of BC proliferation and metastatic capabilities. The NSUN2 in BCBM cells were knocked down in vitro to investigate the relationship between NSUN2 and differentially expressed genes. First, the expression of NSUN2 in BCBM cells was knocked out, and then the knockout efficiency of NSUN2 was verified through GFP-positive images (Fig. 10E). It was confirmed that NSUN2 was inhibited in BCBM using western-blot and qPCR (Fig. 10F-H). After knocking down NSUN2 in BCBM, three genes with upregulated methylation lncRNAs expression ENST00000477316, ENST00000478098 and uc002gtt.1, were positively correlated with NSUN2. In contrast, ENST00000493668, ENST00000544651, ENST00000600912 and ENST00000464989, which were associated with downregulated methylation expression, were negatively correlated ($P < 0.05$), suggesting NSUN2 plays an essential role in the process of lncRNA methylation (Fig. 10E).

Discussion

BC is one of the most common cancers among women worldwide, with a mortality rate ranking second among female cancers [26], and cancer metastasis is the main cause of death [38, 39]. In the exploration of new treatment approaches for BC, tumor mutational burden (TMB) has attracted much attention as a biomarker for immunotherapy, but a high TMB score is associated with a reduced survival rate in BC [39]. Immune-related genes such as CCL18 are markers of poor prognosis, while

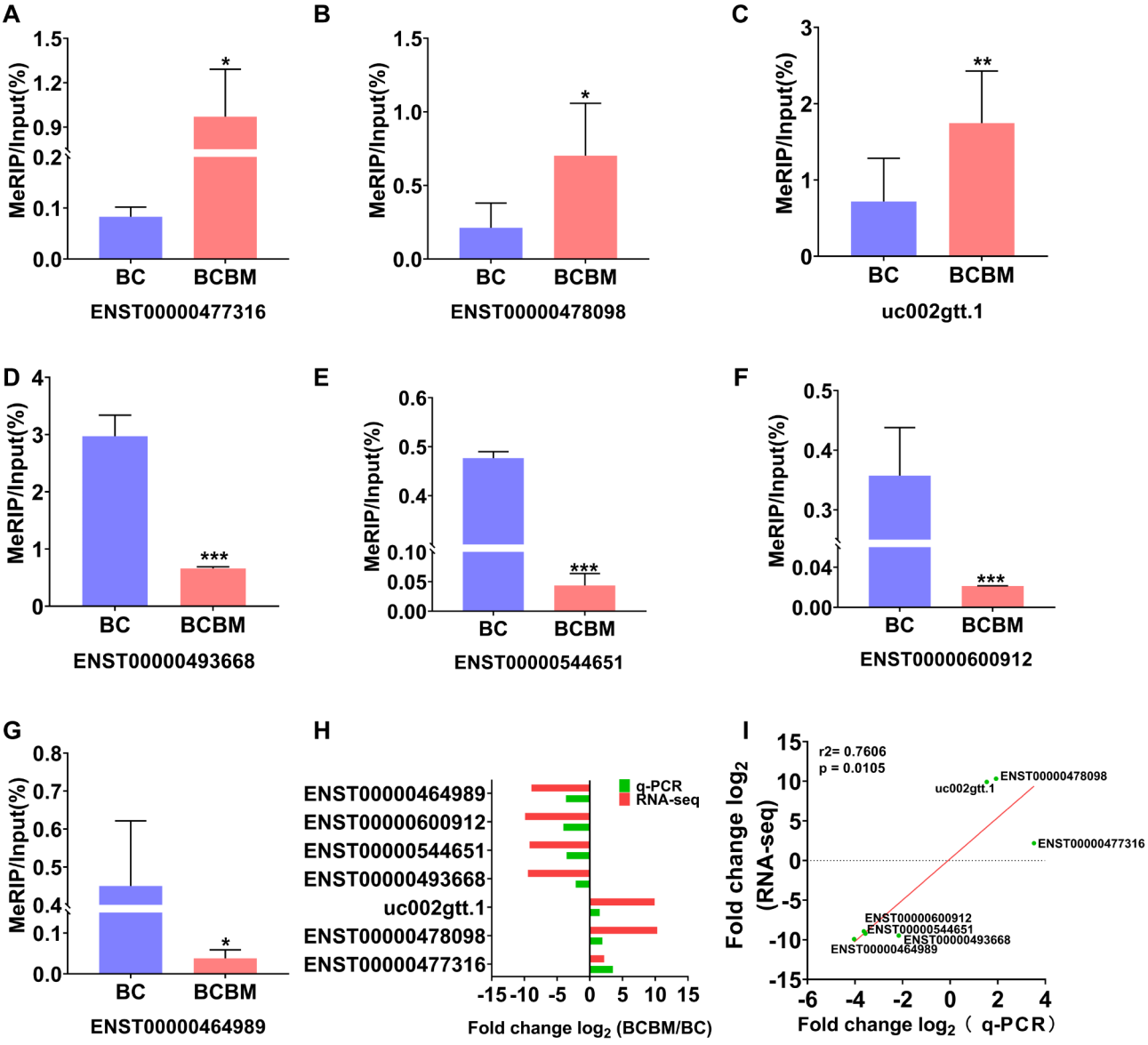

Fig. 7 (**A**-**G**) MeRIP-qPCR analysis of the three up-regulated genes and four down-regulated genes following methylation in the BCBM and BC. ($n = 3$; data are presented as the mean ± SD; $P < 0.05$). (**H**) Comparison of mean fold change (log2 conversion) between MeRIP-qPCR and RNA MeRIP-seq. (**I**) The correlation of the mean fold changes (log2 transformed) between MeRIP-qPCR and the RNA MeRIP-seq data

B-cell infiltration is a marker of good prognosis [39]. Another study has shown that bone metastases, compared to primary breast tumors, exhibit a large number of stromal cells and an inactive immune microenvironment. This suggests that the application of combined immunotherapy in specific bone metastasis patients warrants further exploration [40]. In addition, a good systemic immune activity and the effect of neoadjuvant chemotherapy may be related to lipid metabolism in triple-negative breast cancer (TNBC) [41]. Research has demonstrated that overexpression or abnormal synthesis of proteins in breast tissue influence the occurrence and development of BC [42]. Therefore, identifying BC-related proteins may provide important insights for improving the diagnosis and treatment of BC patients.

BC is a significant health challenge due to its propensity for brain metastasis, which drastically reduces patient prognosis [43, 44]. Thus, it is imperative to study the mechanism driving brain metastasis in BC to generate ideas for developing effective treatments [45]. Epigenetic modifications, including RNA modifications, have emerged as key players in cancer metastasis [46, 47]. To date, over 250 RNA modifications have been identified, with m6A being the most prevalent internal modification of mRNA. Disruption in its regulation has been closely linked to carcinogenesis [48, 49]. For instance, m6A

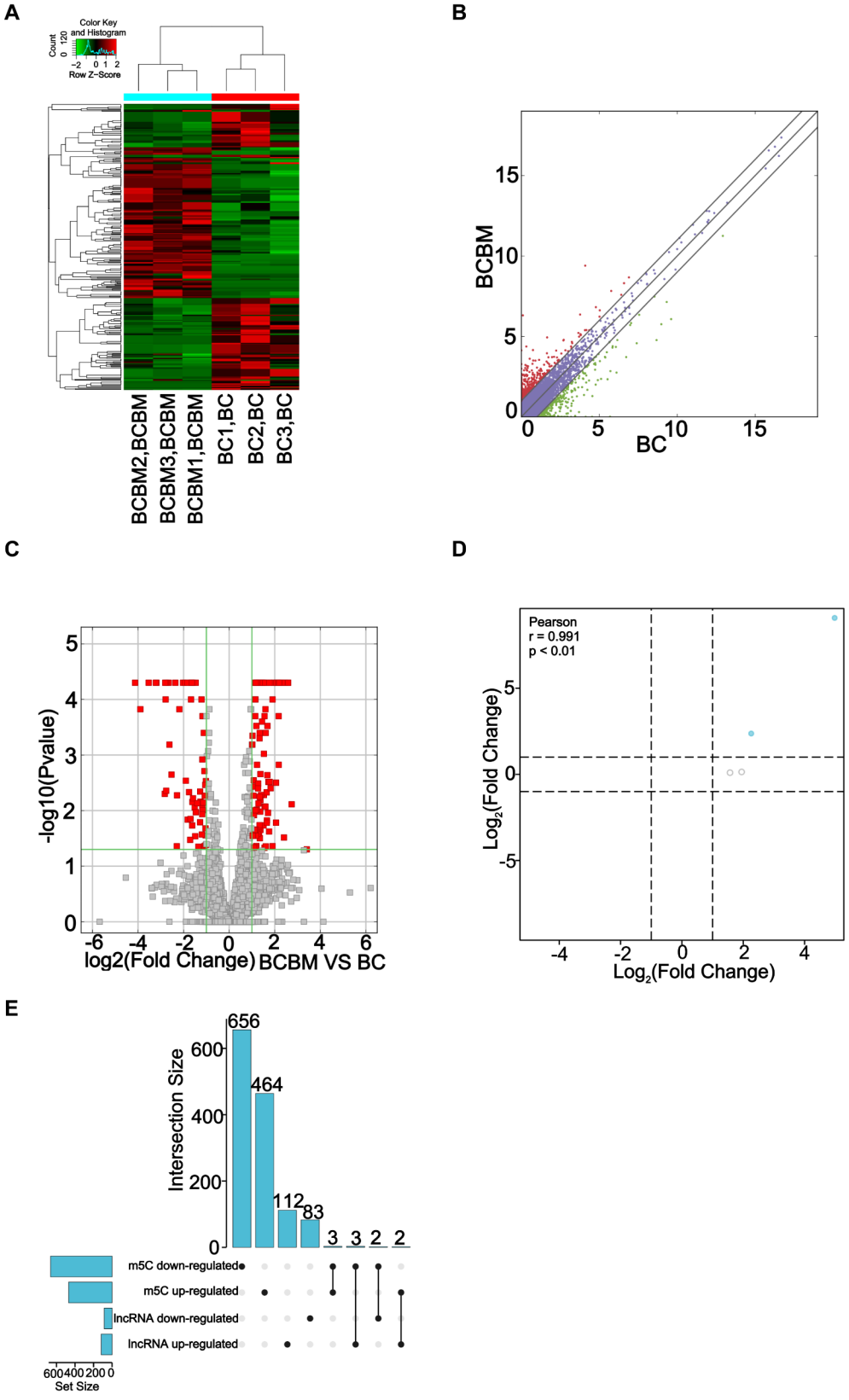

Fig. 8 Integration analysis of m5C lncRNA methylation and lncRNA transcript expression. (**A**) Cluster analysis of the lncRNA levels of BCBM and BC. (**B**) Scatter Plot analysis of differential lncRNA expression in BC and BCBM. Up-regulated lncRNA are shown in red, while down-regulated lncRNA are indicated in green. (**C**) A volcano plot showing significant differences in lncRNA expression between the BCBM and BC (Fold change > 2.0 and $P < 0.05$). (**D**) Nine-quadrant diagram for m5C methylation and lncRNA expression. (**E**) Up-set graph for different m5C methylation and lncRNA expression profiles

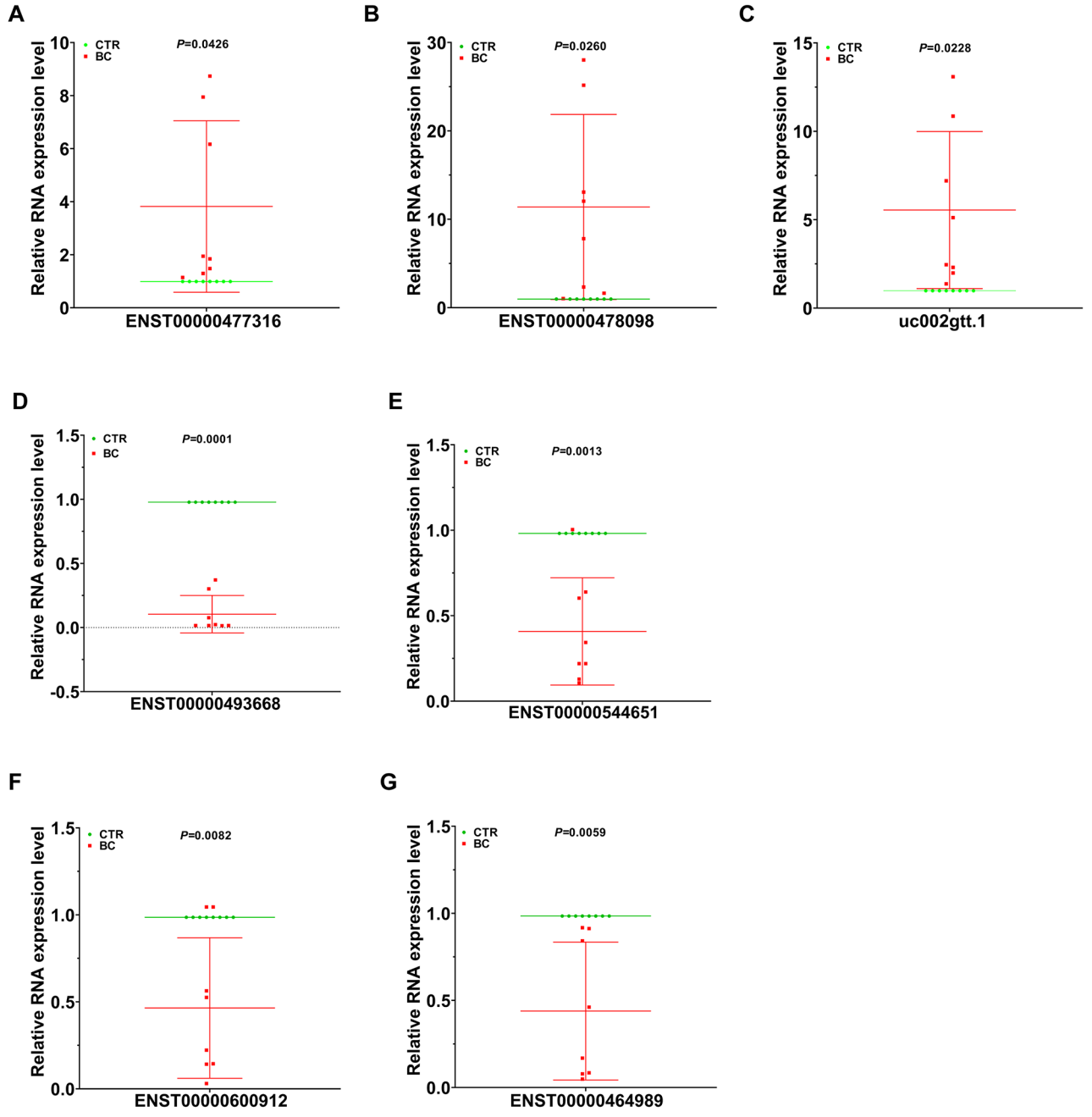

Fig. 9 Differential expression of methylation genes in BC and adjacent tissues. (**A**-**G**) Relative expression levels of 8 different methylated genes in BC and adjacent tissues by qPCR. adjacent tissues: Control (CTR) ($n = 8$; data are presented as the mean ± SD; $P < 0.05$)

reader YTHDF3 was found to enhance the translation of m6A-enriched transcripts of brain metastasis-related genes [46].

The m5C is another common RNA modification in human [9, 50]. While m6A modification has received considerable attention, the role of m5C modification in cancer, particularly in BCBM, is relatively understudied. This study aimed to address this gap by comprehensively analyzing m5C methylation profiles in both BCBM and BC cells by MeRIP-seq.

Distinct patterns of m5C methylation were observed between BCBM and BC cells, indicating a potential role for these m5C modifications in driving metastatic progression. We hypothesize that m5C modifications in BCBM lncRNAs contribute to enhanced RNA stability under conditions of oxidative stress and nutrient deprivation in the brain microenvironment. For example, m5C peaks in NEAT1, a lncRNA essential for nuclear paraspeckle formation, may prevent RNA degradation, thereby facilitating the continuation of pro-survival

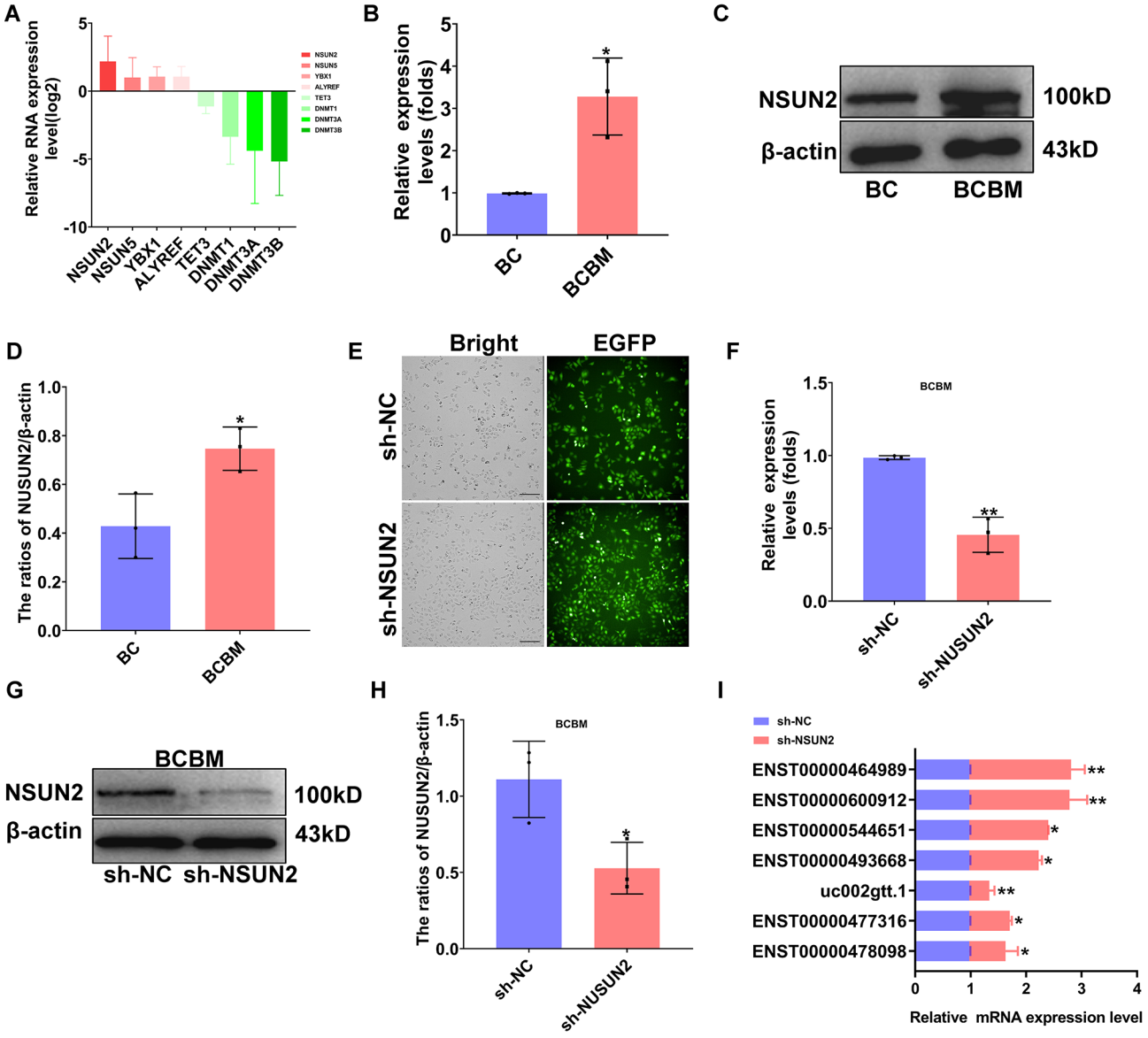

Fig. 10 Analysis of m5C RNA methylation regulators. (**A**) Analysis of the relative expression of eight RNA methylation regulators in BC and adjacent tissues by qPCR. The expression of NSUN2 in BC and BCBM was quantified by qPCR (**B**) and western-blot (**C**-**D**, Supplementary Original western blots). NSUN2 knockdown efficiency in BCBM cells measured by immunofluorescent (**E**, scale bars: 100 μm), qPCR (**F**), and Western-blot (**G**-**H**, Original blots/gels are shown in Supplementary Original western blots). (**I**) The relative expression levels of 7 different methylation genes in BCBM following NSUN2 knockdown. ($n = 3$; Data are presented as the mean ± SD; $P < 0.05$)

signaling [51]. This hypothesis aligns with previous studies that have associated m5C modifications with cellular adaptation to stress. The higher number of m5C sites in BCBM cells, particularly within lncRNAs and genes associated with m5C sites, suggests a relationship between m5C methylation and BCBM. Our sequencing analysis revealed that TGFβ1 is among the genes associated with highly methylated LncRNA. Given its role in tumor biology, we found that m5C modification could influence the cytokine profile secreted by tumor cells. This, in turn, appears to regulate the migration and invasion of BC cells, potentially promoting the development of BCBM. Analysis of m5C distribution across chromosomes revealed significant differences between BCBM and BC cells, showing wide-ranging effects of m5C in BCBM and the potential importance of spatial regulation in cancer epigenetics. The differential distribution of m5C methylation sites on chromosomes may alter the features of BCBM cells by regulating gene expression and cellular functions. m5C methylation may not only regulate gene expression by altering the spatial structure of chromosomes but also influence gene expression by modulating the interaction between RNA and chromatin. For instance, m5C reader proteins (such as YBX1)

can recognize m5C-modified RNA, thereby orchestrating its binding to chromatin to regulate gene expression and affect tumor progression [52]. The differences in motif preferences for m5C peaks between BCBM and BC cells may be attributed to the concerted action of multiple molecular mechanisms and regulatory factors. The observed motif preference for "CCAGSCUG" in BCBM may be linked to the upregulation of methyltransferases, such as NSUN2. Our validation confirmed elevated NSUN2 expression in BCBM, which preferentially targets cytosine residues within specific sequence contexts. Cluster analysis uncovered consistent methylation patterns within groups but significant differences between BCBM and BC cells. Verification of methylated lncRNAs in BCBM corroborated sequencing results.

LncRNAs are implicated in diverse biological processes and pathological processes [53]. A previous study found that lncRNAs also regulate cancer occurrence, development, and metastasis [54]. In BC mouse models, inactivating the purported metastasis-promoting lncRNA MALAT1 enhances lung metastasis. Conversely, knocking out MALAT1 in BC cells increases their metastatic potential [55]. The exosomes of BCBM cells breach the blood-brain barrier by transferring lncRNA GS1-600g8.5 [56]. The role of lncRNAs in cancer metastasis remains unclear despite their effect in m5C-mediated BCBM. Our findings revealed dysregulated expression of m5C-methylated lncRNA genes in BCBM compared to BC cells. We hypothesize that m5C modification may influence the expression of lncRNAs in BCBM by modulating their stability or function, thereby facilitating BCBM. Previous studies have demonstrated that NSUN2 promotes the upregulation of lncRNA NR_033928 in gastric cancer (GC) through its stabilization via m5C modification. NR_033928 promotes GC proliferation and inhibits apoptosis by enhancing glutaminase (GLS) expression [57]. The precise mechanisms underlying the differential expression of lncRNAs in BCBM need to be further investigated.

The differentially expressed lncRNAs may influence tumor metastasis and progression by regulating specific oncogenic pathways, such as the MAPK signaling pathway and epithelial-mesenchymal transition (EMT). For example, the lncRNA SNHG25, which is linked to the MAPK signaling pathway, promotes this pathway by activating MAP2K2 upon overexpression. This activation enhances the invasive capacity of glioma cells, thereby facilitating tumor progression [58]. LncRNA PVT1 promote tumor cell invasion and metastasis by regulating the expression of EMT-related transcription factors, such as TGF-β1 [59]. In this study, we found two significantly upregulated lncRNAs (ENST00000483869 and ENST00000564176), which were associated with MAPK3 and TGFβ1 genes, and involved in the MAPK signaling pathway and EMT process, respectively. In BCBM, the interaction between lncRNAs and m5C methylation significantly affects tumor metastasis and progression by regulating these oncogenic pathways. Through the integration of the lncRNA transcriptome profile with m5C methylation sequencing data, we identified a notable correlation between the two. Specifically, m5C methylation appears to influence the interaction between lncRNA and RNA-binding proteins, such as YBX1, by modifying the secondary structure of lncRNA. This alteration may, in turn, regulate its functional activity [60]. The interaction between lncRNA and m5C methylation may carry some therapeutic benefits in controlling BCBM. Specifically, inhibition of metastasis-promoting lncRNAs (such as DIAPH2-AS1) and simultaneously targeting m5C methyltransferases (such as NSUN2) may more effectively suppress tumor metastasis [61].

The m5C is also regulated by corresponding writers, readers, and erasers [13, 62, 63]. These regulatory factors are involved in the pathological process of BC by dynamically modulating the m5C modification level, affecting the activity, stability and function of lncRNAs [60].The analysis revealed differential expression patterns of certain m5C RNA methylation regulators in BC and adjunct tissues, supporting the relevance of m5C methylation in cancer progression. The elevated expression of NSUN2, NSUN5, YBX1, and ALYREF in BC tissues suggests their potential to regulate metastatic processes. The NSUN2 methyltransferase is a typical nucleolar protein for tRNA, mRNA, and lncRNA [64]. NSUN2 was implicated in various cellular processes such as proliferation [65], ageing, the cell cycle, differentiation of epidermal stem cells, differentiation of neural stem cells, and testicular differentiation [66, 67]. Furthermore, NSUN2, a major m5C-modifying methyltransferase (writer), plays a key role in the methylation of mRNA and lncRNA. Its upregulation has been linked to cancer development. In BC, NSUN2 enhances the stability of lncRNA through m5C modification, facilitating its binding to target genes and thereby promoting the invasion and metastasis of tumor cells [68]. NSUN2 forms functional complexes with m5C-recognizing proteins, such as YBX1, to regulate the expression of target genes. This collaboration further enhances the proliferation and metastatic ability of BC cells [69]. The up- or down-regulation of the different types of regulators and their corresponding changes in NSUN2-knockdown BCBM cells implicates their potential role in driving metastatic processes. Previous studies have shown that differentially expressed genes (DEGs) are significantly enriched in extracellular matrix (ECM)-receptor interaction, TGF-β, and PI3K-Akt signaling pathways after NSUN2 knockout. For instance, the down-regulation of genes such as TGFB1, THBS1 and LOXL2 results in the suppression of the ECM

remodeling capacity, thereby inhibiting tumor invasion [70]. Combined with our research results, we infer that NSUN2 deficiency induces significant dysregulation of multiple metastasis-related genes to suppress tumor cell growth. Using m5C sequencing analysis, we identified two oncogenic lncRNAs in BC, ENST00000461842 and ENST00000564176, which are highly expressed in tumor tissues. Their expression levels are significantly negatively correlated with patient prognosis, suggesting their potential markers for BC metastasis.

The m5C modification contributes to the RNA nucleation, cell differentiation processes [71], regulating stem cell function and stress [72]. Functional enrichment analysis identified the diverse cellular functions and pathways modulating m5C methylation. The enrichment of hypermethylated genes in cancer-related pathways, such as the MAPK and EMT signaling pathway, underscores the potential oncogenic role of m5C methylation in BCBM. Conversely, the association of hypo-methylated genes with pathways such as the Hippo signaling pathway suggests a regulatory role in tumor suppressor pathways. This indicates that the coordinated regulation of key pathways by methylation balance plays a significant role in tumor metastasis. However, the underlying mechanism requires further study.

As an important mechanism of the epitranscriptome, the m5C methylation modification of RNA plays a key role in the progression of breast cancer, particularly during the metastatic stage, offering a new direction for targeted therapy. Studies have shown that NSUN2, the main m5C methyltransferase, is abnormally overexpressed in BC. It enhances the mRNA stability of the target gene HGH1 (FAM203) through m5C modification, thereby promoting tumor cell proliferation, migration, and invasion [58]. Targeting the NSUN2/YBX1 axis, developing m5C-related biomarkers, and integrating stratified treatment strategies offer new perspectives for epigenetic therapy. Small molecule inhibitors that disrupt this complex may inhibit BC metastasis. Furthermore, repurposing drugs and administering various vitamins, such as vitamin D, as prophylactic agents with modulatory effects could have a positive impact on BC [73]. The dysregulation of lncRNA expression observed in BCBM cells underscores the importance of lncRNAs in cancer progression. The dysregulated lncRNAs are potential biomarkers for monitoring metastatic progression and developing drug targets for BCBM.

Conclusion

This study highlights the significance of m5C methylation in lncRNAs within the context of BCBM. It emphasizes the necessity for further mechanistic investigations to elucidate the functional implications of its dysregulation in cancer metastasis. These findings lay the foundation for developing targeted therapies for preventing BC metastasis and progression.

Abbreviations

BC	Breast Cancer
BCBM	Breast Cancer Brain Metastases
m5C	5-Methylcytosine
LncRNA	Long Non-coding RNA
MeRIP-seq	Methylated RNA Immunoprecipitation Next-generation Sequencing
RT-PCR	Quantitative Real-time PCR
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
BP	Biological Processes
CC	Cell Components
MF	Molecular Functions
ANKMY1	Ankyrin Repeat and MYND Domain Containing 1
KCNAB2	Potassium Voltage-gated Channel, Shaker-related Subfamily, Beta Member 2
HECW1	HECT, C2 and WW Domain Containing E3 Ubiquitin Protein Ligase 1
SMARCD3	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamilyd, Member 3
CERS4	Ceramide Synthase 4
LLGL2	LLGL Scribble Cell Polarity Complex Component 2
SHROOM2	Shroom Family Member 2
ACACB	Acetyl-CoA Carboxylase Beta
SPEG	Striated Muscle Enriched Protein Kinase
NSUN2	NOP2/Sun RNA methyltransferase 2
NSUN5	NOP2/Sun RNA methyltransferase 5
YBX1	Y-Box Binding Protein 1
ALYREF	Aly/REF Export Factor
DNMT3B	DNA Methyltransferase 3 Aeta
DNMT3A	DNA Methyltransferase 3 Alpha
DNMT1	DNA Methyltransferase 1
TET3	Tet Methylcytosine Dioxygenase 3
NSUN3	NOP2/Sun RNA methyltransferase 3
NSUN4	NOP2/Sun RNA methyltransferase 4
NSUN5	NOP2/Sun RNA methyltransferase 5
NSUN6	NOP2/Sun RNA methyltransferase 6
TET2	Tet Methylcytosine Dioxygenase 2

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

S W, B F, J.R.G: Conceptualization, Formal analysis, Writingoriginal draft. D Y, X.M.X, M L, Y.F.Z: Data Collection, Methodology, Software. G.H.G, Y.J.L, L P, S C, X.H.Z: Formal analysis and interpretation. S W, M A, B F: Conceptualization, Funding acquisition, Project administration. S W, A.Q.Z, B F: Writing - review & editing, Resources. All the authors agreed with the final version of the manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in the NCBI GEO Archive with the submission ID GSE246721.

Declarations

Ethics approval and consent to participate
All human samples studies have been approved by the Ethics Committee of Liaocheng People's Hospital (2022261), and all participants have signed informed consent forms.

Consent to for publication
All authors have read, approved, and agreed to publish this manuscript.

Competing interests
The authors declare no competing interests.

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