Article

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VIRMA Facilitates Triple-Negative Breast Cancer Progression via Increasing m6A-Dependent KIF15 Expression

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Background: Vir like N6-methyladenosine (m6A) methyltransferase associated protein (VIRMA) is associated with various tumors, but the specific role of VIRMA in triple-negative breast cancer (TNBC) and the mechanisms are still unclear. Thus, in this study, in addition to the effect of VIRMA on TNBC, the underlying mechanisms were also explored.

Methods: *In vitro*, VIRMA expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot; VIRMA lentiviral overexpression vector (LV-VIRMA) and lentiviral vector connected with the shRNA targeting VIRMA (LV-shVIRMA) were constructed to explore the functional role of VIRMA; RNA immunoprecipitation and qRT-PCR were performed to assess the relationship between VIRMA and kinesin family 15 (KIF15). *In vivo*, female Balb/C mice (n = 6) were subcutaneously injected with TNBC cells transfected with LV-shRNA + LV-NC (negative control), LV-shVIRMA + LV-NC, and LV-shVIRMA + LV-KIF15, tumor volume, weight and immunohistochemistry staining of Ki-67 were employed to assess breast tumor growth; immunohistochemistry of VIRMA and KIF15 were performed to examine VIRMA and KIF15 expression in breast tumor tissues.

Results: Compared to normal breast epithelial cells, VIRMA was increased in TNBC cells ($p < 0.01$ and $p < 0.001$). LV-VIRMA elevated proliferation, metastasis and invasion of TNBC cells in comparison with LV-NC ($p < 0.001$), while VIRMA knockdown resulted in the opposite effects in comparison with LV-shRNA NC ($p < 0.01$ and $p < 0.001$). Also, compared to LV-shRNA NC, LV-shVIRMA downregulated KIF15 expression by reducing KIF15 mRNA stability ($p < 0.05$ and $p < 0.001$), which was dependent on m6A. Furthermore, compared to LV-shVIRMA + LV-NC, LV-shVIRMA + LV-KIF15 not only reversed the reduced proliferation, metastasis and invasion of TNBC cells ($p < 0.05$, $p < 0.01$, and $p < 0.001$), but also reversed the decreased tumor weight and volume ($p < 0.05$, $p < 0.01$, and $p < 0.001$).

Conclusions: The above results indicated that VIRMA promoted TNBC progression by upregulating m6A-dependent KIF15 expression, providing a better understanding of the pathogenesis of TNBC.

Keywords: VIRMA; KIF15; m6A; TNBC

Introduction

Compared with other types of breast cancer, triple-negative breast cancer (TNBC) exhibits more aggressive behaviors and poorer outcomes [1,2]. Thus, finding new therapeutic targets will play a positive role in the clinical management strategy of TNBC.

An RNA modification called N6-methyladenosine (m6A) exhibits a crucial role in tumor progression, which is controlled by the interaction among m6A writer, eraser, and reader proteins [3–5]. The dysregulation of the associated regulatory proteins of m6A may function as a new prognostic marker in cancer [6,7]. Methyltransferase-like (METTL) 3 facilitated prostate cancer progression by upregulating lymphoid enhancer-binding factor-1 (LEF1) m6A methylation [8]. METTL14 inhibited hepatocellular carcinoma metastasis by inducing microRNA-126 (miR-126) maturation [9]. The downregulation of METTL14 and zinc finger CCCH domain-containing protein 13 (ZC3H13) suppressed breast cancer invasion [10]. Also, METTL3 was obviously reduced in TNBC, and overexpressing METTL3 inhibited TNBC metastasis by inducing collagen type III alpha 1 (COL3A1) m6A modification [11].

Kinesin family 15 (KIF15) is a member of the driver protein superfamily and several studies have shown that KIF15 regulated tumor development [12–14]. Sheng *et al.* [15,16] found that KIF15 was significantly upregulated in TNBC, and TNBC patients with high KIF15 expression exhibited a poorer prognosis, identified KIF15 as a candidate risk gene in TNBC. Gao *et al.* [17] reported that KIF15 facilitated proliferation and migration of breast cancer cells. A study performed by Zeng *et al.* [18] revealed that silencing zinc finger transcription factor 367 (ZNF367) inhibited the invasion and migration of breast

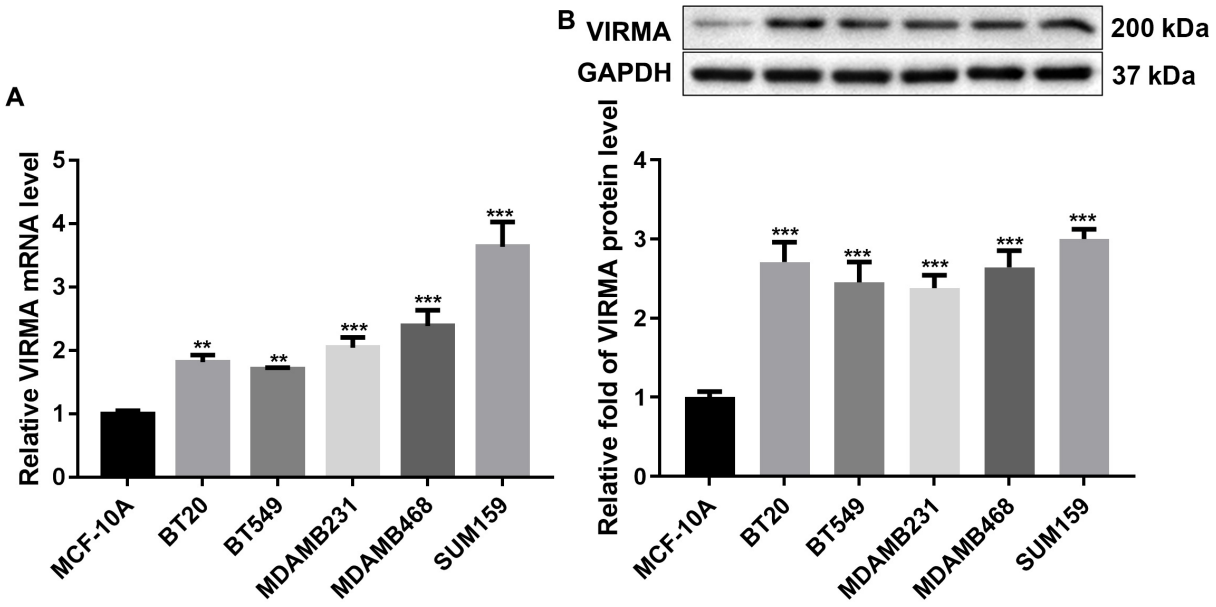

Fig. 1. Vir like N6-methyladenosine (m6A) methyltransferase associated protein (VIRMA) expression was upregulated in triple-negative breast cancer (TNBC) cells. Quantitative real-time polymerase chain reaction (qRT-PCR) (A) and western blot (B) were employed to examine VIRMA expression in TNBC cells and MCF-10A cells. N = 3. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. MCF-10A. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

cancer cells, while which was eliminated by overexpressing KIF15. The above findings revealed the tumor-promoting effect of KIF15 in breast cancer. Bioinformatics analysis revealed multiple m6A modification sites on the KIF15 gene sequence, and the prediction analysis further showed that KIF15 mRNA could combine with multiple m6A-modified enzymes, including Vir like m6A methyltransferase associated protein (VIRMA) (KIAA1429). VIRMA played a key role in bridging METTL3/METTL14/Wilms tumor 1-associated protein (WTAP) and RNA substrates [19,20]. It has also been reported that VIRMA was obviously increased in breast cancer [21]. Thus, herein, in addition to the role of VIRMA in TNBC, the relationship between VIRMA and KIF15 was also addressed. We firstly examined the expression of VIRMA in TNBC cell lines. Then, the functional role of VIRMA in TNBC was explored by the overexpression or knockdown of VIRMA in TNBC cells, and the relationship between VIRMA and KIF15 was determined by RNA immunoprecipitation, quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. Finally, the *in vivo* experiments were performed to assess the effect of VIRMA and KIF15 on TNBC progression.

Methods

Animals

Balb/C mice (female, 6 weeks old, 19–21 g) employed in this study (animal license No. SYXK Su 2022-0048) were purchased from Cavens Laboratory Animal Co. (Changzhou, China). The feeding of experimental animals was in accordance with the Guide for the Care and Use of Laboratory Animals, and the mice were euthanized by injecting overdose barbiturates.

Cell Lines

TNBC cell lines BT20 (CL-0324, Pricella, Wuhan, China), BT549 (CL-0041, Pricella), MDAMB231 (CL-0150B, Pricella, Wuhan, China), MDAMB468 (CL-0290B, Pricella, Wuhan, China), SUM159 (CL-0622, Pricella, Wuhan, China) and normal breast epithelial cell line MCF-10A (CL-0525, Pricella, Wuhan, China) were all identified correctly by STR, and mycoplasma testing was performed for all cell lines.

Lentivirus Infection

The shRNA targeting VIRMA (shVIRMA) and the negative control (NC) synthesized by Gene Pharma (Shanghai, China) were connected to lentiviral vector to obtain lentiviral vector connected with the shRNA targeting VIRMA (LV-shVIRMA) and LV-shRNA NC. The coding sequences of VIRMA and the negative control were amplified and cloned into lentiviral the vector to obtain VIRMA lentiviral overexpression vector (LV-VIRMA) and the negative control lentiviral overexpression vector (LV-NC) (Gene Pharma), then the above vectors were packaged to overexpress or interfere lentiviruses. TNBC cells (3×10^4 cells/well) at logarithmic growth stage were inoculated on 6-well plates, and infected with lentiviruses when the cells grew to a fusion degree of 20%~30%. Cell status was

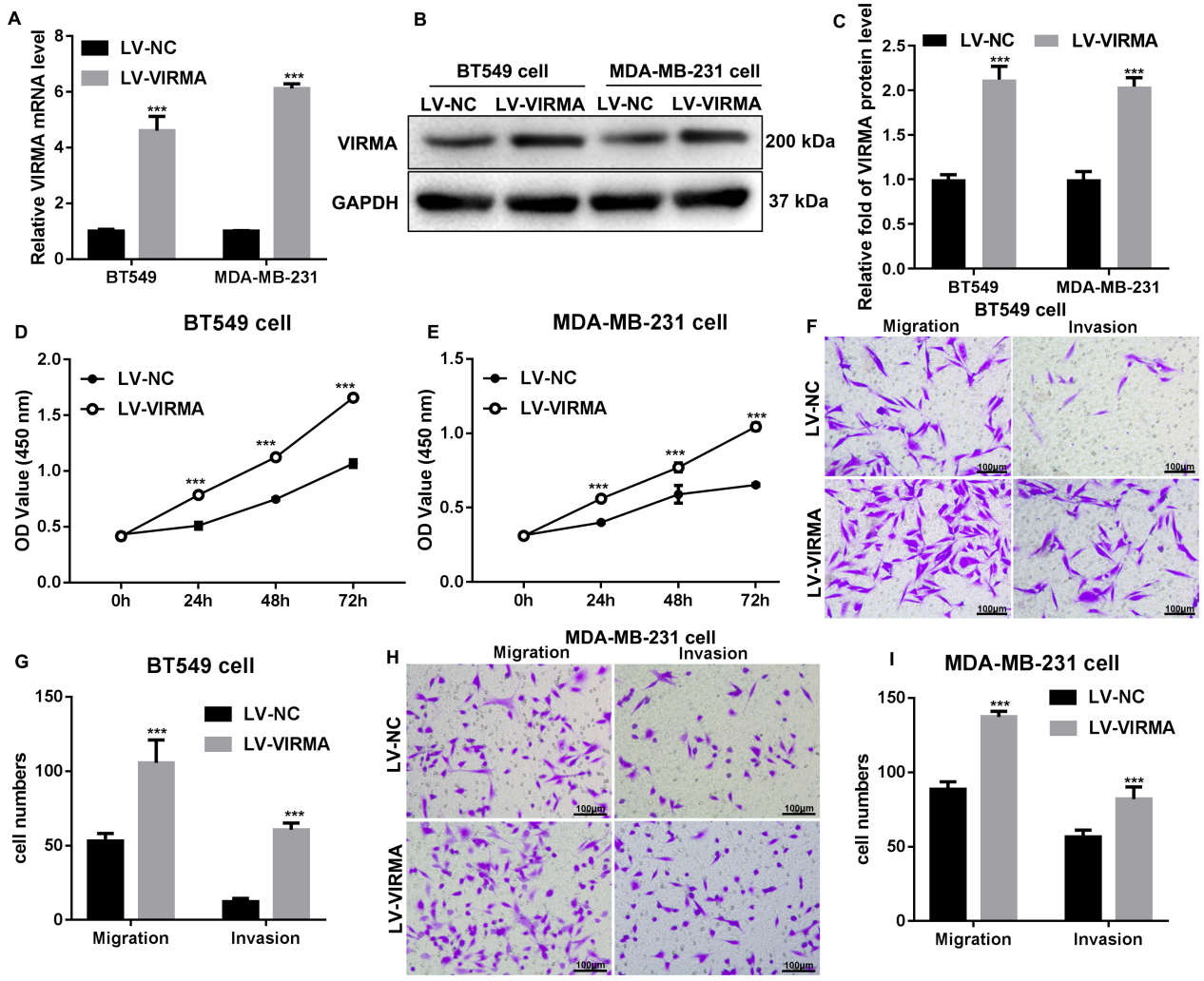

Fig. 2. Vir like m6A methyltransferase associated protein (VIRMA) overexpression elevated the proliferation, migration and invasion of TNBC cells. VIRMA lentiviral overexpression vector (LV-VIRMA) was transfected into BT549 and MDA-MB-231 cells, and the negative control lentiviral overexpression vector (LV-NC) was the control. VIRMA expression was detected using quantitative real-time polymerase chain reaction (qRT-PCR) (A) and western blot (B,C). (D,E) Cell proliferation was determined by cell counting kit-8 (CCK-8). (F–I) Cell migration and invasion was determined by transwell assay, and representative pictures were exhibited and quantified (Scale bar = 100 µm). N = 3. $^{***}p < 0.001$ vs. LV-NC.

observed, and the fresh medium was added 24 h later. The infection efficiency was confirmed by qRT-PCR and western blot 72 h after the infection. The related shRNA sequences were listed as follows:

shVIRMA: 5′-TTGCTGATCACGTATCATCTT-3′
shRNA NC: 5′GCCATATGTTCGAGACTCT3′

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

We employed TRIzol (15596026, Invitrogen, Carlsbad, CA, USA) to obtain RNA and a reverse transcription kit (4992911, Tiangen Biotech, Beijing, China) to obtain complementary DNA (cDNA). Then, we used an ABI StepOnePlus real-time PCR System (4376600, Thermo Fisher Scientific, Waltham, MA, USA) with gene primers (which were shown below) and SYBR Green PCR Master Mix (FP205, Tiangen Biotech, Beijing, China) to perform qRT-PCR. The mRNA expression was normalized to β-actin, which was calculated using the $2^{-\Delta\Delta CT}$ method.

VIRMA (forward): 5′-GAGTAAGAGCCCATAGCAGT-3′

VIRMA (reverse): 5′-TAGCACCAGACCATCAGTATTCAC-3′

KIF15 (forward): 5′-CTCTCACAGTTGAATGTCCTTG-3′

KIF15 (reverse): 5′-CTCCTTGTCAGCAGAATGAAG-3′

β-actin (forward): 5′-AGCGAGCATCCCCCAAAGTT-3′

β-actin (reverse): 5′-GGGCACGAAGGCTCATCATT-3′

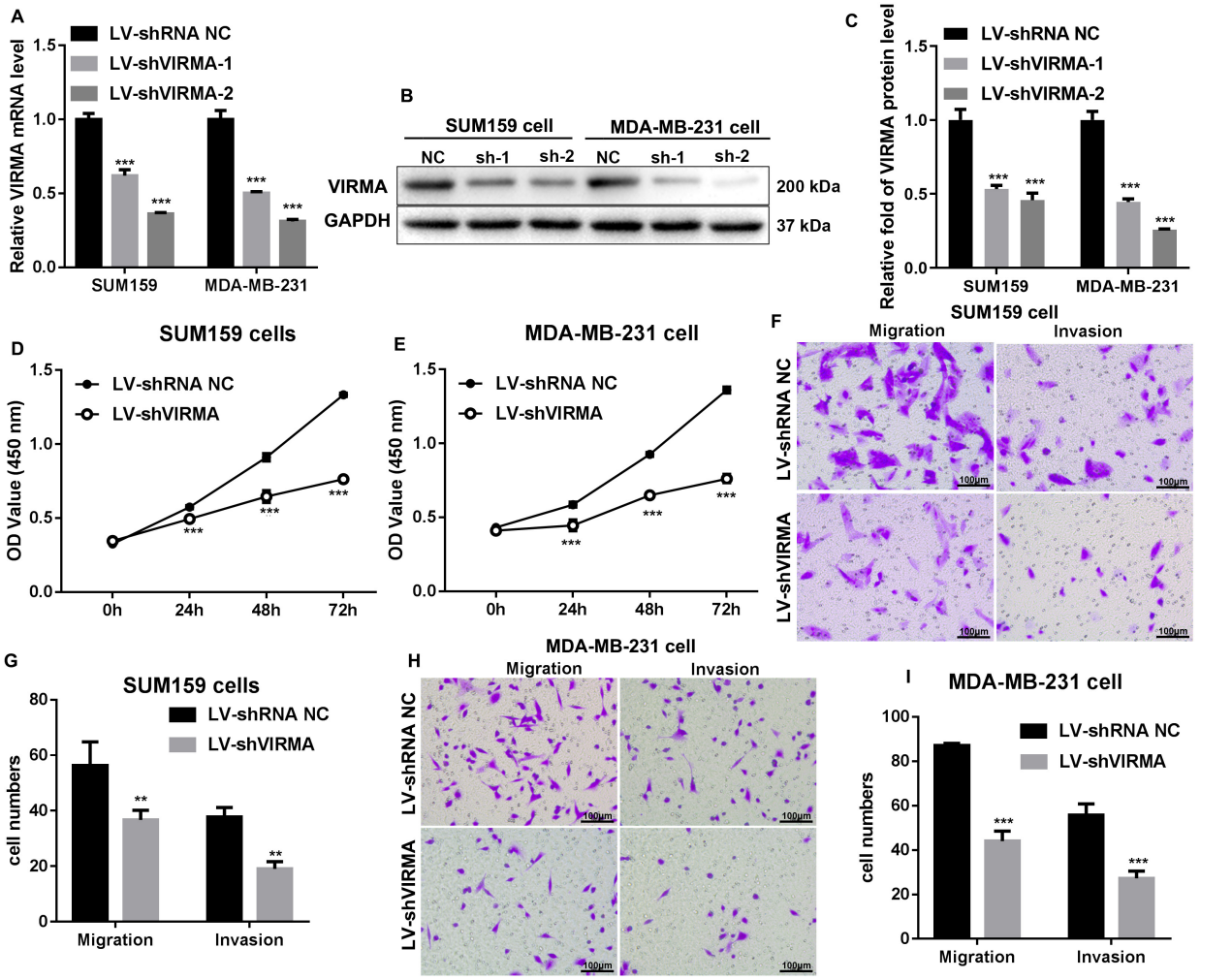

Fig. 3. Vir like m6A methyltransferase associated protein (VIRMA) knockdown reduced the proliferation, migration and invasion of triple-negative breast cancer (TNBC) cells. SUM159 and MDA-MB-231 cells were employed for the knockdown study. VIRMA expression was detected using quantitative real-time polymerase chain reaction (qRT-PCR) (A) and western blot (B,C). (D,E) Cell proliferation was determined by cell counting kit-8 (CCK-8). (F–I) Cell migration and invasion was determined by transwell assay, and representative pictures were exhibited and quantified (Scale bar = 100 μm). N = 3. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LV-shRNA NC. LV-shVIRMA, lentiviral vector connected with the shRNA targeting VIRMA; NC, negative control.

Western Blot

Briefly, the separated proteins were loaded onto 10% SDS-PAGE gels and transferred to PVDF membranes, and then the membranes were incubated with the following primary antibodies: VIRMA (ab271136, 1:1000, Abcam, Cambridge, MA, USA), KIF15 (sc-100948, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245, 1:2500, Abcam, Cambridge, MA, USA), as well as anti-rabbit and anti-mouse secondary antibodies (7074 and 7076, 1:5000, Cell Signaling Technology, Danvers, MA, USA). Then, the membranes were incubated with a SuperFemto ECL Chemiluminescence Kit (E423-01, Vazyme, Nanjing, China) to visualize the protein bands. The signals were detected using a ChemiDoc XRS + system (1708265, Bio-Rad, Hercules, CA, USA), and the optical density was analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). GAPDH was the internal control.

Cell Counting Kit-8 (CCK-8) Assay

Briefly, CCK-8 (96992, Sigma, Saint Louis, MO, USA) was added and incubated with TNBC cells for different time, a microplate reader (1681000, Bio-Rad) was used to measure the optical density at 450 nm in TNBC cells.

Cell Migration and Invasion Assays

The migrated and invasive cells were detected as previously reported [21], and four random fields were photographed under a microscope (Nikon E100, Nikon Corp, Tokyo, Japan).

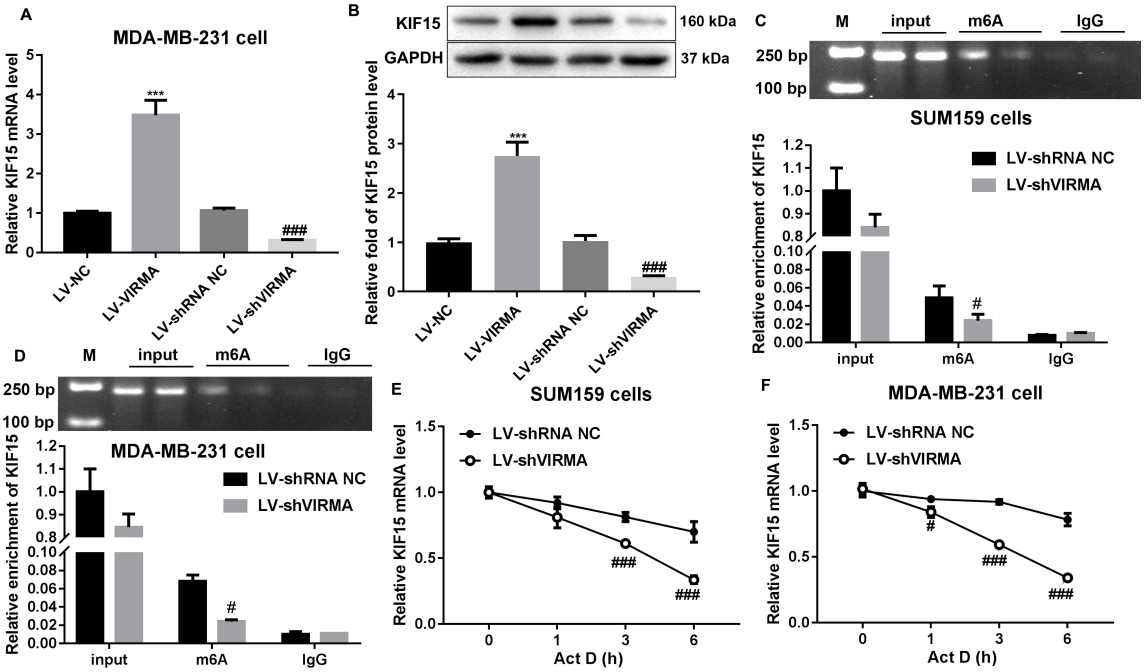

Fig. 4. Vir like m6A methyltransferase associated protein (VIRMA) regulated m6A-dependent kinesin family 15 (KIF15) mRNA expression. VIRMA expression was detected by quantitative real-time polymerase chain reaction (qRT-PCR) (A) and western blot (B) in MDA-MB-231 cells. Immunoprecipitation was employed to examine the effect of VIRMA knockdown on KIF15 mRNA modified by m6A in SUM159 (C) and MDA-MB-231 cells (D). qRT-PCR was performed to examine the half-life of the KIF15 transcript in SUM159 (E) and MDA-MB-231 cells (F). N = 3. $^{***}p < 0.001$ vs. LV-NC; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. LV-shRNA NC. IgG, immunoglobulin G.

RNA Immunoprecipitation (RIP)

RIP assay was performed using Magna RIP kit (Millipore, Billerica, MA, USA). Briefly, cells were lysed by completed RIP lysis buffer (containing RNase Inhibitor and protease inhibitor), and anti-m6A antibody (ab208577, Abcam, Cambridge, MA, USA) or immunoglobulin G (IgG) were incubated with cell lysates coated with magnetic beads at 4 °C for 4 h. Then RNA-protein complexes were washed, and immunoprecipitated RNA was purified. We extracted coprecipitated RNA and performed qRT-PCR, which was normalized to input.

In Vivo Experiments

6-week-old mice (19–21 g) with subcutaneous injection into the right flank of different MDA-MB-231 cells (1×10^7 cells in 0.1 mL PBS) were divided into 3 groups (n = 6): LV-shRNA + LV-NC, LV-shVIRMA + LV-NC, and LV-shVIRMA + LV-KIF15. 7 days after the injection, the volume of the breast tumor was measured every 3 days for 3 weeks via vernier caliper, and calculated by the formula: volume (cm^3) = ($L \times W^2$)/2, L indicated the length of the tumor, W indicated the width of the tumor. The breast tumor was isolated on Day 25, and tumor weight was measured.

Immunohistochemistry (IHC) Staining

For IHC staining, the paraffin-embedded tissue was dehydrated, transparentized, wax-dipped, and embedded again. Then the tissue was cut into 4-µm-thick tissue sections by microtome, and deparaffinized, rehydrated, followed by antigen retrieval in a microwave oven. After blocking endogenous peroxides with 3% hydrogen peroxide solution, the sections were incubated overnight at 4 °C with the following primary antibodies: VIRMA (ab246982, 1:50, Abcam, Cambridge, MA, USA), KIF15 (ab272615, 1:50, Abcam, Cambridge, MA, USA) and Ki-67 (ab16667, 1:200, Abcam, Cambridge, MA, USA) and then with anti-rabbit secondary antibody (ab6721, 1:1000, Abcam) at 37 °C. The sections were photographed under a microscope (Olympus BX41, Tokyo, Japan).

Statistical Analysis

We analyzed the differences between groups using unpaired Student's *t* tests or one-way analysis of variance using GraphPad Prism 7.0 (GraphPad software, La Jolla, CA, USA). The difference was considered significant if the *p* value was less than 0.05.

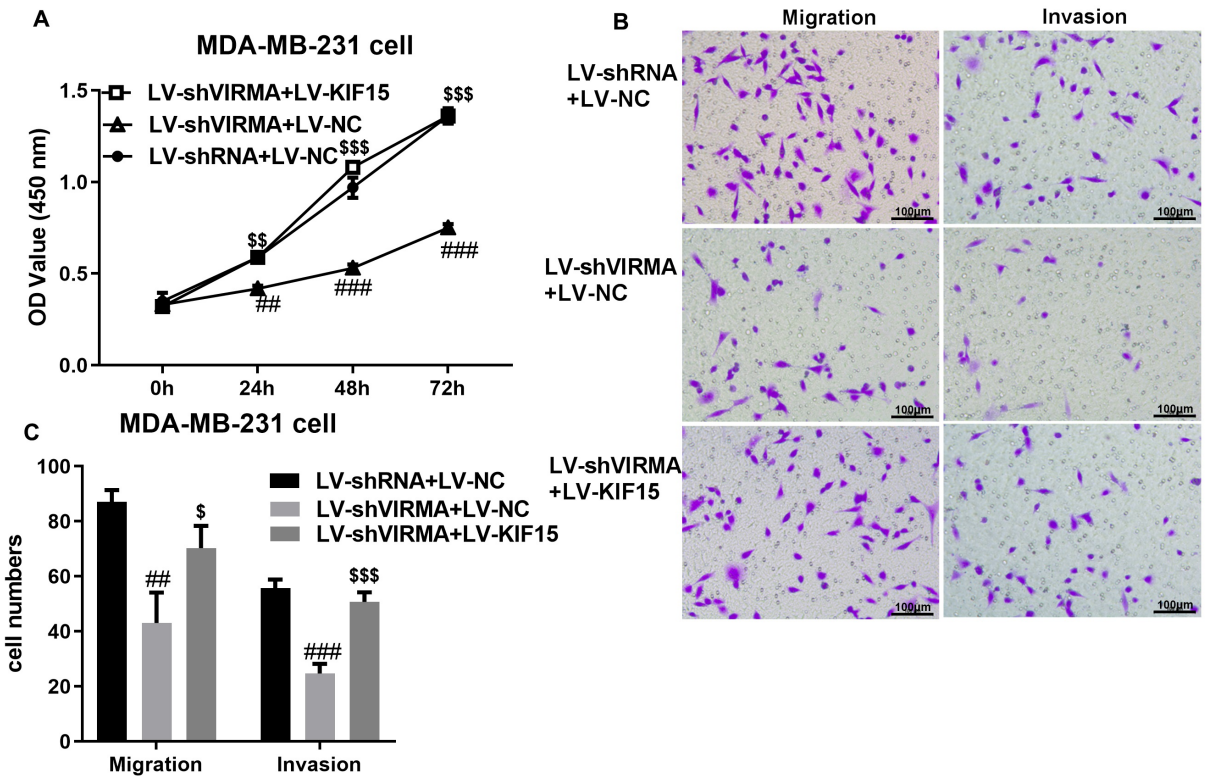

Fig. 5. Vir like m6A methyltransferase associated protein (VIRMA) functioned in triple-negative breast cancer (TNBC) by regulating kinesin family 15 (KIF15). MDA-MB-231 cells were employed. (A) Cell proliferation was determined by cell counting kit-8 (CCK-8). (B,C) Cell migration and invasion were examined by transwell assay, and representative pictures were exhibited and quantified (Scale bar = 100 μm). N = 3. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. LV-shRNA + LV-NC; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. LV-shVIRMA + LV-NC.

Results

VIRMA was Upregulated in TNBC Cells

A comparison of VIRMA expression in different TNBC cell lines and MCF-10A cells revealed that, compared to MCF-10A cells, TNBC cells exhibited higher expression of VIRMA, both in mRNA (Fig. 1A, $p < 0.01$, and $p < 0.001$) and protein (Fig. 1B, $p < 0.001$) level.

VIRMA Elevated Cell Proliferation, Migration and Invasion in TNBC Cells

First, qRT-PCR (Fig. 2A, $p < 0.001$) and western blot (Fig. 2B,C, $p < 0.001$) analysis confirmed that LV-VIRMA was transfected successfully in BT549 and MDA-MB-231 cells. The proliferation, migration and invasion of TNBC cells were markedly elevated by LV-VIRMA in comparison with LV-NC (Fig. 2D–I, $p < 0.001$). Furthermore, VIRMA knockdown was performed successfully in SUM159 and MDA-MB-231 cells (Fig. 3A–C, $p < 0.001$), and compared to LV-shRNA NC, the proliferation, migration and invasion of TNBC cells were markedly reduced by LV-shVIRMA (Fig. 3D–I, $p < 0.01$, and $p < 0.001$).

VIRMA Regulated m6A-Dependent KIF15 mRNA Expression

It was predicted that VIRMA combined with KIF15 through m6A2Target database, we examined the effect of VIRMA on KIF15 expression. We observed that VIRMA overexpression elevated KIF15 expression in comparison with LV-NC, while VIRMA knockdown downregulated KIF15 expression in comparison with LV-shRNA NC (Fig. 4A,B, $p < 0.001$). The immunoprecipitation results further revealed that VIRMA knockdown obviously reduced KIF15 mRNA modified by m6A in TNBC cells (Fig. 4C,D, $p < 0.05$). Moreover, compared to LV-shRNA NC, LV-shVIRMA reduced the half-life of the KIF15 transcript in TNBC cells (Fig. 4E,F, $p < 0.05$ and $p < 0.001$). The above results suggested that VIRMA upregulated m6A-dependent KIF15 mRNA expression by increasing the stability of KIF15 mRNA.

VIRMA Promoted TNBC Tumorigenesis in a KIF15-Dependent Manner

To explore whether KIF15 was involved in TNBC development influenced by VIRMA, LV-shVIRMA and LV-KIF15 were co-transfected into MDA-MB-231 cells. We

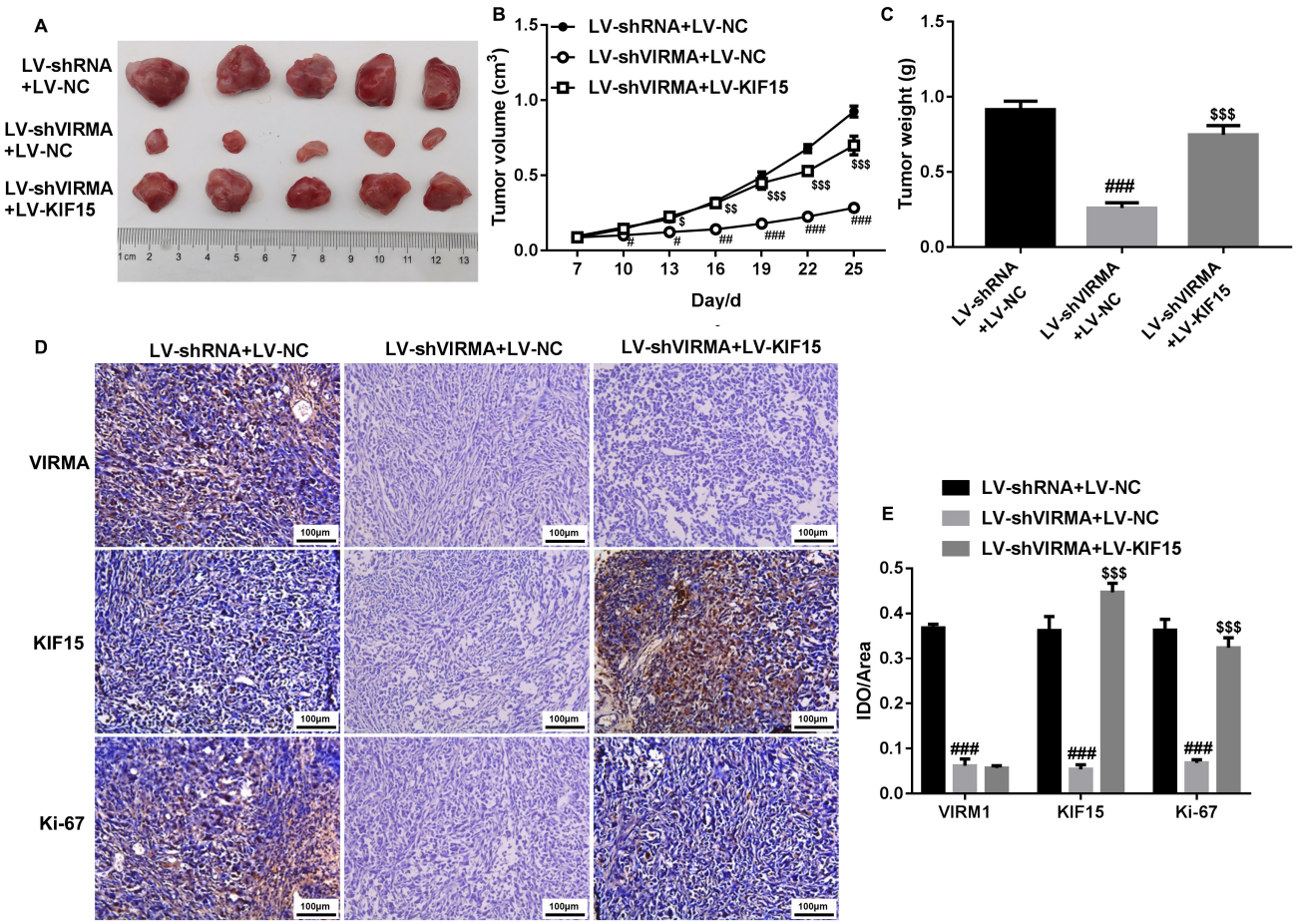

Fig. 6. Vir like m6A methyltransferase associated protein (VIRMA) promoted triple-negative breast cancer (TNBC) tumorigenesis in a kinesin family 15 (KIF15)-dependent manner. Nude mice were injected with MDA-MB-231 cells transfected with LV-shRNA + LV-NC, LV-shVIRMA + LV-NC, and LV-shVIRMA + LV-KIF15 (N = 6). (A,B) Seven days later, the tumor volume was recorded every three days, and the growth curve was illustrated. (C) The tumor was isolated, and tumor weight was measured on Day 25. (D,E) The paraffin-embedded tumor tissues were incubated with VIRMA, KIF15 and Ki67 antibodies for immunohistochemical staining, and representative pictures were exhibited and quantified (Scale bar = 100 μm). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. LV-shRNA + LV-NC; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. LV-shVIRMA + LV-NC.

found that LV-shVIRMA + LV-KIF15 surprisingly reversed the reduced proliferation, migration and invasion of MDA-MB-231 cells induced by LV-shVIRMA + LV-NC (Fig. 5A–C, $p < 0.05$, $p < 0.01$, and $p < 0.001$). Additionally, *in vivo* experiments were performed, and we observed that LV-shVIRMA + LV-NC inhibited tumor growth (Fig. 6A,B, $p < 0.05$, $p < 0.01$, $p < 0.001$) and reduced tumor weight (Fig. 6C, $p < 0.001$), but these effects were reversed in the LV-shVIRMA + LV-KIF15 group ($p < 0.05$, $p < 0.01$, and $p < 0.001$). Additionally, IHC staining revealed the relationship between VIRMA and KIF15 expression (Fig. 6D). Ki67 was reduced with VIRMA knockdown, which was further reversed after KIF15 overexpression (Fig. 6E, $p < 0.001$). We concluded that VIRMA promoted tumorigenesis in a KIF15-dependent manner in TNBC.

Discussion

Many m6A-associated regulatory proteins participate in the development of many cancers. As the largest component of 'writers', VIRMA is associated with the modification, mRNA splicing and processing of m6A [19,22]. It has been reported that VIRMA promoted the progression of multiple tumors, including liver cancer [23], breast cancer [21], gastric cancer [24], and head and neck squamous cell carcinoma [25]. Qian *et al.* [21] reported that VIRMA expression was abnormal in breast cancer. Thus, in addition to exploring the role of VIRMA in TNBC, the underlying mechanisms were also explored.

The kinesin family (KIF) is a group of proteins that share highly conserved motility regions, and most of which have ATP-dependent activity and catalyze microtubule-dependent tail-loading reactions. KIF plays an important role in the occurrence and development of tumors. KIF11

facilitated the invasion, proliferation and self-renewal of glioblastoma [26]. KIF14 promoted the progression and metastasis of gastric cancer and was closely related to the poor prognosis of patients [27]. KIF4A served as a prognostic biomarker and therapeutic target for lung cancer [28]. KIF15 is mainly involved in mitosis and neuronal development, and is closely associated with a variety of malignant tumors, such as prostate cancer [29], hepatocellular carcinoma [30], pancreatic carcinoma [31], lung adenocarcinoma [32]. KIF15 also facilitated cell proliferation and migration in breast cancer [17]. Furthermore, Sheng *et al*. [15,16] revealed that KIF15 was significantly upregulated in TNBC, and TNBC patients with high KIF15 expression exhibited a poorer prognosis. Further functional experiments revealed that KIF15 knockdown induced cell apoptosis but inhibited cell proliferation of TNBC cells [15,16]. Bioinformatics analysis revealed multiple m6A modification sites on the KIF15 gene sequence, suggesting that KIF15 mRNA may be regulated by m6A-related modification enzymes. Further prediction analysis showed that KIF15 mRNA could combine with multiple m6A-modified enzymes, including VIRMA. Thus, we speculated that VIRMA may promote TNBC by regulating KIF15 expression in a m6A-dependent manner.

As expected, in this study, VIRMA overexpression significantly upregulated KIF15 expression, while VIRMA knockdown downregulated KIF15 expression, suggesting the positive relationship between VIRMA and KIF15. We also found that VIRMA knockdown reduced KIF15 mRNA enrichment modified by m6A and the half-life of KIF15 transcript, suggesting that VIRMA upregulating m6A-dependent KIF15 mRNA expression by increasing KIF15 mRNA stability. Interestingly, bioinformatics analysis also showed that KIF15 mRNA could bind to Insulin-Like Growth Factor Binding Protein 3 (IGFBP3), a m6A reader protein. Thus, we suspect that VIRMA may increase KIF15 mRNA stability via the interaction with IGFBP3. However, whether VIRMA can regulate KIF15 mRNA stability through the direct action on IGFBP3 requires the further study. Moreover, our experiments revealed that KIF15 overexpression significantly rescued the inhibited TNBC development induced by LV-shVIRMA, suggesting that VIRMA promoted tumorigenesis in a KIF15-dependent manner in TNBC.

Conclusions

In summary, this study demonstrated that VIRMA upregulated m6A-dependent KIF15 expression by enhancing KIF15 mRNA stability, thereby promoting TNBC tumorigenesis. This study may provide a novel insight into the promotion effect of VIRMA in TNBC.

Availability of Data and Materials

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

Author Contributions

CC designed the research study. CC, YW, YL and CZ performed the research. CZ analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the animal care and use committee of Bengbu Medical College (No. [2021]-246).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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