Cancer-Associated MORC2-Mutant M276I Regulates an hnRNPM-Mediated CD44 Splicing Switch to Promote Invasion and Metastasis in Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is the most lethal subtype of breast cancer, with a high propensity for distant metastasis and limited treatment options, yet its molecular underpinnings remain largely unknown. Microchirida family CW-type zinc finger 2 (MORC2) is a newly identified chromatin remodeling protein whose mutations have been causally implicated in several neurologic disorders. Here, we report that a cancer-associated substitution of methionine to isoleucine at residue 276 (M276I) of MORC2 confers gain-of-function properties in the metastatic progression of TNBC. Expression of mutant MORC2 in TNBC cells increased cell migration, invasion, and lung metastasis without affecting cell proliferation and primary tumor growth compared with its wild-type counterpart. The M276I mutation enhanced binding of MORC2 to heterogeneous nuclear ribonucleoprotein M (hnRNPM), a component of the spliceosome machinery. This interaction promoted an hnRNPM-mediated splicing switch of CD44 from the epithelial isoform (CD44v) to the mesenchymal isoform (CD44s), ultimately driving epithelial–mesenchymal transition (EMT). Knockdown of hnRNPM reduced the binding of mutant MORC2 to CD44 pre-mRNA and reversed the mutant MORC2-induced CD44 splicing switch and EMT, consequently impairing the migratory, invasive, and lung metastatic potential of mutant MORC2-expressing cells. Collectively, these findings provide the first functional evidence for the M276I mutation in promoting TNBC progression. They also establish the first mechanistic connection between MORC2 and RNA splicing and highlight the importance of deciphering unique patient-derived mutations for optimizing clinical outcomes of this highly heterogeneous disease.

Significance: A gain-of-function effect of a single mutation on MORC2 promotes metastasis of triple-negative breast cancer by regulating CD44 splicing. Cancer Res; 78(20); 5780–92. ©2018 AACR.

Introduction

Triple-negative breast cancer (TNBC) is a distinct subtype of breast cancer with low or no expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), which accounts for approximately 15% to 20% of all breast cancers (1, 2). In contrast to other breast cancer subtypes, TNBC occurs most frequently in younger women, exhibits an extremely aggressive phenotype with higher rates of early relapse and distant metastasis, and lacks the responsiveness to endocrine or HER2-targeted therapies, thus contributing to the worst clinical outcome (1, 2). Due to the lack of clinically available targeted therapies, cytotoxic chemotherapy remains the mainstay of treatment for TNBC. However, treatment options are very limited upon the development of chemoresistance and distant metastasis (3). These clinical challenges are further reinforced by its genetic heterogeneity. Recently, several genomic and transcriptomic sequencing studies have revealed extensive mutational heterogeneity among TNBC tumors (4–6). In this context, in addition to a few common recurrent mutations that restrict primarily to p53 and the phosphoinositide 3-kinase (PI3K) pathway, a large number of genes with low mutational frequency were found in TNBC tumors (4–6). Thus, deciphering the patterns of mutations in individual patient with TNBC is critical for personalized cancer therapy and optimizing clinical outcomes (5). However, the functional consequences and related mechanistic underpinnings for the majority of these identified unique patient-derived mutations in TNBC progression remain unknown.

Microchirida family CW-type zinc finger 2 (MORC2) is a member of the evolutionarily conserved MORC nuclear protein superfamily, which is characterized by the presence of a conserved GHKL (Gyrase, Hsp90, Histidine kinase, and MutL)-type ATPase domain, a CW-type zinc finger domain, and several distinct coiled-coil domains (7–9). Although MORC2 is ubiquitously expressed in mammalian cells, its biological functions remain...
largely unknown. Recently, we and others have defined MORC2 as a chromatin remodeling protein with emerging roles in DNA repair (10) and gene transcription (11, 12). Interestingly, mutations in MORC2 have been causally linked with several neurologic disorders, such as Charcot–Marie–Tooth disease (11, 13, 14), cerebellar ataxia, axonal polyneuropathy, and nocturnal hypoventilation (15). In TNBC, a microarray-based gene-expression profiling study revealed that the expression levels of MORC2 are associated with the recurrence risk of patients with TNBC (16).

In addition, an exome sequencing analysis of primary TNBC tumors revealed that 1 of 65 patients with TNBC carried a conserved mutation of G to C at nucleotide 828 of the MORC2 gene, resulting in its encoding protein harboring a substitution of methionine to isoleucine at residue 276 (M276I; ref. 5). However, the functional and mechanistic roles for MORC2 M276I mutation in TNBC development and progression remain unexplored. The heterogeneous nuclear ribonucleoprotein M (hnRNPM) is a component of the spliceosome machinery and plays key roles in suppression of precursor mRNA (pre-mRNA) splicing through antagonizing the recognition of splice sites (17). Indeed, hnRNPM has been shown to regulate alternative splicing of several cancer-associated genes, including fibroblast growth factor receptor 2 (FGFR2; ref. 18) and cell-surface molecule CD44 (19). FGFR2 pre-mRNA is alternatively spliced to form the epithelial- and mesenchymal-specific IIIb and IIIc isoforms, respectively, and the FGFR2 isoform switch potentially affects epithelial–mesenchymal transition (EMT) program and cancer progression (20). Similarly, human CD44 pre-mRNA contains nine variable exons between its constitutive exons. Inclusion of one or more of the variable exons generates CD44 variant isoforms (CD44v), whereas skipping all of the variable exons produces CD44 standard isoform (CD44s; ref. 19). Generally, expression of CD44v is common in epithelial cells, while CD44s is expressed by mesenchymal cells (21). Emerging evidence shows that hnRNPM promotes the splice isoform switch from CD44v to CD44s, which is essential for EMT and breast cancer metastasis (19, 21). More importantly, upregulation of hnRNPM correlates with distant metastasis, poor prognosis, and increased CD44s in patients with breast cancer (19, 22). However, the upstream regulatory signals for hnRNPM-mediated splicing program in TNBC remain to be elucidated.

In this study, we report for the first time that the M276I mutation of MORC2 is a gain-of-function mutation that promotes metastatic progression of TNBC through regulating hnRNPM-mediated CD44 splice isoform switch and EMT. These findings provide novel mechanistic insights into MORC2 M276I mutation in promoting TNBC metastatic progression and highlight the importance of dissecting unique patient-derived mutations and their functional consequences for personalized treatments of this highly heterogeneous disease.

Materials and Methods

Cell culture and reagents

Human breast cancer cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-468, HS578T, BT20, BT549, HCC1937, and BT474), normal breast epithelial cell line MCF10A, and human embryonic kidney 293T (HEK293T) cell line were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. MDA-MB-231-derived LM2-4173 and LM2-4175 cells were kindly provided by Guohong Hu (University of Chinese Academy of Sciences, Shanghai, China). Both cell lines have enhanced lung metastasis potential when compared with their parental cells (23). SUM149 and SUM159 cell lines were obtained from Asterand. All cell lines were authenticated by short tandem repeat profiling. Mycoplasma contamination was tested by a PCR-based method as described previously (24). Cells were expanded and frozen immediately into numerous aliquots after arrival in 2014. The cells revived from the frozen stock were used within 10 to 15 passages and not exceeding a period of 6 months. MCF10A cells were cultured in DMEM/F12 supplemented with 5% donor horse serum, 10 μg/mL insulin, 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, and 100 ng/mL cholera toxin. The culture medium for SUM159 and SUM149 is Ham’s F12 containing 10% fetal bovine serum (FBS), 5 μg/mL insulin, and 1 μg/mL hydrocortisone. Other cell lines were maintained in high-glucose DMEM or RPMI1640 media supplemented with 10% FBS. Culture media and supplements were obtained from BasalMedia.

Horse serum and FBS were from Gibco. All chemicals and regents were purchased from Sigma-Aldrich unless otherwise noted.

Expression vectors

Myc-DDK-MORC2 cDNA was purchased from Origene and subcloned into the lentiviral vector pCDH-CMV-MCS-EF1-Puro (System Biosciences) to generate Flag-MORC2 construct. Myc-DDK-MORC2 cDNA was also subcloned into the lentiviral vector pLVX-IRE-Neo (Clontech) for reexpression of MORC2 (G418 resistance) in MORC2 knockout (KO) cells (puromycin resistance). M276I mutation was generated by PCR-based mutagenesis and verified by DNA sequencing. Flag-His-hnRNPM cDNA was obtained from VigeumBio and subcloned into pCDH-CMV-MCS-EF1-Puro vector to generate HA-hnRNPM expression vector. GIPZ lentiviral short hairpin RNA (shRNA) vectors expressing hnRNPM shRNA (shhnRNPM) or nontargeting negative control (shNC) were obtained from GE Healthcare.

Detailed information concerning expression constructs and the primers used for molecular cloning is provided in Supplementary Tables S1 and S2. Small interfering RNA (siRNA) targeting hnRNPL (shhnRNPL) and nontargeting negative control (siNC) were purchased from GenePharma (Supplementary Table S3).

Plasmid transfection and lentiviral infection

Transient plasmid transfection was performed using NeoFect DNA transfection reagent (TengyiBio) according to the manufacturer’s protocol. To generate stable cell lines expressing shRNAs or cDNAs, HEK293T cells were transfected with each lentivirus expression vector and packaging plasmid mix using Neofect DNA transfection reagents. The supernatant containing viruses was collected 48 hours after transfection, filtered, and used for infecting target cells in the presence of 8 μg/mL of polybrene prior to drug selection with 2 μg/mL of puromycin (Cayman Chemical) or 5 μg/mL of G418 (Sigma) for 1 to 2 weeks. MORC2 KO cell lines were generated using the CRISPR/Cas9 system as described previously (25) with LentiCas9-Blunt and lentiGuide-Puro vectors (Addgene). The short guide RNA sequence for MORC2 (5’-AGTAGACTGAGTGTTGCT-3’) was chosen according to the Web-based CRISPR design tool from the Zhang lab (http://www.genome-engineering.org/) and was cloned into lentiGuide-Puro vector following the standard protocol (25). Successful MORC2 KO in the established cell lines was validated by immunoblotting. SiRNA transfection was performed using Lipofectamine 2000 (Invitrogen). The efficiency of silencing was assessed by immunoblotting 48 hours after transfection.
Cell viability, colony-formation assay, and cell-cycle analysis

Cells were seeded in 96-well plates (2,000 cells/well) in triplicate, and cell viability was determined by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories). For colony-formation assay, cells were seeded in 6-well plates (1,000 cells/well) in triplicate and cultured under normal growth conditions for 2 to 3 weeks. Colonies were stained with 1% crystal violet and counted. For cell-cycle analysis, cells were harvested and fixed in 75% ethanol overnight. After PBS wash, cells were stained with cell-cycle staining kit (MultiSciences Biotech), and analyzed on a BD FACSCanto II flow cytometer (BD Biosciences).

Cell migration and invasion assays

Migration and invasion assays were conducted using 8-μm pore noncoated polycarbonate transwell inserts (BD Biosciences) and BioCoat Matrigel Invasion Chambers (Corning), respectively. Briefly, 5 × 10⁴ cells in 200 μL of serum-free growth medium were seeded in the top chamber. Growth medium containing 10% FBS was used as a chemoattractant in the lower chamber. After 24 hours, migrated and invaded cells were fixed and stained with 1% crystal violet. Cells were counted in 10 random fields under microscope.

Tumorigenicity and metastasis assays

All animal studies were approved by the Institutional Animal Care and Use Committee of Shanghai Cancer Center, Fudan University. For primary tumor and spontaneous metastasis assays, 2 × 10⁶ LM2-4175 cells stably expressing empty vector pLVX, wild-type (WT), and M276I-mutant MORC2 were injected into diabetic/severe combined immunodeficient (NOD/SCID) mice (n = 6; State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai, China). The tumors were measured twice a week after appearance of tumors, and the tumor volume was calculated by the formula of (length × width²)/2. The mice were killed after 6 weeks of the inoculation, and primary tumor and the lung tissues were harvested for histologic analysis. As cancer cells formed diffuse metastases in the lungs of mice in an orthotropic xenograft model of spontaneous breast cancer metastasis, lung metastases were quantified by determining the percent metastatic lung surface area relative to total lung surface area as described previously (26). For experimental metastasis experiments, 2 × 10⁶ MDA-MB-231 cells stably expressing empty vector pCDH, WT, and M276I-mutant MORC2 in 200 μL of PBS were injected in the tail vein of 6-week-old BALB/c female nude mice (n = 6; State Key Laboratory of Oncogenes and Related Genes). After 7 weeks of injection, the lungs were excised, fixed in Bouin solution overnight, and lung colonies were counted under a Nikon SMZ1500 stereomicroscope (Nikon). In addition, paraffin-embedded sections of lung tissues were stained by hematoxylin–eosin (H&E) staining to examine the presence of micrometastases.

Antibodies, immunoblotting, immunoprecipitation, and immunofluorescence

The detailed information for primary antibodies used in this study is provided in Supplementary Table S4. For immunoblotting analyses, cells were lysed in the modified RIPA buffer (50 mmol/L Tris–HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mmol/L EDTA) containing 1× protease inhibitor cocktail (Roche) and 1× phosphatase inhibitor cocktail (Bimake). Proteins were quantified using the bicinchoninic acid assay (Yeasen), resolved by SDS–PAGE, and transferred onto PVDF membrane (Millipore). Antibody detection was conducted using enhanced chemiluminescent substrate kit (Yeasen). For immunoprecipitation (IP) analysis, cells were lysed in NP-40 lysis buffer (50 mmol/L Tris–HCl, pH 8, 150 mmol/L NaCl, 0.5% NP-40, 10% glycerol, 2 mmol/L MgCl₂, and 1 mmol/L EDTA), and total 1 to 2 mg of exogenously expressed proteins was incubated anti-Flag or anti-HA magnetic beads (Bimake) overnight at 4°C to pull down the protein–antibody complex. The resulting complexes were subjected to immunoblotting analysis. For immunofluorescence staining, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 10% normal goat serum in PBS. Cells were incubated with primary antibodies, washed three times in PBS, and then incubated with the appropriate secondary antibody conjugated with 555-Alexa (red) or 488-Alexa (green; Cell Signaling Technology), respectively. DNA staining was performed using Fluoroshield mounting medium with DAPI (Abcam). Microscopic analyses were performed using a Leica SP5 confocal laser scanning microscopy (Leica Microsystems).

Proteomic analysis

To analyze MORC2-interacting proteins, total cellular lysates from HEK293T cells stably expressing pCDH, Flag-MORC2 WT, and Flag-MORC2 M276I were subjected to IP assays with anti-Flag magnetic beads (Bimake). After extensive washing, the bound proteins were eluted by boiling in SDS sample buffer, resolved by SDS–PAGE, visualized by Coomassie Blue staining and subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis as described previously (27). Data from LC-MS/MS analysis were searched against Swiss-Prot database by SEQUEST. Trans Proteomic Pipeline software (Institute of Systems Biology, Seattle) was used to identify proteins based on the corresponding peptide sequences with ≥95% confidence. A Protein Prophet 3 probability of 0.95 was used for the protein identification results. The false positive rate was less than 1% (27).

Quantitative PCR

Total RNA was isolated from cultured cells and xenograft tumors from mice using TRIzol reagent (Invitrogen) and converted to cDNA using PrimeScript RT Master Mix (Takara). qPCR analyses were performed in triplicate using SYBR Premix Ex Taq (Takara) on an Eppendorf Mastercycler ep realplex4 instrument (Eppendorf). All real-time data were normalized to GAPDH. Primer information is described in Supplementary Table S5.

Targeted exon sequencing

Genomic DNA was extracted using Rapid Animal Genomic DNA Isolation Kit (Sangon Biotech), and 100 ng of DNA was used to amplify MORC2 fragments surrounding the M276I mutation (c.828G>c) that span the exon 10 region and adjacent two introns by PCR using the indicated primers in Supplementary Table S6. Cycling conditions were one cycle at 95°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were purified using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech). DNA sequencing reaction was carried out using 10 ng of purified PCR product with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosciences) under the following conditions: an initial denaturation at 96°C
Results
Cancer-associated mutations of MORC2 in breast cancer

The development and progression of human cancer is driven by the accumulation of pathogenic somatic mutations that confer oncogenic properties such as growth advantage, tissue invasion and metastasis, angiogenesis, and evasion of apoptosis (29). To identify cancer-associated mutations of MORC2 in human cancer, we analyzed publicly available cBioPortal for Cancer Genomics (http://www.cbioportal.org/) and Catalogue of Somatic Mutations in Cancer (COSMIC; https://cancer.sanger.ac.uk/cosmic) databases. In the cBioPortal database, total 379 mutations (330 missense, 47 truncating, and 2 other mutations) in MORC2 have been reported in various types of human cancer. The positions of missense, truncating, and other mutations are documented in the cBioPortal database.

MORC2 M276I mutation in TNBC Metastatic Progression

MORC2 M276I mutation is dispensable for cell proliferation and cell-cycle progression but promotes cell migration and invasion in vitro

To determine biological functions of the M276I mutation in TNBC development and progression, we carried out a series of

Statistical analysis
All data are presented as the mean ± standard deviation from at least three independent experiments. The Student t test was used for assessing the difference between individual groups and P ≤ 0.05 was considered statistically significant.
functional assays, including CCK-8, colony growth assay, and cell-cycle analysis. Results showed that stable expression of both WT and M276I-mutant MORC2 in MDA-MB-231, Hs578T, and MCF10A cells did not significantly affect cell proliferation (Supplementary Fig. S7A), colony formation (Supplementary Fig. S7B and S7C), and cell-cycle progression (Supplementary Fig. S7D) as compared with its empty vector control. As one of the hallmarks of TNBC is its highly invasive and metastatic behavior (2), we next carried out Transwell migration and Matrigel invasion assays to evaluate the migratory and invasive capability of MDA-MB-231, Hs578T, and MCF10A cells stably expressing empty vector pCDH, Flag-MORC2, and Flag-MORC2 M276I were treated with 100 µg/mL of cycloheximide (CHX) for the indicated times and then subjected to immunoblotting with the indicated antibodies (top). Relative expression levels of exogenous MORC2 (Flag-MORC2/Vinculin) are shown at the bottom. C, Cells stably expressing Flag-tagged WT and M276I-mutant MORC2 were treated with 100 µg/mL of cycloheximide (CHX) for the indicated times and then subjected to immunoblotting with the indicated antibodies (top). Relative expression levels of exogenous MORC2 (Flag-MORC2/Vinculin) are shown at the bottom. D, Cells stably expressing empty vector pCDH, Flag-MORC2, and Flag-MORC2 M276I were subjected to immunofluorescence staining with an anti-Flag antibody. Cell nuclei were counterstained with DAPI.

To rule out the potential effects of endogenous MORC2 on the biological functions of exogenous expression of MORC2, we knocked out endogenous MORC2 in MDA-MB-231 and Hs578T cells using the CRISPR/Cas9 system (33) and then reexpressed empty vector pLVX, WT, and M276I-mutant MORC2 in these MORC2 KO cells. Using MDA-MB-468 cells, which express relatively high levels of endogenous MORC2 (Fig. 1A) as an internal control, we selected the stable clones whose exogenous MORC2 expression levels are comparable to endogenous MORC2 expression levels of MDA-MB-468 cells for subsequent functional studies (Fig. 2A). Consistently, a series of functional assays demonstrated that stable expression of both WT and M276I-mutant MORC2 in MDA-MB-231 and Hs578T cells did not affect cell proliferation (Fig. 2B), colony formation (Fig. 2C), and cell-cycle progression (Fig. 2D). In contrast, expression of M276I-mutant MORC2 enhanced the migratory and invasive potential of MDA-MB-231 and Hs578T cells as compared with its WT counterpart (Fig. 2E and F, respectively). Together, these results suggest that the M276I mutation promotes migration and invasion of TNBC.
MORC2 M276I mutation does not affect primary tumor growth but enhances spontaneous and experimental lung metastasis in vivo

To examine whether expression of mutant MORC2 affects primary tumor growth and spontaneous lung metastasis of TNBC cells in vivo, we chose LM2-4175 cells as a model system as this cell line is highly metastatic to lung (23). First, we knocked out endogenous MORC2 in LM2-4175 cells by the CRISPR/Cas9 system (33) and then reexpressed empty vector pLVX, WT, and M276I-mutant MORC2 in MORC2 knockout cells (Fig. 3A and B). Second, we established an orthotopic xenograft model by incubation of these established cell lines into the mammary fat pads of NOD/SCID mice. Results showed that expression of M276I-mutant MORC2 had no significant effects on the growth of primary LM2-4175 tumors (Fig. 3C–E), but enhanced spontaneous lung metastasis of breast tumors as compared with its WT counterpart (Fig. 3F and G).

To further assess the effects of expression of M276I-mutant MORC2 on TNBC metastatic colonization, we established an experimental lung metastasis model using MDA-MB-231 cells stably expressing pCDH, WT, and M276I-mutant MORC2 by injection into tail vein of nude mice. After 7 weeks of injection, we observed that mutant MORC2-expressing cells significantly increased the number of metastatic nodules in the lungs of mice as compared with empty vector- and WT MORC2-expressing cells (Fig. 3H and I). These results are also supported by histologic examination of lung sections of these mice via H&E staining (Fig. 3J). Together, these results suggest that MORC2 M276I mutation acts as a gain-of-function mutation that promotes TNBC metastatic progression.

MORC2 M276I mutation enhances the binding ability of MORC2 to hnRNPM

To address the molecular mechanisms by which the M276I mutation promotes TNBC invasion and metastasis, we next examined whether the M276I mutation could affect MORC2 interactome by IP coupled with LC-MS/MS analysis (27). To do this, we generated stable HEK293T cell lines expressing pCDH,
Flag-MORC2, and Flag-MORC2 M276I by lentiviral infection (Fig. 4A). Then, total cellular lysates from these established cell lines were subjected to IP analysis using anti-Flag magnetic beads (Fig. 4B), and the IP complex were subjected to the LC-MS/MS analysis (27). To optimize specificity, proteins detectable in pCDH-expressing sample (negative control) were classified as nonspecific contaminants and eliminated from the MORC2-interacting protein list. On the basis of these analyses, we found that total 38 and 23 proteins specially interacted with WT and M276I-mutant MORC2, respectively, while 48 proteins interacted with both WT and mutant M276I MORC2 (Fig. 4C). As expression of both WT and M276I-mutant MORC2 promotes invasion and metastasis of TNBC cells as compared with empty vector control (Figs. 2 and 3), we further analyzed the shared 48 proteins in both groups (Supplementary Table S7). Gene ontology analysis using the Protein Analysis Through Evolutionary Relationships (PANTHER) program (http://www.pantherdb.org/) revealed the molecular functions of those 48 proteins are involved in poly(A) RNA-binding, RNA-binding, cadherin binding involved in cell–cell adhesion, protein binding involved in cell–cell adhesion and cell adhesion (Fig. 4D). Among them, the molecular functions of 22 proteins are enriched in poly(A) RNA-binding and RNA-binding (Supplementary Table S8). Of specific interest, it was noticed that 5 of 22 proteins are involved in RNA alternative splicing, including hnRNPM, splicing factor 3B subunit 1 (SF3B1), serine/arginine-rich splicing factor 3 (SRSF3), splicing factor proline and glutamine rich (SFPQ), and U2 small nuclear RNA auxiliary factor 1 (U2AF1).

To validate the above proteomic results, we next carried out a series of sequential IP and immunoblotting analyses. As shown in Fig. 4E, Flag-MORC2 was associated with endogenous hnRNPM in HEK293T, MDA-MB-231, and Hs578T cells. Moreover, this noted interaction between MORC2 and hnRNPM was enhanced in M276I-mutant MORC2-expressing cells as compared with its WT counterpart, suggesting that the M276I mutation enhances the binding ability of MORC2 to hnRNPM. In contrast, there was no difference in the interaction of SRSF3 with both WT and M276I-mutant MORC2 (Fig. 4F). In addition, we did not observe any interaction of both WT and mutant MORC2 with SRSF1 (Fig. 4G), U2AF1 (Fig. 4H), and SFPQ (Fig. 4I).

Figure 3. MORC2 M276I mutation does not affect primary tumor growth but enhances spontaneous and experimental lung metastasis of TNBC cells in vivo. A and B, Endogenous MORC2 was knocked out in LM2-4175 cells by the CRISPR/Cas9 system (A) and then empty vector pLVX, WT, and M276I-mutant MORC2 were reexpressed in MORC2 knockout cells by lentiviral infection (B). The expression status of endogenous and exogenous MORC2 in these established cell lines was validated by immunoblotting. C–G, LM2-4175 cells stably expressing empty vector pLVX, WT, and M276I-mutant MORC2 were transplanted into the mammary fat pads of NOD/SCID mice. After 6 weeks of incubation, mice were killed and primary tumors and lung tissues were harvested. Images of harvested xenograft tumors (C), tumor weight (D), growth curves of xenograft tumors (E), and representative images of H&E-stained lung sections of mice harboring orthotopic LM2-7175 tumors (F) are shown. G, Quantitative results of spontaneous lung metastasis determined by the percent metastatic lung surface area relative to total lung surface area. H–J, MDA-MB-231 cells stably expressing pCDH, WT, and M276I-mutant MORC2 were injected into nude mice through the tail vein, and the lungs were harvested after 7 weeks of injection. Representative images of lung metastasis (H), quantitative results of lung nodules (I), and representative images of H&E-stained sections of lung tissues (J) are shown.
Consistently, immunofluorescent staining showed that WT and mutant MORC2 colocalized with hnRNPM in MDA-MB-231 and Hs578T cells (Fig. 4J). Further, the enhanced interaction between mutant MORC2 and hnRNPM was validated in cotransfected HEK293T cells with exogenously expressed Flag-MORC2 and HA-hnRNPM plasmids (Fig. 4K).

In addition to hnRNPM (19), other abundant members of the hnRNP protein family, such as hnRNPA1 (34) and hnRNPL (35), are also involved in alternative splicing events driving tumorigenesis and cancer progression. To examine whether MORC2 interacts with either hnRNPA1 or hnRNPL, HEK293T cells stably expressing pCDH, Flag-MORC2, and Flag-MORC2 M276I were subjected to IP analysis using anti-Flag magnetic beads. Immunoblotting analysis showed that there were no detectable interactions of MORC2 with either hnRNPA1 or hnRNPL (Supplementary Fig. S9A). As a positive control, we repeatedly demonstrated an enhanced interaction between mutant MORC2 and hnRNPM as compared with its WT counterpart. To further examine the specificity of the MORC2–hnRNPM interaction, we knocked down hnRNPL in HEK293T cells stably expressing pCDH, Flag-MORC2, and Flag-MORC2 M276I by specific siRNAs targeting hnRNPL. Results showed that knockdown of hnRNPL did not affect the interaction of MORC2 with hnRNPM (Supplementary Fig. S9B). These results suggest that MORC2 interaction with hnRNPM is specific.

hnRNPM is required for M276I-mutant MORC2-mediated invasion and metastasis of TNBC cells

hnRNPM has been shown to promote breast cancer invasion and metastasis (19, 22). To examine whether hnRNPM is involved in mutant MORC2-mediated metastatic progression of TNBC cells, we knocked down endogenous hnRNPM in WT and M276I-mutant MORC2 expressing MDA-MB-231 and Hs578T cells using two specific shhnRNPMs (Fig. 5A). Transwell...
migration and Matrigel invasion assays demonstrated that knockdown of hnRNPM reduced cell migratory (Fig. 5B and C) and invasive (Fig. 5D and E) potential of WT and mutant MORC2-expressing MDA-MB-231 and Hs578T cells when compared with control shNC-infected cells. Consistently, in vivo experimental lung metastasis assays by injection of these established cell lines into tail vein of immunodeficient mice also demonstrated that knockdown of hnRNPM reduced WT- and M276I-mutant MORC2-induced lung metastatic potential (Fig. 5F–H). Collectively, these results suggest that MORC2 M276I mutation promotes invasion and metastasis of TNBC cells through, at least in part, hnRNPM-mediated signaling.

MORC2 M276I mutation regulates hnRNPM-mediated CD44 splicing switch

hnRNPM has been shown to regulate alternative splicing of FGFR2 and CD44, which are implicated in EMT and cancer progression (18, 19). We next examined whether the M276I mutation promotes TNBC progression through regulating hnRNPM-mediated alternative RNA splicing and EMT. To address this question, we chose MCF10A cell line as a model system, which can undergo EMT in response to various extracellular and intracellular signals. MCF10A cells stably expressing pCDH, WT, and M276I-mutant MORC2 were generated by lentiviral infection and overexpression of MORC2 was validated by immunoblotting (Fig. 6A). qPCR analysis showed that mutant MORC2-expressing cells had a significant upregulation of CD44s with a concomitant downregulation of CD44v5/6 (Fig. 6B). In contrast, expression of both WT and mutant MORC2 did not significantly affect the alternative splicing of FGFR2 (Fig. 6C). To verify these results, we next examined the expression levels of CD44s and CD44v5/6 by qPCR in primary tumors from the LM2-4175 orthotopic xenograft models (Fig. 6D). qPCR analysis showed that mutant MORC2-expressing cells had a significant upregulation of CD44s compared with its WT controls (Fig. 6D). Furthermore, knockdown of...
hnRNPM by shRNAs in mutant MORC2-expressing MCF10A cells showed decreased CD44s mRNA with a concomitant increase in CD44v5/6 mRNA (Fig. 6E), indicating that hnRNPM is involved in mutant MORC2-mediated CD44 splicing switch. Following these observations, we carried out RIP assays (36) to examine the binding of MORC2 to CD44 pre-mRNA. RNA that was bound to immunoprecipitated MORC2 was analyzed by qPCR. Results showed that the M276I mutation enhanced the binding ability of MORC2 to CD44 pre-mRNA as compared with its WT counterpart (Fig. 6F). Moreover, knockdown of hnRNPM by a specific shRNA targeting hnRNPM (shhnRNPM #C) in MCF10A cells stably expressing Flag-MORC2 and Flag-MORC2 M276I (Fig. 6G) attenuated the noted binding of MORC2 to CD44 pre-mRNA (Fig. 6H). These results suggest that hnRNPM mediates MORC2 recruitment to the variable exon region of CD44 pre-mRNA.

The CD44 isoform switching is required for breast cancer cells to undergo EMT (19, 21). EMT is characterized by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype with enhanced migratory and invasive properties (37). To determine whether MORC2 M276I mutation regulates hnRNPM-mediated EMT, we examined the expression levels of 6 EMT-associated marker molecules, including E-cadherin, N-cadherin, snail, Slug, Twist, and Zeb1, in MCF10A cells stably expressing pCDH, WT, and M276I-mutant MORC2. Immunoblotting...
(Fig. 6I) and qPCR (Fig. 6J) analyses showed that the mesenchymal markers N-cadherin and Slug were upregulated, while the epithelial marker E-cadherin was downregulated, in M276I-mutant MORC2-expressing cells as compared with pCDH and WT MORC2 expressing cells. Interestingly, expression of M276I-mutant MORC2 in MCF10A cells did not significantly affect the protein and mRNA levels of Snail, Twist, and Zeb1 as compared with its WT counterpart (Fig. 6I and J). These results suggest that Slug may be involved in the MORC2–hnRNPM–CD44 pathway-mediated EMT event. Furthermore, knockdown of hnRNPM in MCF10A cells expressing mutant MORC2 led to an increase in E-cadherin and a decrease in N-cadherin at both protein and mRNA levels (Fig. 6K and L). Together, these results indicate that the M276I-mutant MORC2 promotes TNBC invasion and metastasis through, at least in part, regulating hnRNPM-mediated CD44 splicing program and EMT.

Discussion
In this study, we present several important findings concerning the functional and mechanistic role of MORC2 M276I mutation in TNBC progression (Fig. 7).

First, the M276I mutation is a gain-of-function mutation that promotes TNBC invasion and metastasis. MORC2 is a newly identified chromatin remodeling protein with emerging roles in the maintenance of genome integrity in response to DNA damage (10) and epigenetic regulation of gene transcription (11, 12). Not surprisingly, genetic alternations of this gene may be linked to human diseases. Indeed, MORC2 has been shown to be upregulated in human cancers (39) and promotes the development and progression of breast (16, 40), gastric (41), and liver cancers (42). More interestingly, mutations of MORC2 have been causally implicated in several neurologic diseases (11, 13–15). Although a total of 379 mutations of MORC2 have been documented in various types of human cancer (Supplementary Fig. S1A), the functional consequences of these identified mutations in cancer development and progression have not yet been explored. In the present study, we provide the first evidence that one of such mutations (M276I) was functionally important for TNBC progression. These findings could advance our understanding of the functional role for MORC2 mutations in human cancer, in addition to the documented functional connection between MORC2 mutations and several neurologic disorders (11, 13–15). In addition, these results provide a clue to further investigate the
biological functions of all identified mutations of MORC2 in cancer progression and therapeutic response using well-established recombination-based mutation barcoding library (43, 44) and genetically engineered mouse models in the near future.

Second, the M276I mutation of MORC2 enhances its binding ability to hnRNPM, an abundant component of human hnRNP complexes. The splicing of pre-mRNAs is an essential step of eukaryotic gene expression, which is tightly controlled by the activities of splicing regulators, such as hnRNPs or serine/arginine-rich (SR) proteins (17). By means of a comparison of biochemical approaches, we demonstrated that the M276I mutation does not affect MORC2 protein expression levels, stability, and nuclear localization (Fig. 1), but enhances the binding ability of MORC2 to hnRNPM (Fig. 4). Previous studies have demonstrated that methionine and isoleucine residues are important for forming the protein–protein interaction interface (45, 46). For instance, it has been reported that the M5311 mutation in CDKN2A loses its ability to interact with cyclin-dependent kinase 4 (CDK4; ref. 31). In support of our findings, a recent study on crystal structure of MORC2 suggests that the M276I mutation would cause a conformational change that may affect its interaction with other proteins. In addition, this mutation would not be expected to substantially alter the stability or half-life of MORC2 (47).

Third, we establish a mechanistic link between the oncogenic activity of the M276I mutation and the hnRNPM-mediated CD44 splicing program. Previous studies have shown that hnRNPM has key roles in regulating pre-mRNA splicing of several cancer relevant genes such as CD44 (19). CD44 splice isoform switching from the splicing variants (CD44v) to the standard form (CD44s) is essential for EMT and breast cancer metastasis (19, 21). Consequently, depletion of CD44s inhibits breast cancer metastasis in mice (48), and expression of CD44s rescues the impaired metastatic phenotype by hnRNPM depletion (19). qPCR analysis demonstrated that expression of M276I-mutant MORC2 promotes a switch of CD44 splice isoform from CD44v to CD44s (Fig. 6). These findings are consistent with a recent report that hnRNPM promotes breast cancer metastasis through activating CD44 splicing program and EMT (19). Moreover, knockdown of hnRNPM attenuates the M276I mutation-mediated CD44 splicing switch, EMT, cell migration, invasion, and metastasis (Figs. 5 and 6). These results indicate that the M276I mutation promotes breast cancer invasion and metastasis through, at least in part, activating hnRNPM-mediated CD44 alternative splicing switch and EMT. Given that alternative splicing events of CD44 have been widely documented in various types of human cancer including melanoma (49), we cannot rule out the possibility that other MORC2 mutations may regulate the development and progression of human cancer through regulating CD44 splicing program. Interestingly, a recent report showed that CD44s activates the expression of EMT-inducing transcription factor Zeb1, which in turn controls CD44s splicing by repression of epithelial splicing regulatory protein 1, thus forming a CD44s–Zeb1 feedback loop to maintain EMT and stemness properties in cancer cells (50).

In conclusion, findings presented here demonstrate a gain-of-function mutation of MORC2 in TNBC progression and provide mechanistic insights into the oncogenic mutation of MORC2 involving hnRNPM-mediated alternative splicing and EMT. These findings broaden our understanding of the genetic heterogeneity of TNBC and highlight the importance of analyzing unique patient-derived mutations for the development of personalized therapies in the era of precision oncology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: F.-L. Zhang
Development of methodology: F.-L. Zhang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F.-L. Zhang, J.-L. Cao, H.-Y. Xie, R. Sun, L.-F. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F.-L. Zhang, J.-L. Cao, H.-Y. Xie, L.-F. Yang
Writing, review, and/or revision of the manuscript: F.-L. Zhang, D.-Q. Li
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F.-L. Zhang, J.-L. Cao, H.-Y. Xie, R. Sun, L.-F. Yang
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Cancer-Associated MORC2-Mutant M276I Regulates an hnRNPM-Mediated CD44 Splicing Switch to Promote Invasion and Metastasis in Triple-Negative Breast Cancer

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