Filamin A Is a Potential Driver of Breast Cancer Metastasis via Regulation of MMP-1

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Recurrent metastasis is a major fatal cause of breast cancer. Regrettfully, the driving force and the molecular beneath have not been fully illustrated yet. In this study, a cohort of breast cancer patients with locoregional metastasis was recruited. For them, we collected the matched samples of the primary tumor and metastatic tumor, and then we determined the mutation profiles with whole-exome sequencing (WES). On basis of the profiles, we identified a list of deleterious variants in eight susceptible genes. Of them, filamin A (FLNA) was considered a potential driver gene of metastasis, and its low expression could enhance 5 years’ relapse survival rate by 15%. To prove the finding, we constructed a stable FLNA knockout tumor cell line, which manifested that the cell abilities of proliferation, migration, and invasion were significantly weakened in response to the gene knockout. Subsequently, xenograft mouse experiments further proved that FLNA knockout could inhibit local or distal metastasis. Putting all the results together, we consolidated that FLNA could be a potential driver gene to metastasis of breast cancer, in particular triple-negative breast cancer. Additional experiments also suggested that FLNA might intervene in metastasis via the regulation of MMP-1 expression. In summary, this study demonstrates that FLNA may play as a positive regulator in cancer proliferation and recurrence. It provides new insight into breast cancer metastasis and suggests a potential new therapeutic target for breast cancer therapy.

Keywords: breast cancer, metastasis, FLNA, MMP-1, EMT

INTRODUCTION

Breast cancer has become the most common cancer and the main cause of cancer death in women. In 2020, there are an estimated 2.3 million new cases of breast cancers worldwide (11.7%), surpassing lung cancer (11.4%) in number for the first time (1). The global incidence rate and mortality rate of breast cancer are still increasing annually, and the increase in the lower sociodemographic index (SDI) countries is larger than that of higher SDI countries (2). The yearly-increasing cancer cases not only put heavy psychological pressure on patients but also raise great economic burdens to society and the country. In the new era of cancer therapy, breast cancers can be classified into four types according to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 (3): Luminal A [ER+ and/or PR+, HER2−, Ki-67 < 14%], Luminal B [ER+...
and/or PR+, HER2+; ER+ and/or PR+, HER2−, Ki-67 > 14%), HER2 positive (HER2+) [ER−, PR−, HER2+], and triple-negative breast cancer (TNBC) [ER−, PR−, HER2−]. The patients of specific cancer will receive individual therapy regimens to achieve the best therapeutic effect.

However, the tumor has the characteristics of heterogeneity, easy mutation of the genome, and strong adaptability to the external environment changes, which make it insensitive or resistant to various drug treatments and vulnerable to local recurrence or distal migration. Previous studies showed that over 25% of early breast cancer patients had metastases at the time of initial diagnosis (4), and about 30% of them would develop metastatic breast cancer in the future (5). The clinical outcome of breast cancer depends on the biology, extent, and location of metastasis. The luminal breast cancer has a higher propensity to develop bone metastases, while TNBC tends to metastasize to the lungs and brain (6, 7). Although the 5-year survival rate of breast cancer is increasing year by year, drug resistance, recurrence, and metastasis are still urgent problems in the treatment of cancer.

The occurrence and development of breast cancer are the results of the interaction of genes and environment, and the effect of the environment can also be manifested through genetic or epigenetic changes (8). Mark et al. found that BRCA plays an important role in breast cancer metastasis. PALB2, a key partner of BRCA1/BRCA2, was involved in DNA damage repair and tumor suppression activity; thus, its mutation can lead to increased susceptibility to breast cancer (9). In addition, the Max team found that loss of p53 in cancer cells promoted Wnt secretion and triggered neutrophil inflammation through stimulating tumor-associated macrophages to produce IL-1β (10). There is a causal relationship between neutrophils and metastasis, in which the high neutrophil-to-lymphocyte ratio could promote the metastasis of breast cancer and reduce the survival rate of patients (11). What is more, PTEN is a tumor suppressor gene (12) related to a variety of human cancers and a major negative regulator of the PI3K/Akt signaling pathway (13). Abdullah et al. found that inhibition of PTEN can promote the activation of the PI3K/Akt pathway and further control the proliferation and development of breast cancer stem cells (CSCs) (14). Although many valuable efforts have been made, the genetic driving force underlying the recurrence and distal metastasis of breast cancers largely remains unexplored.

In this study, we collected nine pairs of primary and recurrent tumors of breast cancer patients, determined the mutation profiles with whole-exome sequencing (WES), identified potential driver genes, and further validated them with both cells and animal experiments. We intended to provide new insights into breast cancer metastasis and suggest potential new therapeutic targets for precise breast cancer therapy.

RESULTS

Identification of Potential Driver Genes to Breast Metastasis

The WES of nine cohort patients (18 tissue samples) yielded a total of 47,407 high-quality and non-redundant somatic variants, including 27,845 single-nucleotide variants (SNVs), 16,679 insertions and deletions (indels), and 1,461 stopgain and stoploss mutations. To identify potential metastatic driver genes to breast cancers, we performed serial bioinformatics analyses (Figure 1A). The analyses were made based on an open assumption of the following: 1) the cohort patients may have different genetic backgrounds of metastasis (Table 1), 2) the metastatic driver gene mutations could be harmful (deleterious) to the cells, and 3) the deleteriousness of gene mutations would be a benefit to metastasis. Accordingly, we first narrowed down the whole mutation profiles to the harmful ones by integrating deleterious prediction results of multiple bioinformatics tools. A list of 2,755 deleterious mutations was obtained in the primary cancer samples consistently, including one synonymous SNV, 2,166 non-synonymous SNVs, 304 non-frameshift indels, 224 frameshift indels, 45 stopgain mutations, and 15 stoploss mutations. These deleterious mutations were distributed on all chromosomes except the Y chromosome, and the majority of them occurred in protein-coding regions (Figure 1B). Similarly, we obtained 2,533 deleterious mutations in the metastatic cancer samples consistently, including 2,068 non-synonymous SNVs, 233 non-frameshift indels, 196 frameshift indels, 24 stopgain, and 12 stoploss mutations. These mutations had similar chromosome distribution as those of primary cancer samples and were also located mainly at protein-coding regions (Figure 1C). These results manifest that primary tumors and metastatic tumors in this study have no genetic difference in general. Furthermore, we extracted the susceptible genes that have deleterious mutations in at least two samples of either primary tumor or metastatic tumor. The criteria eventually identified eight susceptible genes shared by primary/metastatic tumors; they were COMP, FLNA, FOXO3, HSPA2, ITPR3, PIK3R2, NF1, and TP53 (Figures 1D, E). Literature surveillance manifested that these genes played multiple roles in cancers, such as cell growth, cell apoptosis, cell migration, and cell invasion (15–22).

To further connect these genes with metastasis, we performed a progression-free survival (PFS) analysis on the deleterious mutants within the nine-member cohort (Figure 2A). Of the eight susceptible genes, only one gene (FLNA) exhibited significant change (two-tail unpaired Wilcoxon rank-sum test, p < 0.1) of PFS when the deleterious mutation occurred in primary cancer, which extended the PFS. In particular, the patients with deleterious mutations in FLNA in primary tumors had an average PFS value (n = 5, average PFS = 56 months) of about 2.5 times larger than that of those without the mutations (n = 4, average PFS = 23.3 months). Many of the deleterious mutations are located at the first few repeats of the immunoglobulin (Ig) domain (Table 2), causing the dysfunction of FLNA protein. In addition, we performed the survival analysis on basis of 392 TNBC patients from 55 independent experiments to examine the gene expression level of susceptible genes on metastasis, assuming that deleterious mutations would reduce the corresponding gene expressions. The result manifested that low expression of FLNA would significantly enhance 5 years’ relapse-free survival rate by 15% compared to that high expression group (Figure 2B). Putting all the data together, we
speculate that FLNA could be one of the positive factors to breast cancer metastasis. Deleterious mutation of FLNA gene, particularly at its first few Ig repeats, would reduce its expression and thus resist metastasis.

Cellular Consequence of Defected FLNA via Knockout Experiments
We examined the protein level of FLNA in breast mammary epithelial cells (MCF-10A) and different breast cancer cell lines mentioned in the Material and Methods with Western blotting. Comparatively, FLNA is highly expressed in MDA-MB-231 (Figure 3A). Hence, we constructed knockout cells of MDA-MB-231. Subsequent Western blotting validated the successful knockout of FLNA in different target cells (Figure 3B). Accordingly, we chose two knockout cell lines of MDA-MB-231, target 1 and target 2, namely, FLNA/KO-1 and FLNA/KO-2, respectively, for cell proliferation and migration assays. The results showed that knockout of FLNA caused a decrease of proliferation for 76.35% in FLNA/KO-1 and 75.61% in FLNA/KO-2 cells at 72 h (Figure 3C), and the wound healing capability of cells
dropped 43.95% and 43.84% at 48 h, respectively (Figures 3D, E). Besides, the migration and invasion ability of FLNA/KO-1 cells decreased 91.17% and 87.06%, and the FLNA/KO-2 cells decreased 76.43% and 75.48%, respectively (Figures 3F, G). Comparatively, the negative control (NC) showed no significant difference from the wild-type MDA-MB-231 in all aspects of cell proliferation, wound healing, migration, and invasion. These results confirm that knockout of FLNA is not fatal to cancer

### TABLE 1 | Detailed information of 9 breast cancer patients.

| Sample ID | Age | TNM of initial diagnosis | ER | PR | HER2 | DFS (months) |
|-----------|-----|--------------------------|----|----|------|--------------|
| Patient1  | 56  | T2N2M0                   | ++ | ++ | –    | 62           |
| Patient2  | 56  | T2N0M0                   | +  | –  | –    | 80           |
| Patient3  | 46  | T3N2M0                   | ++ | ++ | –    | 28           |
| Patient4  | 44  | T1N0M0                   | ++ | ++ | –    | 72           |
| Patient5  | 68  | T1N0M0                   | +++| –  | +    | 22           |
| Patient6  | N.A.| T1N0M0                   | –  | +  | ++   | 38           |
| Patient7  | N.A.| T1N0M0                   | –  | –  | ++   | 42           |
| Patient8  | 58  | T2N0M0                   | ++ | +  | –    | 24           |
| Patient9  | 30  | T1N0M0                   | –  | +  | –    | 5            |

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; DFS, disease-free survival.

N.A., Not Available.

FIGURE 2 | Exploring the relationship between eight susceptible genes and prognosis of breast cancer patients. (A) A progression-free survival (PFS) analysis on the deleterious mutants within the nine-member cohort. (B) The survival analysis was on basis of 392 triple-negative breast cancer (TNBC) patients from 55 independent experiments.
Knockout of FLNA Decreases Xenograft Tumor Growth and Metastasis

To further study the functional role of FLNA, we used wild-type MDA-MB-231, FLNA/NC, FLNA/KO-1, and FLNA/KO-2 stably transfected cell lines to establish xenograft models. Each model had five repeated cases. We monitored the expression of FLNA in mouse in situ tumors and found that FLNA/KO groups decreased by 46.25% and 46.91% (Figure 4A). Compared to wild type and NC, the FLNA/KO mice had significantly slower tumor growth rate and smaller tumor volume (declined 61.72% and 68.30%, respectively) by 28 days (Figure 4B). The tumor volume of two cases with ipsilateral chest wall metastasis was recorded in Figure 4E. H&E stain of the xenograft tumor showed that there may exist two morphologies of cancer cells in the orthotropic tumor (Figure 4C): the cancer cells near the margin of in situ tumor were large, with obvious atypia large nucleus, common mitosis, and basophilic cytoplasm (indicated by yellow arrows). In contrast, the cancer cells in the center of in situ tumor were small or medium-sized, more consistent in shape, mostly round or oval, and loosely arranged and had fewer mitosis (indicated by black arrows). This phenomenon may be owing to the tumor growth exceeding the growth rate of the blood vessels providing nutrition, resulting in tissue necrosis or even liquefaction of some central tissues due to insufficient energy supply. We also observed that the morphology of lung metastatic cancer cells had large cells, rich chromatin, and common mitotic images. The liver metastasis cells from breast cancer were small and loosely arranged. GATA3 colored the nucleus light brown, indicating that the tumor was of breast origin. Furthermore, we also detected the expression of Ki-67 in tumors in situ and metastases. The result manifested that Ki-67 is expressed low in the FLNA/KO groups and high in the other groups, suggesting a strong ability of cell proliferation (Figures 4G, H).

FLNA Regulated the Expression of MMP-1

Previous studies have shown that tumor metastasis is closely related to epithelial-to-mesenchymal transition (EMT) (24) and extracellular matrix (ECM) (25), and they were recognized as critical factors in governing metastatic colonization. In the process of EMT, the cells showed decreased adhesion and increased motility, which led to metastasis of malignant tumor cells (26). ZO-1 is indispensable for tight junction formation and function (27), in which mutation can induce EMT (28). Slug is a widely expressed transcriptional repressor protein that, when combined with the integrin promoter, inhibits integrin expression and leads to decreased cell adhesion (29). β-Catenin can activate slug, which is related to tumorigenesis (30). Vimentin is highly expressed in a variety of tumors, which is closely related to promoting tumor growth, invasion, and poor prognosis (31). Therefore, we first detected the expression of EMT-related proteins and found that FLNA/KO had no significant effect on EMT (Figure 5A). Therefore, we concluded that FLNA may not affect the metastasis of breast cancer through the EMT pathway, and there may exist other ways. After that, we determined the mRNA levels of several conventional ECM components such as MMP-1, MMP-2, and MMP-9 in response to FLNA knockout with RT-qPCR. Interestingly, of these major ECM components, only MMP-1 cells; however, it can repress cell proliferation, migration, and invasion. Immunofluorescence (IF) assay showed that FLNA was mainly distributed in the cytoplasm and nucleus (Figure 3H). Compared with wild-type and NC group cells, FLNA/KO cells had smaller sizes and poor cytoskeleton development (Figure 3H).

| Sample | Start | End | Ref | Alt | Type | AA change | FLN repeat |
|--------|-------|-----|-----|-----|------|-----------|------------|
| Patient1-M | 154360534 | 154360570 | GCGGGGCGGGGAGGCCCCAGCCTCCTGCAACGCC | Frameshift deletion | P1075fs | 9 |
| Patient2-M | 154362486 | 154362491 | TGGCAT | Non-frameshift deletion | S31833del | 6 |
| Patient3-M | 154359888 | 154359891 | TGGC | Frameshift deletion | A1274fs | 11 |
| Patient4-M | 154362486 | 154362491 | TGGCAT | Non-frameshift deletion | S31833del | 6 |
| Patient5-M | 154362486 | 154362491 | TGGCAT | Non-frameshift deletion | S31833del | 6 |
| Patient6-M | 154361688 | 154361688 | GCCACAGCA | Frameshift deletion | V976fs | 8 |
| Patient7-M | 154366374 | 154366374 | C | Non-synonymous SNV | G3968S | 2 |
| Patient8-M | 154362486 | 154362491 | TGTCAT | Non-frameshift deletion | S31833del | 6 |
| Patient9-M | 154352600 | 154352600 | GCCAGACA | Frameshift deletion | V976fs | 8 |
| Patient10-M | 154359888 | 154359891 | TGGC | Frameshift deletion | A1274fs | 11 |
| Patient11-M | 154361688 | 154361688 | GCCACAGCA | Frameshift deletion | V976fs | 8 |

SNV, single-nucleotide variant.
decreased after FLNA knockout (Figure 5B). This result was further confirmed in protein level (Figure 5C). We detected the expression of MMP-1 in situ and metastatic tumors of breast cancer xenograft in mice, and we found that MMP-1 decreased by 44.23% and 47.23% in FLNA/KO-1 and FLNA/KO-2 groups, respectively (Figures 5D, E). These results indicated that FLNA could affect the metastasis of breast cancer cells by regulating the expression of MMP-1.
FIGURE 4  | (A) Representative immunohistochemistry (IHC) images (scale bar, 100 μm) of tissue sections of in situ tumor from the four groups. FLNA was stained brown in cytoplasm and nucleus. Representative IHC images (scale bar, 100 μm) of tissue sections of in situ tumor from the four groups. FLNA was stained brown in cytoplasm and nucleus. Beside it is the average optical density (AOD) value of FLNA in situ tumor tissues. (B) The volume (mm³) of in situ tumor in each group was recorded every 3 days. (C) Representative H&E (scale bar, 100 μm) staining of tissue sections of different organs from the four groups (n = 5). The cells near the margin of in situ tumor are indicated by yellow arrows, and the center cancer cells were indicated by black arrows. (D) Representative IHC images (scale bar, 50 μm) of tissue sections of different organs from the four groups (n = 5). GATA3 was stained light brown in the nucleus, which was mainly expressed in the nucleus and often used for detecting the breast origin tumor. (E) The volume (mm³) of ipsilateral chest wall metastatic tumors of two mice in MDA-MB-231 and FLNA/KO groups. (F) Pictures of ipsilateral chest wall metastasis and peritoneal metastasis in nude mice in MDA-MB-231 and FLNA/NC groups. Black arrows indicate tumor location. In the H&E (scale bar, 100 μm) staining results, the marginal cells of metastatic tumor are indicated by yellow arrows, and the central cells are indicated by black arrows. GATA3 (scale bar, 50 μm) colored the nucleus light brown. (G) The expression level of Ki-67 in different tissues (scale bar, 100 μm). Ki-67 colored the nucleus brown. (H) The Ki-67 AOD value of different tumor tissues. Data are presented as mean ± SD. *p < 0.05, **p < 0.001, ***p < 0.0001, ns, no significance.
FIGURE 5  |  (A) Epithelial-to-mesenchymal transition (EMT)-related pathway protein expression level and the relative density normalized to GAPDH. (B) The mRNA expression level of MMP-1, MMP-2, and MMP-9 in different group cells. (C) The protein expression level of MMP-1, MMP-2, and MMP-9 in different group cells and the quantitative and statistical analysis results of proteins. (D) Expression of MMP-1 in different tissues (scale bar, 100 μm). (E) The MMP-1 AOD value of in situ tumor tissues in four groups. ****p < 0.0001, ns, no significance.
Overexpression of MMP-1 Promotes Cell Growth and Migration

We overexpressed MMP-1 in two FLNA knockout stably transfected cell lines, FLNA/KO-1 and FLNA/KO-2, in an attempt to explore whether MMP-1 can reverse the antitumor effect. We used PCR and Western blotting to monitor the transfection efficiency and expression level of MMP-1. PCR results showed that the overexpression efficiency of KO-1/P1 and KO-2/P1 was 13.3 times and 38.85 times higher than that of KO-1/NC and KO-2/NC, respectively (Figure 6A). Western blotting showed that the expression levels of MMP-1 in KO-1/P1 and KO-2/P1 were respectively 23.55 and 11.67 times higher than those in the NC (Figure 6B). Overexpression of MMP-1 could promote the proliferation of FLNA/KO cell lines, which increased by about 1.24 times at 72 h (Figure 6C). The wound healing capability of the two cell lines increased by 3.17 and 5.89 times at 48 h, respectively (Figures 6D, E). In addition, we also observed changes in migration and invasion. Transwell experiment showed that the number of cell migration of KO-1/P1 and KO-2/P1 was respectively 3.23 and 3.08 times higher than that of NC groups (Figures 6F, G), and the invasion ability was increased by 2.6 and 2.75 times (Figures 6F, H), respectively. These results suggest that overexpression of MMP-1 can reverse the antitumor effect of FLNA knockout to a certain extent.

DISCUSSIONS

Early studies reported that the genetic variants in TP53 (32), BRCA1 (33), and EGFR (34) could intervene in tumorigenesis and tumor development. Regretfully, none of these mutations were observed in this study. Instead, this study identified several novel deleterious variants likely associated with the recurrence of breast cancers in a small cohort. Of them, filamin A (FLNA)
showed the most potential in regulating breast cancer metastasis and PFS, in particular in TNBCs. FLNA is a 280-kDa protein that can be cleaved into two fragments of 170 kDa (ABD + Rep.1–15) and 110 kDa (Rep.16–24). The latter one is near the C-terminal region, which can be further cleaved into a 90-kDa fragment (Rep.16–23, FLNA-C) (35). Previous studies had demonstrated FLNA could intervene in cancer development via promoting or inhibiting the expression of some genes. For instance, a metadata analysis on basis of 392 TNBC samples from 55 separate experiments suggested that low expression of FLNA could significantly enhance the 5-year relapse survival rate compared to that of high expression. A large-scale clinical study revealed that the overphosphorylation of FLNA Ser2152 was associated with a poor prognosis of hepatoma, which may be a potential prognostic biomarker of primary liver cancer (36). Bojan et al. found that microRNA-200c could reduce FLNA by inhibiting the transcription factors c-Jun and MRTF/SRF and thereby affect the polarization of breast cancer cells, resulting in the cell morphology changes and decreased motor ability (37). Another study showed that ADP ribosylation factors like 4C (Arl4C) could interact with FLNA rep.22 in a GTP-dependent manner to induce filopodium formation and promote cell migration (38). Therefore, we speculate that FLNA plays an important role in tumor metastasis. Although FLNA was reported to be highly expressed in cancers (39–41), its connection with breast cancer metastasis has not been well investigated previously.

In this study, we proposed that FLNA could be a positive factor in breast cancer metastases for the first time. The in vitro cell assays confirmed the fundamental function of FLNA as a scaffold in constructing the actin cytoskeleton. Knockout of FLNA did not sacrifice cells; however, it impaired cell cytoskeleton and largely reshaped the cells to a smaller size. Thereby, the proliferation, migration, and invasion of cancer cells were significantly weakened. The in vivo xenograft mouse model further consolidated that knockout of FLNA largely repressed the local and distal metastases of transplanted tumors. All shreds of evidence strongly support that FLNA is a positive driver gene of breast cancer metastasis.

In addition, we conducted preliminary research to investigate the possible mechanism underlying FLNA-regulated metastasis. We monitored the expression changes of four common EMT markers vimentin, β-catenin, Slug, and ZO-1 proteins after FLNA knockout. Previously, vimentin was reported to promote tumor metastasis through positive regulation of Axl (AXL Receptor Tyrosine Kinase) in breast cancer (42). However, we did not find any significant changes in these EMT phenotypic proteins after FLNA knockout. We considered that there might exist an alternative route like ECM, to promote tumor metastasis other than the EMT. Matrix metalloproteinas (MMPs) are a group of calcium-dependent zinc-containing endopeptidases, which mainly function in degrading ECM. MMP-1 is a ubiquitously expressed collagenase in ECM that can degrade type I, II, and III collagen (43). In this study, we found that knockout of FLNA significantly reduced the expression of MMP-1 but did not affect the other two ECM members MMP-2 and MMP-9. However, how FLNA regulates MMP-1 has not been fully elucidated. Bandaru et al. found that FLNA-C can be cleaved off by calpain to stimulate adaptive angiogenesis by transporting multiple transcription factors into the nucleus (44). Here, we found FLNA expressed in both the nucleus and cytoplasm of TNBC cell MDA-MB-231. Therefore, we speculated that FLNA-C might act as a transcription factor and directly or indirectly promote the expression of MMP-1 mRNA. Alternatively, prior works also found that FLNA could physically interact with integrin beta-1 (ITGB1) (45). ITGB1 can bind to various ECM components, which participate in multiple extracellular effects such as adhesion, ECM degradation, and cell invasion (46). Rizwan et al. found that stimulation of ITGB1 resulted in higher MMP activities in metastatic cancer cells (47). Accordingly, we monitored the expression of MMP-1, MMP-2, and MMP-9 in response to FLNA knockouts. The results manifested that only MMP-1 was significantly repressed in FLNA knockout cells. Previously, several works have suggested MMP-1 as a promoter of metastasis. For instance, overexpression of MMP-1 could promote the growth of xenograft tumors and the formation of brain metastasis (48). MMP-1 combined with ADAMTS1 can activate osteoclast differentiation by modulating the bone microenvironment in favor of osteoclastogenesis, to promote breast cancer bone metastasis (49). In summary, we speculate that FLNA likely promotes breast cancer metastasis in two different ways (Figure 7). FLNA-C interferes with the nucleo-cytoplasmic transportation of transcription factors to regulate MMP-1 expression, or FLNA regulates MMP-1 activities via interacting with the ITGB1-mediated signaling. To validate the mechanisms, extensive studies are desired in the future.

MATERIAL AND METHODS

Patients and Specimens
In this study, a cohort of nine breast cancer patients was recruited from the Cancer Hospital of Harbin Medical University. The study was approved by the Ethics Committee of Cancer Hospital of Harbin Medical University and Xiang’an Hospital of Xiamen University (XAHLL2020013) and abided by the Declaration of Helsinki principles. All patients were confirmed with recurrence of breast cancer, and the recurrent tumors were locoregional metastases (chest wall). The medical information of patients was briefly summarized in Table 1, and the individual information was replaced by anonymous digital codes. For every member in the cohort, paired tissue samples of the primary tumor and recurrent tumor were collected by surgery operation. The tumor tissues were then routinely formalin-fixed and paraffin-embedded (FFPE).

DNA Extraction and Whole-Exome Sequencing
For each tissue sample, 3–5 μg of genomic DNA was applied for quality control, and its integrity was checked by the agarose electrophoresis. The whole exome was captured using the MGIEasy Exome Library Prep Kit (BGI, Shenzhen, China), and the library for sequencing was prepared according to the
manufacturer’s instructions. The WES was performed by the Beijing Genome Institute (BGI, Shenzhen, China) using the BGIseq-500 platform in a 100-base pair (bp) paired-end mode.

**Exome Data Preprocessing, Variant Calling, and Variant Annotation**

Before variant calling, the quality control of the exome data was conducted by FastQC (v.0.11.9, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Trimmomatic (v.0.39; parameters: LEADING = 5, TRAILING = 5, SLIDING WINDOW:5:20, MINLEN = 50) (50) to remove adapter sequences and discard low-quality reads. The clean reads were mapped to the human reference genome (GRCh38.p13) using the Burrows-Wheeler Aligner (BWA, v.0.7.17; parameters: mem -t 4 -M -R) (51). The Genome Analysis Toolkit (GATK, v.4.1.2.0) (52) and Samtools (v.1.9) (53) were used for basic processing, duplicate marking, and base quality score recalibration (BQSR). Calling of somatic mutations was conducted with GATK Mutect2 (default parameters). The variants were further annotated with ANNOVAR (v2019sep29) (54). The datasets produced by this study were available in the Genome Variation Map portal repository at the following URL: https://ngdc.cnbc.ac.cn/gvm/ (accession number: GVM000287).

**Determination of Deleterious Variants**

The deleterious variants for recurrent tumors were determined by satisfying several criteria: 1) the variant genotype was supported by a sequencing depth of >10. 2) Only four types of non-synonymous mutations at the exon region were involved in this study, including SNV, frameshift indel, non-frameshift indel, and stopgain and stoploss. 3) The occurrence of mutation in the Eastern Asian population was ≤1% as recorded in the ExAC_EAS database (55). 4) The variant was deleterious to protein. The deleteriousness of these non-synonymous variants was evaluated with multiple tools by different variant types. For SNVs, 15 tools were used to quantify the deleteriousness, including SIFT (56), Polyphen-2 HDIV (57), Polyphen-2 HVAR (57), LRT (58), MutationTaster (59), MutationAssessor (60), FATHMM (61), PROVEAN (62), VEST3 (63), MetaSVM (64), MetaLR (64), M_CAP (65), CADD (66), FATHMM-MKL (67), and fitCons (68). The variants were taken as deleterious variants if they were predicted pathogenic by more than twelve tools. For variants of frameshift Indel and stopgain, the deleteriousness was mainly assessed by checking the haploinsufficiency in the clinGen database (69). In addition, VEST-Indel (70) was also adopted to evaluate the deleteriousness of frameshift Indel and non-frameshift Indel mutations. The mutations with VEST Score ≥0.85 and VEST p-value ≤0.01 were considered as deleterious mutations. All stoploss variants were retained, as they were obviously harmful by adding part of a protein sequence.

**Survival Analysis**

The survival analysis was conducted based on the database (71), which included 7,830 unique samples from 55 Gene Expression Omnibus (GEO) independent datasets to assess the impact of gene expression on breast cancer metastasis. Accordingly, overall 392 TNBC samples were involved in this analysis. The survival analysis was performed with the Kaplan–Meier Plotter web server (71).

**Cell Culture**

All cell lines (including the normal breast mammary epithelial cell line MCF-10A, luminal A breast cancer cell lines MCF-7 and T-47D, luminal B breast cancer cell line BT-474, TNBC cell lines MDA-MB-231 and BT-549, and HER2+ cell line SK-BR-3) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). MCF-10A were grown in MEGM kit (Lonza/Clonetics, CC-3150) with cholera toxin (Sigma, St. Louis, MO, USA; C8052) of 100 ng/ml. MCF-7 were
grown in MEM (GIBCO, Grand Island, NY, USA; 41500034) with NaHCO3 1.5 g/L, sodium pyruvate 0.11 g/L, and 0.01 mg/ml of bovine insulin. T47D and SK-BR-3 were grown in DMEM (GIBCO by Life Technologies, C11995500BT). BT474 was grown in Roswell Park Memorial Institute (RPMI) 1640 (GIBCO by Life Technologies, C11875500BT). MDA-MB-231 and MDA-MB-549 were grown in DMEM. All cell culture media were supplemented with 10% fetal bovine serum (FBS; GIBCO, 42A0378K) and 1% penicillin/streptomycin (GIBCO, 15140122). All cells were grown at 37°C and 5% CO2.

Gene Knockout With CRISPR/Cas9 Technology

CRISPR/cas9 plasmid was synthesized by the Jikai Gene Company (Shanghai, China). The GV392 CRISPR-Cas9 vector had three gene-specific regions of the guide RNA (gRNA) sequences. The three gRNA sequences for FLNA were as follows: target 1: 5′-CAC CGGCCGCGTTACCAATGCGCGAG-3′; target 2: 5′-CACCG CGAGGTGACCGGGACTCATA-3′; and target 3: 5′-CACCAG AAGCGGGCAGAGTTCACTG-3′. The sequence 5′-CGCTTCC GCGGCCGCTTCAA-3′ of empty plasmid was used for NC (FLNA/NC). Transfection experiments were carried out in six-well plates. When the cell confluence reached 30%~40%, the transfection solution was added (V = MOI × Cell number/Virus concentration). After 24 h, stable FLNA knockout of MDA-MB-231 cells was obtained with 1 μg/ml of puromycin selection. FLNA knockout efficiency was evaluated by Western blot.

The overexpression MMP-1 plasmid was synthesized by the Jikai Gene Company (Shanghai, China), and it was anti-Blasticidin S. The sequencing results after successful plasmid construction are been shown in Supplementary Materials 3.

Western Blot Analysis and Antibodies

The cells were fully lysed with RIPA (Lablead, Beijing, China; R1090), and the protein concentration was detected by bicinchoninic acid (BCA) kit (YEASEN, Shanghai, China; 20201ES76). The supernatant was then treated with 1/4 volume of 5× SDS-PAGE (YEASEN, 2031ES05), and cooked at 100°C for 10 min. Because the FLNA protein was large in molecular weight (280 kDa), gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA; RIDB96261) at 250 mA for 3 h. The other protein transfer conditions were 80 V, 1.5 h. The primary antibody was incubated at 4°C overnight, and the secondary antibody was incubated at room temperature for 1 h. The details of antibodies are presented in Supplementary Table 1. To analyze the pictures, ImageJ was chosen.

Quantitative Real-Time PCR (RT-qPCR)

The cells were fully lysed with TRIzol (ambion, Austin, TX, USA; 210805) to extract total RNA. Genomic DNA was removed, and the mRNAs were reverse transcribed into cDNA using Takara reverse transcription kit (Takara, Mountain View, CA, USA; RR047A). The PCR was conducted in a 20-μl system, including 2 μl of cDNAs, along with 0.4 μl of forward and reverse primers, 10 μl of SYBR (YEASEN, 11201ES03), and 7.2 μl of water. The specific primers for target RNA detection are given in Supplementary Table 2. Relative expression of each target gene was normalized to GAPDH mRNA level and calculated with the 2−ΔΔCt method (72).

Cell Proliferation

Cells were seeded onto 96-well (3 × 103 cells/well) plates. Before measuring the optical density (OD), the cells were incubated with 10 μl/well of Cell Counting Kit-8 (CCK-8) (APEXBio, Houston, TX, USA; K1018320180830) for 2 h. The OD value was measured at 450-nm spectrum by intervals of 0, 24, 48, and 72 h. The cell growth curve was drawn according to the OD value. Cell growth rate = (control group OD – experimental group OD)/control group OD × 100%.

Cell Movement, Migration, and Invasion

Wound-Healing Assay

The wound-healing assay was initiated with 1 × 106 cells/well in the six-well plate. When the cell confluence was greater than 95% or just full, a straight line was drawn in the hole. Then the cells were continuously cultured in the serum-free medium to reduce the effect of cell proliferation on wound healing. The scratch changes were recorded by taking photos at 0, 12, 24, 36, and 48 h. The scratch area at each time point is defined with ImageJ by setting the parameter of Wound-healing percentage = (Initial area – each time point area)/Initial area × 100%.

Migration Assay

The cells were starved with the serum-free medium for 8 h and inoculated into transwell chambers. Each upper chamber was seeded with 2 × 104 cells in 100 μl of serum-free medium (3.5 × 105 cells of overexpressing MMP-1 were seeded into the upper chamber). A total of 800 μl of complete medium containing 10% FBS was added to the lower chamber. After 24 h, the cells were fixed with 4% paraformaldehyde (PFA; Biosharp, anhui, China, 71041800) and stained with crystal violet (Solarbio, G1063), and the upper cells were carefully wiped off with a cotton swab. Three visual fields were randomly selected to take photos and count under the microscope.

Invasion Assay

Cells were starved for 8 h before planking. Matrix glue measuring 90 μl (300 ng/ml) was to the upper chamber before plating 3 × 104 cells in each upper chamber (4.5 × 105 cells of overexpressing MMP-1 were seeded into the upper chamber). All the upper chambers were added with 100 μl of serum-free medium, whereas the lower chamber was added with a medium containing 10% FBS. After 24 h, the cells were fixed and stained, and three visual fields were randomly selected under the microscope for photographing and counting.

Immunofluorescence

The cells were fixed with 4% PFA for 30 min, permeabilized with 0.5% Triton (Beyotime, Shanghai, China; ST795) for 10 min, blocked with 5% bovine serum albumin (BSA; YEASEN,
Subsequently, dyed at room temperature for 60 min, especially avoiding light. Phalloidin (YEASEN, 40734ES75) into each culture dish and nucleus blue. Fl for F-actin staining as pink. FLNA was stained green with under the microscope and photographed. Phalloidin was used for F-actin staining as pink. FLNA was stained green with fluorescently conjugated secondary antibody. DAPI stained the nucleus blue.

Xenograft Model
All procedures of the mouse model were approved by the Xiamen University (AP: XMULAC20200119) and conformed to the guidelines for the care and maintenance of laboratory animals. Breast cancer cells (5 × 10⁶ cells/mouse) were injected into the fourth pair of mammary glands on the right side of 6-week-old female Balb/c nude mice according to the above groups (73, 74). There were 5 mice in each group. The length and width of the tumor in situ were monitored with a vernier caliper. The calculation formula of tumor volume in athymic nude mice is

\[ V = 0.5 \times \text{Length} \times \text{Width}^2 \text{(mm}^3) \]

(W, smaller diameter; L, larger diameter) as described previously (75). After 4 weeks, the mice were sacrificed, and the liver, kidney, lung, and brain of mice were collected to evaluate the metastatic state.

H&E Stain and Immunohistochemistry
The tissue sections were dewaxed in xylene and hydrated in alcohol. The nucleus and cytoplasm were stained by hematoxylin (Beyotime, C0105S) and eosin, respectively. The stained tissues were dehydrated and sealed, and they were observed and image-captured under a microscope.

The immunohistochemical assay was performed on FFPE sections of xenograft mouse tissues. Tumor sections measuring 5 μm were incubated with primary antibody at 4°C overnight and secondary antibody at room temperature for 2 h. Subsequently, all fields were observed under light microscopy. ImageJ was used to calculate the integrated OD (IOD), the distribution area of IHC staining images, and the average OD (AOD). AOD = IOD/Area.

Statistical Analysis
GraphPad Prism 8.0.1 software was used for statistical analyses. All data were presented as mean ± SD of at least three independent experiments. One-way ANOVA was selected for more than two groups. *p < 0.05, **p < 0.01, or ****p < 0.0001 was labeled for statistical significance.

DATA AVAILABILITY STATEMENT
The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT
The animal study was reviewed and approved by Xiamen University (AP: XMULAC20200119). Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS
JZ designed and performed the experiments and analyzed the data. LW analyzed the sequencing data of clinical samples. JZ and LW drafted the manuscript and made the tables. XK designed and supervised all the experiments, participated in the revision of manuscript. ZJ provided guidance for sequencing data analysis and revised the manuscript. PX collected clinical samples. LY participated in animal experiments and revised the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.836126/full#supplementary-material

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