METTL14 and miR-1247 are associated with poor outcomes in Triple-Negative Breast Cancer

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Research article

Keywords: METTL14, m6A, has-miR-1247, TNBC, poorer survival

Posted Date: January 21st, 2020

DOI: <https://doi.org/10.21203/rs.2.21437/v1>

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Abstract

Background

Triple-negative breast cancer (TNBC) doesn't have targets for therapy, and accounts for 15% of all breast cancers. 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.

Methods

The count data of miRNA and mRNA of breast cancer patients with both tumor tissues and matched normal tissues were downloaded from the TCGA data portal. The expression of METTL14 was determined by immunohistochemistry and western blot. The expression of has-miR-1247 was determined by qRT-PCR. The Cox regression analysis was executed for the expression levels of METTL14 and DE miRNAs. The Kaplan-Meier survival analysis were performed to establish the correlation between the expression level and survival of BC patients.

Results

We discovered that METTL14 was significantly downregulated in TNBC tissues, and low expression of METTL14 is correlated with worse differentiation, higher ki67 proliferation and poorer survival suggesting its potential as an independent prognostic biomarker for TNBC. We also found the positive correlation between the expression levels of METTL14 and has-miR-1247. Moreover, has-miR-1247 was significantly downregulated in TNBC tissues, and lead to poorer survival.

Conclusion

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

Introduction

Breast cancer is a heterogeneous disease. Despite this, clinical treatments are 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 breast cancers. Due to the lack of these receptors as targets for therapy, the main treatment for patients with triple-negative breast cancer is chemotherapy[2]. However, women with TNBC have a higher recurrence rate and greater disease progression after treatment[3]. The high proliferation, high histological grade, and metaplastic characteristics of TNBC lead to poor outcomes[4], making the discovery of the molecular mechanisms behind TNBC progression an urgent research requirement.

m⁶A is the most common reversible modification of human mRNA, rRNA, tRNA, microRNA, and lncRNA[5-7]. Studies have suggested that m⁶A has an effect on cell meiosis and differentiation[8, 9], which is related to its role in cancer development. Two types of key catalytic proteins are involved in m⁶A modification: METTL3, METTL14, and WTAP, which form a critical methyltransferase complex that assembles multiple methyl groups onto RNA, and FTO and ALKBH5, which can reverse the methylation as they are demethylases[10]. m⁶A modification is dynamically regulated by the interaction of m⁶A methyltransferase and demethylase. METTL14 has been reported to inhibit hematopoietic/progenitor differentiation, thus promoting leukemogenesis[11]. Ma et al (2017) discovered that METTL14 promotes hepatocellular carcinoma metastasis via repression of microRNA-126 expression, which implies that METTL14 contributes to cancer progression via different microRNAs[12]. However, the function of METTL14 in breast cancer remains unknown.

MicroRNAs (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 post-transcriptional level. Studies have shown that miRNAs are involved in tumor progression and that they function as oncogenes and tumor suppressor genes[13, 14]. In the present study, we primarily used the TCGA database to explore the expression of METTL14 in different ER, PR, and HER2 status patients, and analyzed the association between its different expression levels and prognosis and pathological parameters. We also identified some miRNAs that are highly correlated with METTL14, which lead to poor BC prognosis. Furthermore, we confirmed the expression of miRNAs using qRT-PCR. Our findings will be helpful for understanding the mechanism behind 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. Raw RNA-seq reads of breast carcinoma and matched normal tissues, along with the corresponding clinical information, of 96 patients were download from the TCGA database (<https://cancergenome.nih.gov/publications/publicationguidelines>). In addition, we collected fresh breast cancer tissues and para-cancerous tissues from patients without other malignancies and not receiving preoperative radiotherapy or chemotherapy. We obtained informed consents from all patients. Tissues were immediately frozen in liquid nitrogen after surgical resection and were stored until protein and RNA extraction.

Acquisition and analysis of miRNA and mRNA expression profiles

The count data of miRNA and mRNA of breast cancer patients with 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 FPKM[15, 16] as a

means of quantitatively expressing transcriptome data. After the deletion of data without expressions, METTL14 expressions were compared between the tumor tissues and normal tissues using FPKM. The miRNAseq data was analyzed by 'edgeR', which is a Bioconductor package based on the R programming language used for differential expression analysis of RNAseq data. miRNAs with FDR <0.01, log² fold change >2, and p <0.01 were defined as differentially expressed genes and were used for further analysis.

Immunohistochemistry

We collected 20 benign breast tissues and 82 breast cancer 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), ki67 (ready-to-use; Gene Tech), heat-mediated antigen retrieval was carried out with 10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, and a pH of 9. Slides were processed with a Envision/HRP kit and with a DAB substrate kit (DAKO). Slides were counterstained with hematoxylin and were 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, low positive; 2, positive; 3, high positive.

Western blot analysis

Total proteins were extracted using RIPA lysis buffer (Sigma), and their concentrations were measured using a BCA detecting kit (Pierce), according to the manufacturer's instructions. Protein samples were separated by 10% polyacrylamide gels and were then transferred onto a nitrocellulose membrane (Hybond). Membranes were blocked with 5% nonfat milk for 1 hour 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 fluorescent-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 from four pairs of TNBC tissue specimens and matched normal tissues were extracted 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 reaction system with a total volume of 10 µl containing: 0.5 µl PCR Forward Primer (10 µM), 0.5 µl PCR Reverse 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, then 95°C (15 sec), 60°C (60 sec), for a total 40 cycles. U6 was used as an endogenous control for quantification of pri-miRNAs and miRNAs. Relative expression levels were calculated using the $\Delta\Delta C_t$ method. The primer of has-miR-1247 was purchased from Tiangen Biochemical Technology CO., Ltd. (Beijing, China) without disclosure of the proprietary primer sequences. U6 was synthesized from Tsingke Biotechnology Co., Ltd., (Beijing, China) and its primer sequences were as follows.

U6: F, 5'-GATGACACGCAAATTCGTGAA-3'

R, 5'-GCTGTCAACGATACGCTACG-3'

Statistical analysis and Graphs

All data were represented as the mean $\pm$ 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 immunofluorescent staining scores in benign breast tissues and breast cancer tissues were performed using χ^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 statistically significant.

Results

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

TCGA database analysis suggested that METTL14 was downregulated in 96 breast cancer tissues compared to their matched normal tissues (Fig. 1A). We further investigated the expression patterns of METTL14 in patients of different ER, PR, and HER2 statuses. Results showed that METTL14 was significantly downregulated in TNBC patients compared to ERPR+/HER2+ and ERPR+/HER2- patients. Differential expression of METTL14 between ERPR+/HER2+ and ERPR+/HER2- patients and ERPR-/HER2+ patients was not statistically significant (Fig. 1B). Consistently, immunohistochemistry and immunoblot results revealed a significant decrease in METTL14 expression in TNBC tumor tissues at the protein level (Fig. 1C- 1F). To analyze the correlations between METTL14 expression and clinicopathological characteristics, we divided BC tissues into high and low METTL14 groups based on median expression level. 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) (Fig. 1G).

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

We downloaded the count data of miRNA, then analyzed the differentially expressed miRNAs between TNBC tissues and matched normal tissues using the cut-off criteria of $FDR < 0.01$, $\log^2$ fold change > 2 , and $p < 0.01$. There were 32 downregulated miRNAs and 46 upregulated miRNAs (Fig. 2A). We primarily focused on the downregulated miRNAs, and regression analyses were executed for the expression levels of METTL14 and DE miRNAs. Positive correlations were found between the expression levels of METTL14 and the following miRNAs: hsa-miR-495, has-miR-432, has-miR-5683, has-miR-1247, and has-miR-10b (Fig. 2B).

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

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

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 effects the processing of pri-miRNAs and determines the generation of mature miRNAs. Ma et al (2017) discovered that METTL14 promotes hepatocellular carcinoma metastasis by repressing microRNA-126 expression¹², and another study found that METTL3 promotes the progression of breast cancer via inhibiting the tumor suppressor let-7g[18]. However, few studies have directly focused on METTL14 and its effect on microRNA expression in breast cancer. Analyses of METTL14 expression status in the TCGA database and breast cancer tissues both 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 breast cancer.

Genome-wide predictions suggest that miRNAs regulate more than 60% of the protein coding genes. The dysregulation of specific miRNAs could be associated with different cancers, including TNBC[19]. Our data showed that the expressions of five miRNAs were positive correlated with METTL14 expression in TNBC, indicating that METTL14 may influence miRNA expression through RNA methyl-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 was significantly associated with patients' 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 had been reported that downregulation of miR-1247-5p is associated with poor prognosis, and that this downregulation facilitates tumor cell growth via DVL1/Wnt/b-catenin signaling in breast cancer[23]. Our data showed that miR-1247 expression was significantly decreased in TNBC tissues, suggesting that miR-1247 can serve as a potential biomarker for TNBC.

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

Declarations

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the Maternal and Child Hospital of Hubei Province.

Competing interests

The authors declare they have no competing interests.

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Table

Table 1. Correlation between METTL14 expression levels and clinicopathological features of breast cancer patients.

Parameters	N(cases)	METTL14 expression		P value
		Low(n=35)	High(n=34)	
Age				0.633
≤50 years	34	16	18	
≥50 years	35	19	16	
Differentiation				0.011*
Grade1	2	1	1	
Grade2	33	11	22	
Grade3	34	23	11	
Size of tumor(cm)				0.413
≤3	51	24	27	
≥3	18	11	7	
Lymph node metastasis				0.633
No	34	16	18	
Yes	35	19	16	
Ki67				0.024*
≤20%	16	4	12	
≥20%	53	31	22	

*P<0.05 indicates statistical significance.

Figures

Figure 1

METTL14 is downregulated in BC tissues, especially in TNBC tissues, and serve as a prognostic factor. (A) A comparison of METTL14 expression between breast tumor tissues and adjacent normal tissues (n=96), or (B) with 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) Immunoblots for METTL14 in TNBC patients (n=4). (F) Kaplan-Meier survival curves of RFS based on METTL14 expression in breast cancer, created using the online bioinformatics tool Kaplan-Meier plotter.

Figure 2

Differentially expressed microRNAs (miRNAs) and the correlations with expression levels of METTL14 in TNBC patients (A) Volcano plot of differentially expressed miRNAs in TNBC tissues. (B-F) positive correlations between expression levels of METTL14 and the following downregulated miRNAs: (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-10b.

Figure 3

Prognostic analysis of representative downregulated miRNAs and validate the expression of miRNAs in TNBC tissues (A-E) Overall survival of the representative downregulated miRNAs: (A) has-miR-495, (B) has-miR-432, (C) has-miR-5683, (D) has-miR-1247, and (E) has-miR-10b. (F) The relative expression of has-miR-1247.