RESEARCH ARTICLE

METTL14 and miR-1247 are associated with poor outcomes in triple-negative breast cancer

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ABSTRACT

BACKGROUND: Triple-Negative Breast Cancer (TNBC) does not have targets for therapy, and accounts for 15% of all Breast Cancers (BCs). N6-methyladenosine (m6A) modification has been reported to play important role in the progression of various cancers. However, the expression and function of the m6A methyltransferase METTL14 in TNBC are unclear.

MATERIALS AND METHODS: The microRNA (miRNA) and mRNA count data of BC patients providing both tumor tissues and matched normal tissues were downloaded from The Cancer Genome Atlas (TCGA) data portal. The expression of METTL14 was determined by immunohistochemistry and western blotting. The expression of hsa-miR-1247 was determined by qRT-PCR. The function of METTL14 was analyzed by colony assays. Cox regression analysis was applied to assess the expression levels of METTL14 and Differentially Expressed microRNAs (DEmiRNAs). Kaplan-Meier survival analysis was performed to establish the correlation between expression levels and BC patient survival.

DISCUSSION: RNA m6A modification has been suggested as another pattern of epigenetic regulation, similar to histone and DNA methylation. Some methyltransferases and demethylases mediate this dynamic modification. METTL3, METTL14, FTO, NSun2, YTHDF2, and ALKBH5 have previously been identified as abnormally methylated molecules in different types of cancers.

RESULTS: We discovered that METTL14 is significantly downregulated in TNBC tissues and that low expression of METTL14 is correlated with worse differentiation, higher proliferation and poorer survival, suggesting its potential as an independent prognostic biomarker for TNBC. We also found a positive correlation between the expression levels of METTL14 and hsa-miR-1247 in TNBC tissues and cell lines. Moreover, hsa-miR-1247 was significantly downregulated in TNBC tissues and this downregulation was related to poorer survival.

CONCLUSION: Our data suggest that METTL14 and miR-1247 could be valuable diagnostic tools, prognostic biomarkers, and therapeutic targets for TNBC.

Key Words: *m6A; METTL14; miR-1247; Breast cancer; Methyltransferase; TCGA*

INTRODUCTION

Breast Cancer (BC) is a heterogeneous disease. Despite this, clinical treatment is based on just three biomarkers: Human Epidermal Growth Factor Receptor (HER2), Progesterone Receptor (PR), and Estrogen Receptor (ER) [1]. Triple-Negative Breast Cancer (TNBC) does not express any of these three biomarkers and accounts for 15% of all BCs. Due to the lack of these receptors as targets for therapy; the main treatment for patients with TNBC is chemotherapy [2]. However, TNBC has a high recurrence rate and is likely to progress greater disease progression after treatment [3]. The high proliferation, high histological grade, and metaplastic characteristics of TNBC lead to poor outcomes [4]; thus, research on the molecular mechanisms behind TNBC progression is urgently needed.

N6-methyladenosine (m6A) is the most common reversible modification of human mRNA, rRNA, tRNA, microRNA (miRNA), and long noncoding (lncRNA) [5-7]. Studies have suggested that m6A has an effect on cell meiosis and differentiation [8, 9] that is related to its role in cancer development. Two types of key catalytic proteins are involved in m6A modification: the first type includes METTL3, METTL14, and WTAP, which form a critical methyltransferase complex that assembles multiple methyl groups onto RNA; the second type includes FTO and ALKBH5, which can reverse methylation as they are demethylases [10]. m6A modification is dynamically regulated by the interaction of m6A methyltransferases and demethylases. METTL14 has been reported to inhibit hematopoietic/progenitor cell differentiation, thus promoting leukemogenesis [11]. Ma et al. discovered that METTL14 promotes hepatocellular carcinoma metastasis via the repression of miRNA-126 expression, which implies that METTL14 contributes to cancer progression via different miRNAs [12]. However, the function of METTL14 in BC remains largely unknown.

miRNAs, small RNAs with a length of approximately 20-24 nucleotides, exist in a variety of mammalian organs and have been found to play an important role in regulating gene expression at the posttranscriptional level. Studies have shown that miRNAs are involved in tumor progression and that they can function as oncogenes and tumor suppressor genes [13, 14]. In the present study, we primarily used The Cancer Genome Atlas (TCGA) database to explore the expression of METTL14 in patients with different ER, PR, and HER2 statuses and analyzed the associations between METTL14 expression level and prognosis and pathological parameters as well as the function of METTL14 in TNBC cell line proliferation. We also identified some miRNAs that are highly correlated with METTL14, which is related to a poor prognosis in BC patients. Furthermore, we confirmed the expression of miRNAs using qRT-PCR. Our findings will be helpful for understanding the mechanism underlying TNBC progression.

MATERIALS AND METHODS

Patients and samples

This study was approved by the Ethics Committee of the Maternal and Children Health Hospital of Hubei Province. The raw RNA-seq reads of breast carcinoma tissues and matched normal tissues and the corresponding clinical information of 96 patients were downloaded from the TCGA database. In addition, we collected fresh BC tissues and paracancerous tissues from patients without other malignancies who did not receive preoperative radiotherapy or chemotherapy. We obtained informed consents from all patients. Tissues were immediately frozen in liquid nitrogen after surgical resection and stored until protein and RNA extraction.

Acquisition and analysis of miRNA and mRNA expression profiles

The miRNA and mRNA count data of BC patients providing both tumor tissues and matched normal tissues were downloaded from the TCGA data portal. The miRNA expression profiles were generated using an Illumina HiSeq 2000 miRNA sequencing platform, and the mRNA expression profiles were produced using an Illumina HiSeq 2000 RNA sequencing platform. We used fragments per kilobase of transcript per million mapped reads (FPKM) [15, 16] as a means of quantitatively expressing transcriptome data. After the deletion of data without expression, METTL14 expression was compared between the tumor tissues and normal tissues using FPKM. The miRNA-seq data were analyzed by 'edgeR', which is a Bioconductor package based on the R programming language used for differential expression analysis; miRNAs with a False Discovery Rate (FDR) <0.01, log2 fold change >2, and $p<0.01$ were defined as differentially expressed miRNAs (DEmiRNAs) and were used for further analysis.

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Immunohistochemistry (IHC)

We collected 20 benign breast tissue specimens and 82 BC specimens in the Pathology of the Maternal and Children Health Hospital of Hubei Province from 2016 to 2019. For METTL14 (1:1000 dilution; Sigma HPA038002-100UL) and Ki67 (ready-to-use; Gene Tech) detection, heat-mediated antigen retrieval was carried out with 10 mM Tris base, 1 mM EDTA and 0.05% Tween 20 at a pH of 9. The slides were processed with the Envision/HRP kit and a 3,3'-diaminobenzidine (DAB) substrate kit (DAKO). The slides were counterstained with hematoxylin and dehydrated using graded alcohols and xylene. All tissues were scored by two experienced pathologists in a blinded manner based on staining intensity. At least five representative fields of each slide were counted using a 20× objective. The level of METTL14 immunofluorescent staining was classified according to the following scale: 0, no staining; 1, weakly positive; 2, positive; and 3, strongly positive.

Western blot analysis

Total protein was extracted using RIPA lysis buffer (Sigma), and the protein concentration was measured using a Bicinchoninic Acid (BCA) detection kit (Pierce) according to the manufacturer's instructions. Protein samples were separated on 10% polyacrylamide gels and were then transferred onto a nitrocellulose membrane (Hybond). The membranes were blocked with 5% nonfat milk for 1 h at room temperature and were then incubated with anti-METTL14 antibodies (1:300 dilution; Sigma HPA038002-100UL) and actin (1:1000; Proteintech) at 4°C overnight. After incubation with IgG fluorescently labeled goat anti-rabbit IRDyeTM secondary antibodies (1:5000), band signals were detected using the Odyssey system (Bio-Rad Life Sciences).

qRT-PCR

Total RNA was extracted from four pairs of TNBC tissue specimens and matched normal tissue specimens with TRIzol reagent (Invitrogen) for qRT-PCR validation. After RNA isolation, M-MLV reverse transcriptase (Invitrogen, USA) was used to synthesize cDNA. Subsequently, we prepared the qRT-PCR system with a total volume of 10 μl containing 0.5 μl forward PCR primer (10 μM), 0.5 μl reverse PCR primer (10 μM), 1 μl cDNA, 5 μl 2× Master Mix, and 3 μl double-distilled water. The reaction conditions were 95°C for 10 min and then 95°C (15 sec) and 60°C (60 sec) for a total of 40 cycles. U6 was used as an endogenous control for quantification of pri-miRNAs and miRNAs. Relative expression levels were calculated using the ΔΔCt method. The hsa-miR-1247 primer was purchased from Tiangen Biochemical Technology Co., Ltd. (Beijing, China) without disclosure of the proprietary primer sequences. The following U6 primers were synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China):

U6: F, 5'-GATGACACGCAAATTCGTGAA-3'

R, 5'-GCTGTCAACGATACGCTACG-3'

Cell culture and transfection

MDA-MB-468 cells were purchased from Procell Life Science & Technology Co., Ltd. and continuously maintained in McCoy's 5A media (Gibco, 16600108) supplemented with 10% Fetal Bovine Serum (FBS) and 1% 100× penicillin/streptomycin (pen/strep) (Gibco, 15, 140) at 37°C in a humidified atmosphere containing 5% CO_2. After culture for 24 h at 70% density, the cell transfection assay was carried out according to the manufacturer's instructions for the METTL14 siRNA (siMETTL14) and siRNA negative control (RiboBio).

Colony formation assays

MDA-MB-468 cells (1000) transfected with siMETTL14 were seeded in 6-well ultralow-attachment plates. The growth medium was replaced every 3 days. The former colonies were photographed for size assessment at 14 days posttreatment. Colonies withy diameters >50 μm were counted in three different wells.

Statistical analysis and graphs

All data are presented as the mean ± SEM. Statistical analyses were performed using Student's t-tests for two group comparisons and one-way ANOVA for multiple comparisons. Pearson correlation coefficients (r) were calculated to assess correlations, and statistical significance was assessed by two-tailed t-tests of r=0. Statistical analyses of METTL14 immunofluorescence staining scores in benign breast tissues and BC tissues were performed using $\chi 2$ tests. GraphPad Prism 5.0 was used to create graphs. Data were analyzed using SPSS 16.0 software, and $p<0.05$ was considered to indicate statistical significance.

RESULTS

METTL14 is downregulated in BC tissues, especially TNBC tissues, and serves as a prognostic factor

TCGA database analysis suggested that METTL14 was downregulated in 96 BC tissues compared to their matched normal tissues (Figure 1). We further investigated the expression patterns of METTL14 in patients with different ER, PR, and HER2 statuses. The results showed that METTL14 was significantly down regulated in TNBC patients compared to ER+/PR+/HER2+ and ER+/PR+/HER2- patients. Differential expression of METTL14 between ER+/PR+/HER2+ and ER+/PR+/HER2- patients and ER-/PR-/HER2+ patients was not statistically significant (Figure 1). Consistently, the IHC and western blotting results revealed a significant decrease in METTL14 expression in TNBC tumor tissues at the protein level (Figure 1). To analyze the correlations between METTL14 expression and clinicopathological characteristics, we divided BC tissues into high and low METTL14 groups based on the median expression level. The results suggested that low METTL14 expression was associated with differentiation and Ki67 proliferation (Table 1). Kaplan-Meier analysis showed that BC patients with low levels of METTL14 had poorer Recurrence-Free Survival (RFS) (Figure 1).

Differentially expressed microRNAs (DEmiRNAs) and their correlations with the expression levels of METTL14 in TNBC patients

We downloaded the count data of miRNAs and then analyzed the differentially expressed miRNAs between TNBC tissues and matched

TABLE 1

Correlations between METTL14 expression levels and the clinicopathological features of BC patients

Parameters	N (cases)	METTL14 expression		p value
		Low (n=35)	High(n=34)	
Age				
≤ 50 years	34	16	18	0.633
>50 years	35	19	16	
Differentiation				
Grade 1	2	1	1	0.011*
Grade 2	33	11	22	
Grade 3	34	23	11	
Size of tumor (cm)				
≤ 3	51	24	27	0.413
>3	18	11	7	
Lymph node mestasis				
No	34	16	18	0.633
Yes	35	19	16	
Ki67				
≤ 20%	16	4	12	0.024*
20%	53	31	22	

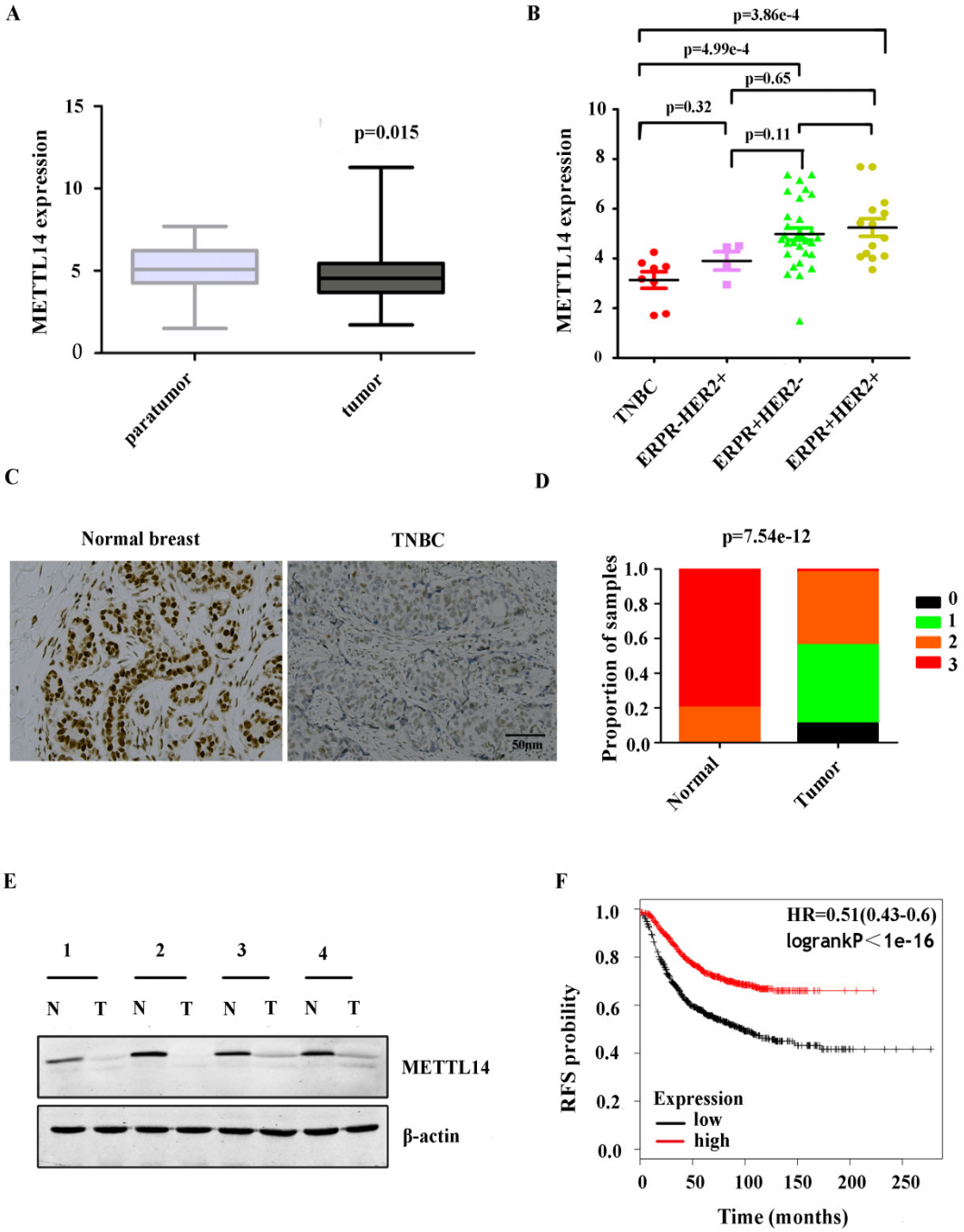


Figure 1) *METTL14 is downregulated in BC tissues, especially in TNBC tissues, and serves as a prognostic factor (A) A comparison of METTL14 expression between breast tumor tissues and adjacent normal tissues (n=96) (B) breast tumor tissues with different ER, PR, and HER2 statuses, including ER-/PR-/HER2- (n=8), ER-/PR-/HER2+ (n=4), ER+/PR+/HER2- (n=32), and ER+/PR+/HER2+ (n=14). Statistical significance was determined using t-tests and one-way ANOVA (C) IHC staining of breast tissues for METTL14 (D) Quantification of IHC staining in normal breast tissues (n=21) and TNBC tissues (n=47) (E) Western blots for METTL14 in TNBC patients (n=4) (F) Kaplan-Meier survival curves of RFS based on METTL14 expression in BC created using the online bioinformatics tool Kaplan-Meier plotter*

normal tissues using the cutoff criteria of FDR<0.01, log2-fold change >2, and p<0.01. There were 32 downregulated miRNAs and 46 upregulated miRNAs (Figure 2). We primarily focused on the downregulated miRNAs, and regression analyses were executed to assess the correlations between for the expression levels of METTL14 and DEmiRNAs. Positive correlations were found between the expression levels of METTL14 and those of the following miRNAs: hsa-miR-495, hsa-miR-432, hsa-miR-5683, hsa-miR-1247, and hsa-miR-10b (Figure 2).

Prognostic analysis of representative downregulated miRNAs and validation of the expression of miRNAs in TNBC tissues

Kaplan-Meier analysis showed that BC patients with low levels of hsa-miR-1247 had poorer OS and that there were no differences in the expression levels of hsa-miR-43, hsa-miR-495, hsa-miR-5683, and hsa-miR-10b (Figure 3). Previous TCGA analysis has already shown that low levels of hsa-miR-1247 lead to a poor prognosis in BC patients, so we validated its expression in four pairs of TNBC tissues and matched normal tissues using RT-PCR. RT-PCR assays suggested that hsa-miR-1247 was downregulated in TNBC tissues compared to normal tissues (Figure 3).

METTL14 modulates miR-1247 expression and drives the malignant proliferation of TNBC cells

Among the downregulated miRNAs, miR-1247 was positively correlated with METTL14, and low levels of hsa-miR-1247 were related to poorer OS. We assumed that reduced METTL14 expression in TNBC might contribute to the downregulation of miR-1247. To verify this, we established MDA-MB-468 cells with stable knockdown of METTL14 by transfection with METTL14 small interfering RNA (siRNA) (Figure 4). As expected, miR-1247

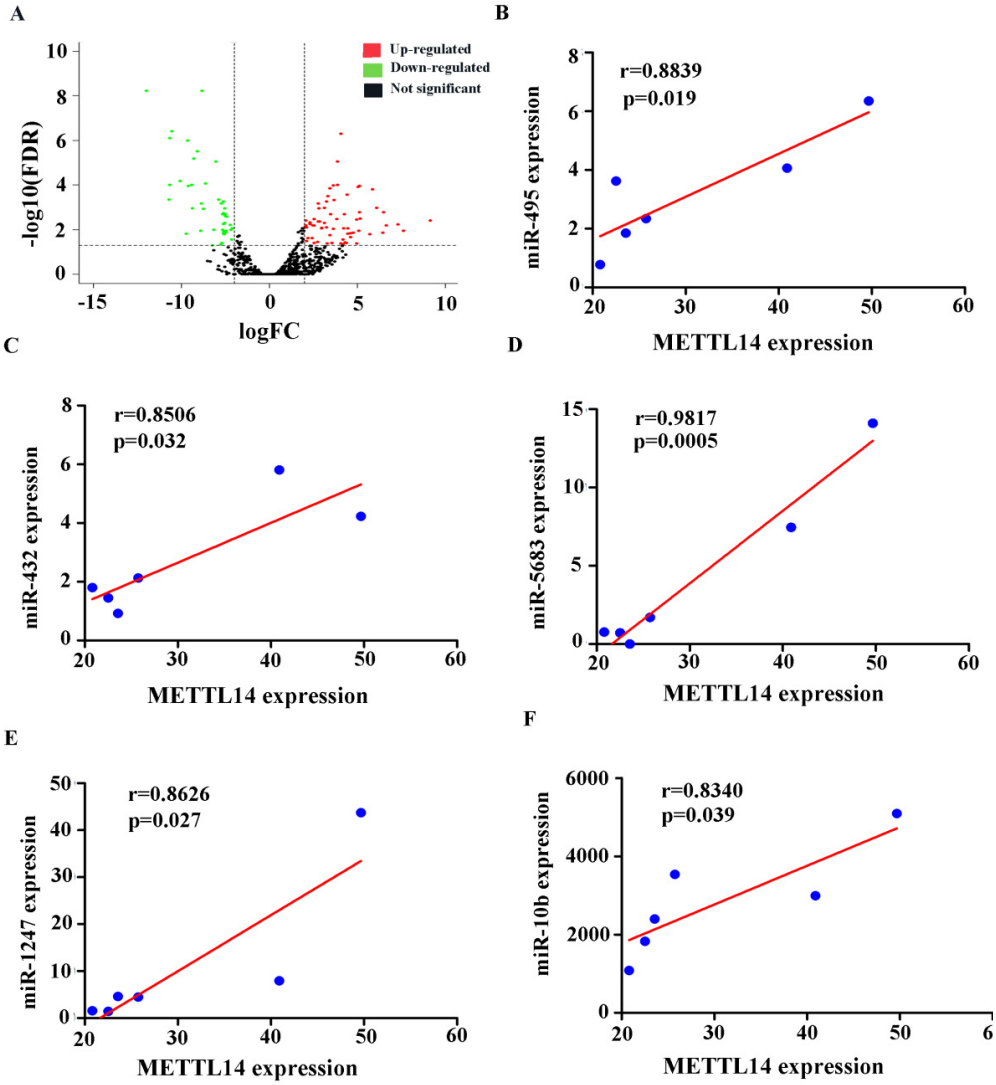
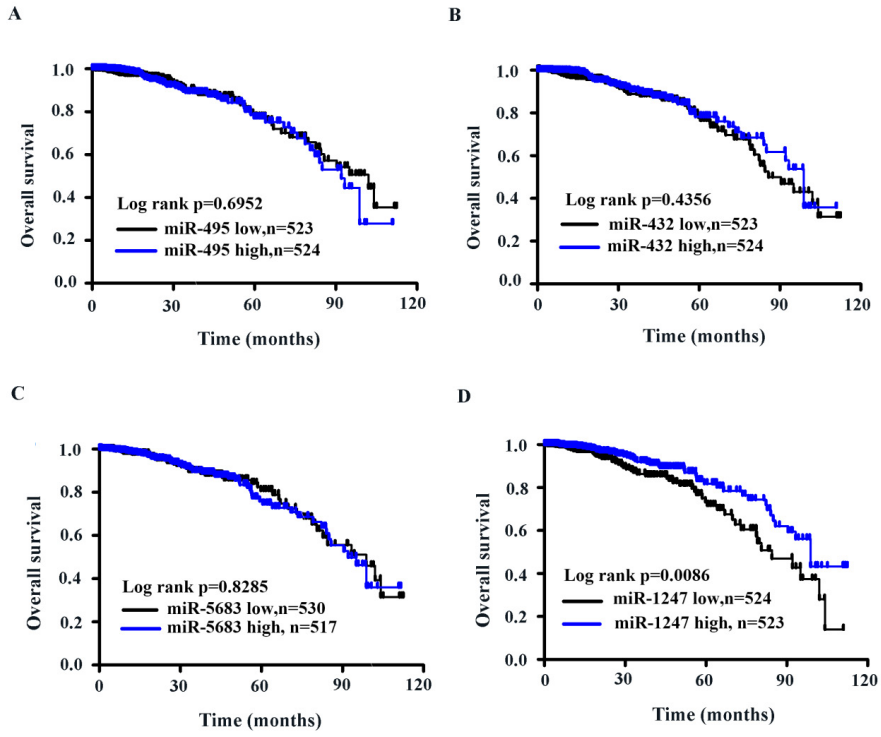


Figure 2) *DEmiRNAs and their correlations with the expression levels of METTL14 in TNBC patients (A) Volcano plot of DEmiRNAs in TNBC tissues (B) METTL14 vs miR-495 (C) METTL14 vs miR-432 (D) METTL14 vs miR-5683 (E) METTL14 vs miR-1247 and (F) METTL14 vs miR-10bM (positive correlations between the expression levels of METTL14 and the following downregulated miRNAs)*



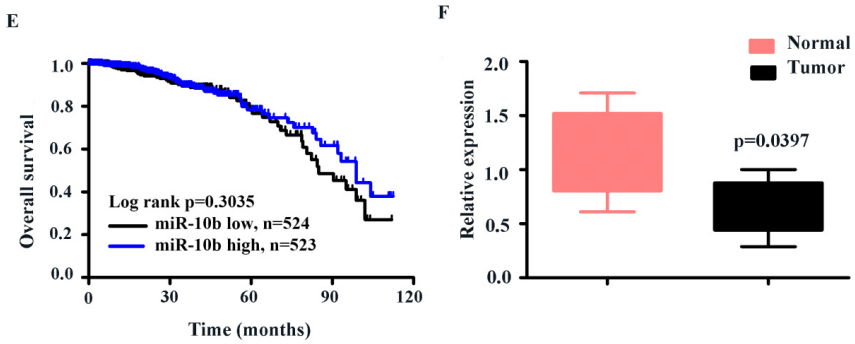


Figure 3) *Prognostic analysis of representative downregulated miRNAs and validation of the expression of miRNAs in TNBC tissues (A) hsa-miR-495 (B) hsa-miR-432 (C) hsa-miR-5683 (D) hsa-miR-1247 (E) hsa-miR-10b (F) The relative expression of hsa-miR-1247 (overall survival of the representative downregulated miRNAs)*

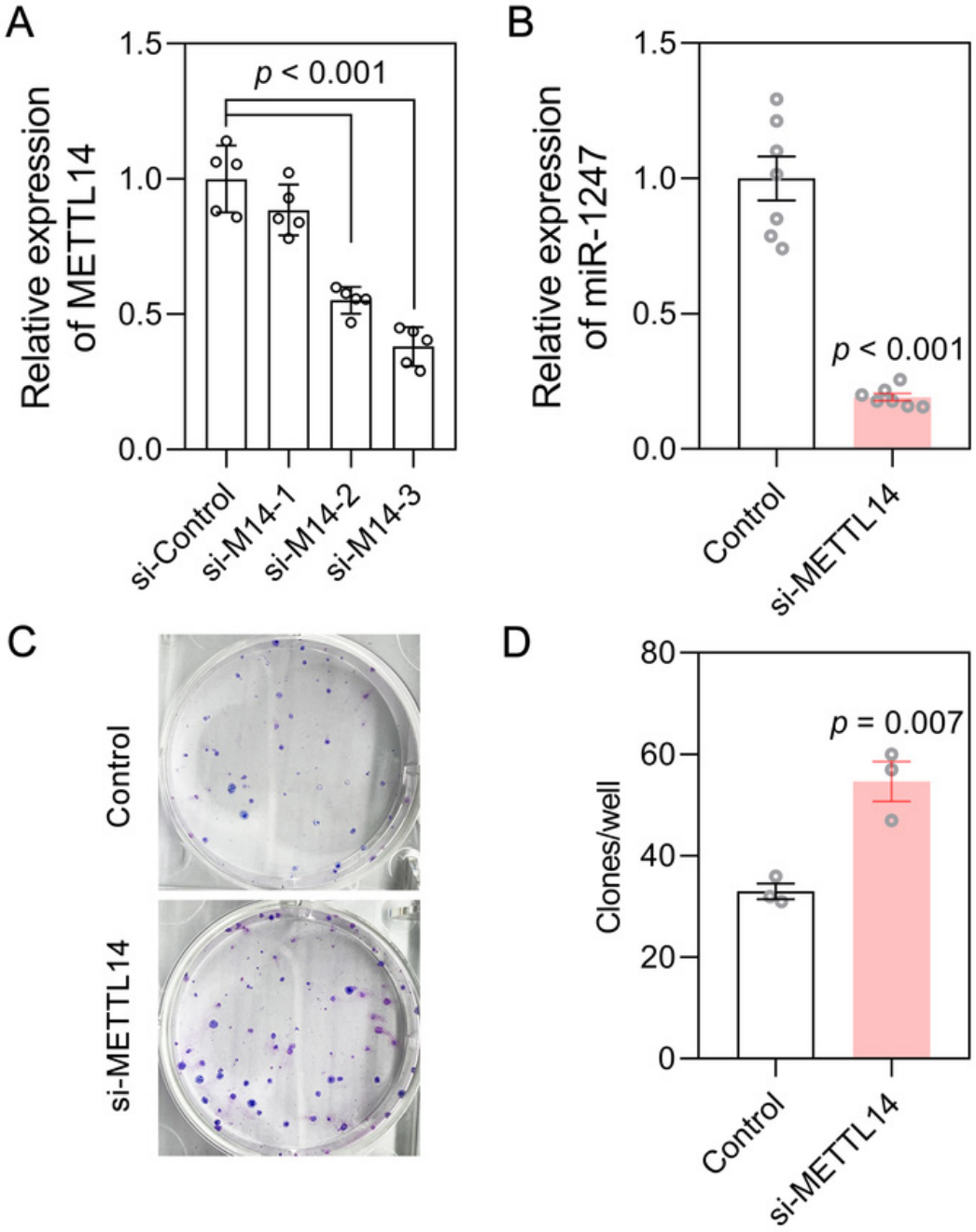


Figure 4) *METTL14 modulates miR-1247 expression and drives the malignant proliferation of TNBC cells (A) METTL14 knockdown in MDA-MB-468 cells (B) miR-1247 was quantified by qRT-PCR upon METTL14 depletion in MDA-MB-468 cells (C) Figure shows the effect of siMETTL14 on the colony formation ability of MDA-MB-468 cells was detected by colony assays (D) Graph shows the effect of siMETTL14 on the colony formation ability of MDA-MB-468 cells was detected by colony assays*

expression was remarkably decreased in METTL14-depleted cells (Figure 4). Importantly, METTL14 knockdown significantly promoted MDA-MB-468 colony formation (Figure 4).

DISCUSSION

RNA m6A modification has been suggested as another pattern of epigenetic regulation, similar to histone and DNA methylation. Some methyltransferases and demethylases mediate this dynamic modification. METTL3, METTL14, FTO, NSun2, YTHDF2, and ALKBH5 have previously been identified as abnormally methylated molecules in different types of cancers [17]. In addition to regulating mRNA expression, m6A methylation also affects the processing of pri-miRNAs and regulates the generation of mature miRNAs. Ma et al discovered that METTL14 promotes hepatocellular carcinoma metastasis by repressing miRNA-126 expression, and another study found that METTL3 promotes the progression of BC by inhibiting the tumor suppressor let-7g [18]. However, few studies have directly focused on METTL14 and its effect on miRNA expression in BC. Analyses of METTL14 expression status in the TCGA database and BC tissues and analysis of the role of METTL14 in TNBC cell line proliferation indicated that METTL14 is significantly correlated with TNBC, revealing that METTL14 can serve as a biomarker for further classification of TNBC and as a prognostic factor for tumor recurrence in BC.

Genome-wide predictions suggest that miRNAs regulate more than 60% of protein-coding genes. The dysregulation of specific miRNAs could be associated with different cancers, including TNBC [19]. Our data showed that the expression of five miRNAs was positively correlated with that of METTL14 in TNBC, indicating that METTL14 may influence miRNA expression through RNA methylation modification. Next, we focused on the prognostic significance of miRNAs that were positively correlated with METTL14. Kaplan-Meier survival curves suggested that miR-1247 downregulation alone is significantly associated with patient overall survival. As a member of the miR-1247 family, miR-1247-5p has been proven to play a crucial role in tumor progression. Its expression has been reported to decrease in human cancers. Some studies have shown that miR-1247-5p, a novel tumor suppressor, can act as a potential biomarker and therapeutic agent for a variety of cancers [20-22]. It has been reported that downregulation of miR-1247-5p is associated with a poor prognosis and that this downregulation facilitates tumor cell growth via DVL1/Wnt/ β-catenin signaling in BC [23]. Our data showed that miR-1247 expression is significantly decreased in TNBC tissues compared to normal breast tissues, suggesting that miR-1247 can serve as a potential biomarker for TNBC.

CONCLUSION

Our results provide novel insights into the mechanisms underlying the pathogenesis of TNBC. Our data may serve as a foundation for further functional research into m6A in TNBC and suggest that METTL14 and miR-1247 could be valuable diagnostic tools, prognostic biomarkers, and therapeutic targets for TNB.

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