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# EZH2 mutation is associated with the development of visceral metastasis by enhancing proliferation and invasion and inhibiting apoptosis in breast cancer cells

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## Abstract

**Background** The prognosis of breast cancer patients with visceral metastasis (VM) is significantly worse than that of patients without VM. We aimed to evaluate EZH2 (enhancer of zeste homolog 2) mutation as a biomarker associated with VM.

**Methods** Data from forty-nine patients with metastatic breast cancer (MBC) pathologically confirmed at our hospital between March 2016 and September 2018 were collected. Metastatic tissue samples were obtained via ultrasound-guided needle biopsy, and paired peripheral blood samples were also collected. Tissue and blood samples were subjected to targeted next-generation sequencing via a 247-gene panel. Stably transfected MDA-MB-231 cells expressing wild-type EZH2 ($EZH2^{WT}$) or a mutant form of EZH2 ($EZH2^{K515R}$) were generated. Cell proliferation, colony formation ability, migration and invasion abilities and apoptosis were assessed using CCK-8 assays, plate colony formation assays, Transwell chamber assays and flow cytometry.

**Results** The incidence of EZH2 mutations in the VM subgroup was greater than that in the non-VM subgroup in the entire cohort ($n=49$, 42.3% vs. 13.0%, $p=0.024$) and in the triple-negative breast cancer (TNBC) subgroup ($n=20$, 50.0% vs. 10.0%, $p=0.05$). Patients carrying EZH2 mutations had a significantly greater risk of developing VM than did those in the non-EZH2 mutation group in the entire cohort (HR 2.9) and in the TNBC subgroup (HR 6.45). Multivariate analysis revealed that EZH2 mutation was an independent prognostic factor for VM (HR 2.99, $p=0.009$) in the entire cohort and in the TNBC subgroup (HR 10.1, $p=0.006$). Data from cBioPortal also showed that patients with EZH2 mutations had a significantly greater risk of developing VM (HR 3.1), and the time to develop VM was significantly earlier in the EZH2 mutation group (31.5 months vs. 109.7 months, $p=0.008$). Multivariate analysis revealed that EZH2 mutation (HR 2.73, $p=0.026$) was an independent factor for VM after breast cancer surgery. There was no correlation between EZH2 mutations and BRCA1/2 mutations. Most of the patients (81.8%) in our cohort who developed VM

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carried the "c.1544A > G (p.K515R)" mutation. Compared with $EZH2^{WT}$ MDA-MB-231 cells, $EZH2^{K515R}$ MDA-MB-231 cells had greater colony formation rates ($p < 0.01$), greater migration and invasion rates ($p < 0.001$), and lower apoptosis rates ($p < 0.01$). The proportion of S + G2/M phase cells in the $EZH2^{K515R}$ group was significantly greater than that in the $EZH2^{WT}$ group.

**Conclusions** EZH2 mutation is associated with VM development in breast cancer patients. The $EZH2^{K515R}$ mutation leads to VM and a poor prognosis by enhancing proliferation and invasion and inhibiting apoptosis in breast cancer cells.

**Keywords** EZH2 mutation, Visceral metastasis, Breast cancer, Targeted next-generation sequencing

## Introduction

Breast cancer remains the most common malignant tumor in women worldwide [1]. The prognosis of MBC patients with visceral metastasis (VM) is significantly worse than that of patients without VM (non-VM) [2–4]. The breast cancer-specific survival (BCSS) and overall survival (OS) rates of patients with VM are significantly lower than those of patients without VM. However, the mechanism of VM in breast cancer is unclear. Further understanding of the biological processes involved in the development of VM in breast cancer will help researchers better understand the heterogeneity of breast cancer and, more importantly, could be of clinical value for distinguishing different subgroups of patients who may benefit from tailored treatment.

EZH2 is a cancer-related gene with histone methyltransferase activity and is the key catalytic subunit of polycomb repression complex 2 (PRC2). PRC2 is an important epigenetic regulator that is mainly composed of four core proteins, EZH2, EED, SUZ12 and RbAp46/48, which play important roles in the occurrence and development of breast cancer [5]. EZH2 can induce trimethylation of lysine 27 of histone H3 (H3K27me3) in a classic PRC2-dependent manner and promote the formation of heterochromatin to silence genes associated with tumor occurrence, progression and maintenance of stem cell characteristics. EZH2 can also exert transcriptional activation in a PRC2-independent manner. In recent years, epigenetic regulation of EZH2 has been found to be closely related to the MAPK signaling pathway. Inhibiting H3K27 methylation can lead to abnormal activation of the kinase MAPK signaling pathway, confirming the interaction between epigenetic regulation and the cellular phosphorylation signaling network [6, 7]. EZH2 can also engage TGFβ signaling to promote breast cancer bone metastasis via integrin β1-FAK activation [8]. Downregulation of EZH2 through siRNAs and shRNAs or through signaling pathways involving EZH2 can inhibit the proliferation and growth of tumors. High expression of EZH2 in breast cancer cells is associated with increased invasiveness and a poor prognosis and is an independent predictor of the development of metastases [9, 10].

However, the role of EZH2 mutation in breast cancer is unclear. Previous studies on EZH2 mutations have mostly focused on hematological tumors [11]. With the development of genomics and sequencing technology, targeted next-generation sequencing (targeted NGS) has become widely used for screening cancer biomarkers for precision medicine [12–14]. In this study, we evaluated EZH2 mutation as a biomarker associated with VM in breast cancer by comparing tissue and blood samples from VM and non-VM MBC patients through targeted NGS. We collected data on 1,392 patients with breast cancer from the cBioPortal datasets to further evaluate whether EZH2 mutation was an independent prognostic factor for VM in primary breast cancer patients via multivariate analysis. Subsequently, we explored the preliminary mechanism by which EZH2 mutation leads to increased cell invasion and metastasis in vitro.

## Methods

### Patient selection

We collected samples from 231 MBC patients pathologically confirmed at our hospital between March 2016 and September 2018. Sixty-six patients were enrolled consecutively according to the following inclusion criteria: (I) underwent a biopsy of the metastatic site, and paired peripheral blood was collected as a normal control; (II) the interval between biopsy and subsequent treatment was shorter than 1 week; and (III) tissue and blood samples were tested by targeted next-generation sequencing of a 247-gene panel from Annoroad Gene Tech. (Beijing) Co. Seventeen patients were subsequently excluded based on the following exclusion criteria: (I) patients with prior or concomitant malignancies; (II) patients who failed to follow-up during the therapeutic process; and (III) patients whose treatment before metastasis was not standardized according to the current guidelines. For example, standard chemotherapy or radiotherapy was not performed, the number of chemotherapy cycles was insufficient, or anti-HER2 targeted therapy was not administered to patients with the HER2-positive subtype (Fig. 1).

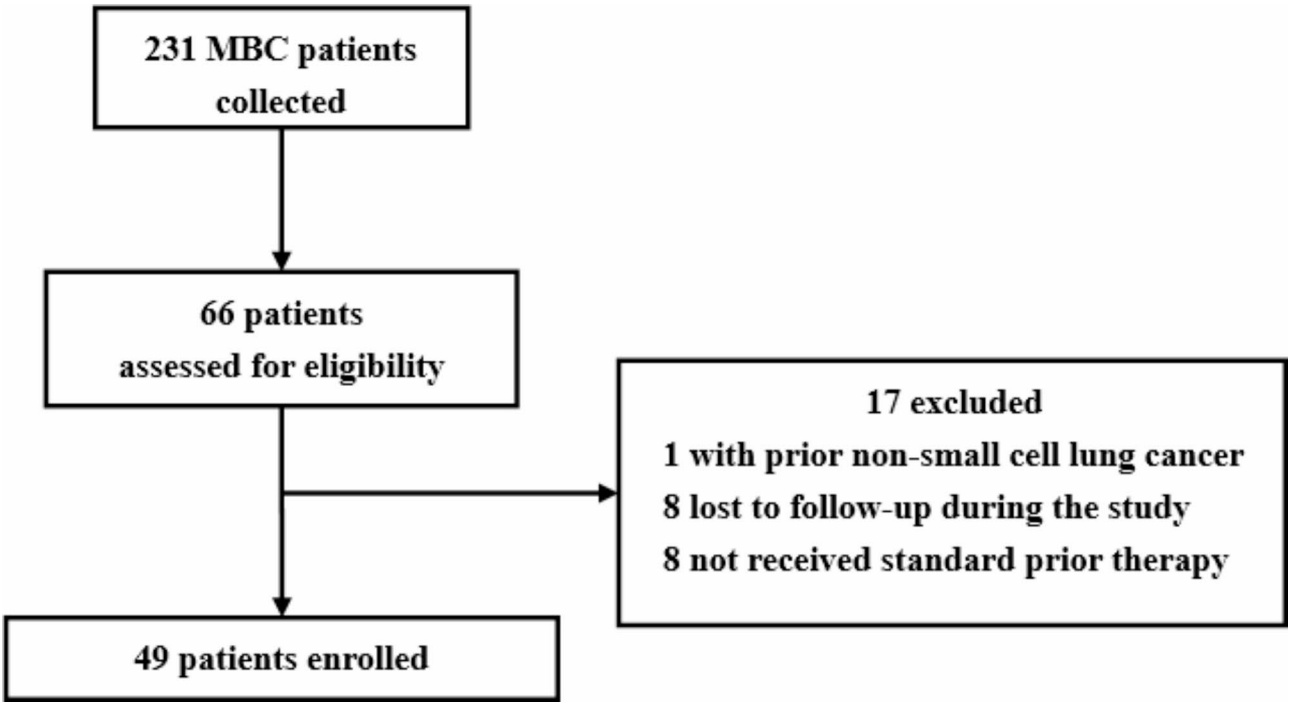


**Fig. 1** The flow chart of patient selection process

#### Sample collection

Tissue samples of metastatic tissue were obtained by ultrasound-guided needle biopsy and preserved in formalin for pathological and immunohistochemical detection or in RNAsafer stabilizer reagent (R0424-02, OMEGA) for targeted NGS analysis. For those whom with only bone metastasis, CT-guided biopsy was used to obtain metastatic tissue. For whom with brain metastasis, tissue samples of extracranial metastatic tissue were obtained by ultrasound-guided needle biopsy. Paired peripheral blood samples were collected and preserved in blood collection tubes (Streck Cell-Free DNA BCT). ER- and PR-positive status was defined as the presence of >1% tumor cells with nuclear staining within invasive breast carcinoma. One patient with ER-negative and PR 2% weakly positive was defined as hormone receptor-negative. HER2 positivity was defined either by protein overexpression as defined by immunohistochemical score (IHC) 3+or equivocal protein expression (IHC 2 +), with evidence of HER2 gene amplification by fluorescence in situ hybridization (FISH) in accordance with the American Society of Clinical Oncology/College of American Pathologists guidelines [15–17].

#### Construction of stably transduced mutant cell lines

We constructed an EZH2 wild-type vector (pcSLenti-EF1-EGFP-P2A-Puro-CMV-EZH2-3xFLAG-WPRE) and used a QuikChange Site-Directed Mutagenesis Kit (Stratagene) point mutation kit to construct an EZH2-K515R mutation vector (pcSLenti-EF1-EGFP-P2A-Puro-CMV-EZH2C.1544 A>G-3xFLAG-WPRE), and the mutation site was verified by Sanger sequencing. PCR was performed on 200 bp near the mutation site of the genomic DNA, and the PCR products were detected by agarose gel electrophoresis. MDA-MB-231 cells were transduced with lentiviral particles to overexpress mutant EZH2-K515R ($EZH2^{K515R}$ group), wild-type EZH2 ($EZH2^{WT}$ group), or the empty vector in tandem with EGFP (control group). After the initial transduction, stably transfected $EZH2^{K515R}$ and $EZH2^{WT}$ MDA-MB-231 cells were screened and selected through puromycin resistance.

Cell proliferation was detected using a Cell Counting Kit-8 (CCK-8) assay (40203ES76, Yeasen). A plate colony formation assay (3516, Corning) was used to detect the colony-forming ability of the MDA-MB-231 cells. Transwell chambers (3422, Corning and 356234, Corning) were used to detect cell migration and invasion ability. Flow cytometry (FCM) was used to analyze cell apoptosis (KGA1026, Keygen and C1052, Beyotime). The above experiments were performed according to the manufacturers' instructions.

#### Targeted next-generation sequencing

The methods and process of targeted next-generation sequencing were previously described in our previous manuscript [18]. In brief, we collected metastatic tissue from patients and peripheral blood ctDNA as a normal control for analysis through targeted NGS. The library was assayed and captured 247 target sequence hotspots in key genes closely related to tumor detection.

### Clinicopathological review

Patients were generally assessed every 2 cycles during treatment and then every 3 months after completing treatment to assess drug efficacy. Patient data, including medical history, concurrent diseases, age at diagnosis, surgical information, pathological evaluation, and imaging findings were retrieved. Efficacy evaluation was based on imaging according to RECIST 1.1. The last follow-up visit was in June 2023 and the median follow-up was 40 months. The primary endpoint was visceral metastasis-free survival (VMFS), defined as the length of time after primary treatment for a cancer, at which point the patient survived without any signs or symptoms of VM. And disease-free survival (DFS) was defined as the length of time after primary treatment for a cancer ends that the patient survives without any signs or symptoms of that cancer [19].

**Table 1** Clinicopathologic characteristics of the patients

| Characteristics | EZH2 | | *P* |
|---|---|---|---|
| | Mutation ($n=14$) | No mutation ($n=35$) | |
| Median age (range), years | 55(49–70) | 50(32–59) | 0.521 |
| Type of histology | | | 0.312 |
| Ductal | 14 | 31 | |
| Lobular | 0 | 4 | |
| ER/PR | | | 0.928 |
| ER or PR positive | 7 | 18 | |
| ER and PR negative | 7 | 17 | |
| HER2 | | | 0.914 |
| Positive | 3 | 8 | |
| Negative | 11 | 27 | |
| Subtype | | | 0.976 |
| Luminal | 7 | 18 | |
| HER2-positive | 1 | 3 | |
| TNBC | 6 | 14 | |
| Grade | | | 0.588 |
| 1/2 | 6 | 18 | |
| 3 | 8 | 17 | |
| Metastatic sites (visceral vs. non-visceral) | | | 0.015 |
| Visceral | 11 | 15 | |
| Lung | 5 | 8 | |
| Liver | 8 | 7 | |
| Brain | 1 | 2 | |
| Non-visceral | 3 | 20 | |
| Bone | 0 | 7 | |
| Chest wall | 1 | 5 | |
| Lymph node | 3 | 16 | |
| Non-targeted treatments | | | 0.917 |
| Endocrine | 7 | 18 | |
| Chemotherapy | 11 | 30 | |
| HER2-targeted treatments | | | 0.997 |
| Monoclonal antibody | 3 | 8 | |
| TKI | 2 | 5 | |
| Monoclonal antibody + TKI | 2 | 5 | |

ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor 2; TKI: Tyrosine Kinase Inhibit

### Statistical analysis

Statistical analysis of clinicopathologic data was performed using a two-tailed Student's t test for continuous variables and Fisher's exact test for categorical variables. *P* values $<0.05$ were considered to indicate statistical significance. K–M survival curves were used to analyze patient survival. Multivariate analysis was performed to identify factors related to PFS using a Cox proportional hazards model. The variables included menstrual status, primary stage, ER/PR status, HER2 status, and EZH2 mutation status. Variables with $p<0.05$ in the univariate analysis were included in the multivariate analysis. All analyses were performed using SPSS 21.0 (IBM, Armonk, NY, USA) and visualized with GraphPad Prism 9.3.0 (GraphPad Software, Inc) and R software v4.0.3 (R Core Team). Based on the formula for investigating the sample size by population rate π, n= $(U\alpha/\delta)^2(1\text{-}P)$ P, we set $\alpha=0.05$, $U\alpha=1.96$, and $\delta=0.05$, where P is the sample rate. According to previous studies, the frequency of EZH2 mutations in breast cancers is approximately 1–3% [20–22]. Thus, we estimated the sample size to be 44 patients, the dropout rate was 10%, and 49 patients were ultimately enrolled.

Expanded information regarding EZH2 mutations and patient survival time in breast cancer patients was downloaded from cBioPortal, an open access database that is available at http://www.cbioportal.org [23, 24]. Using the Breast Cancer dataset (MSK, Cancer Cell 2018), data from the targeted sequencing of tumor/normal sample pairs from 1,918 breast cancer patients were retrieved.

## Results

### EZH2 mutation was an independent prognostic factor for VM in the whole patient cohort and in the TNBC subgroup

There were 20 TNBC patients, 25 luminal patients and 4 HER2-positive patients. EZH2 mutation was associated with VM ($p=0.015$) but was not associated with age, type of histology, ER or PR status, HER2 status, subtype, tumor grade, or treatment regimen (Table 1). In the whole patient cohort ($n=49$), the incidence of EZH2 mutations in the VM group (11/26=42.3%) was significantly greater than that in the non-VM group (3/23=13.0%) ($p=0.024$). For different subtypes of breast cancer, the incidence of EZH2 mutations in the VM subgroup (5/10=50.0%) was greater than that in the non-VM subgroup (1/10=10.0%) ($p=0.05$) in the TNBC subtype but not in the HER2-positive or luminal subtype ($p>0.05$). Patients carrying EZH2 mutations had a significantly greater risk of developing VM (HR 2.9, 95% CI 1.1–7.5) and shorter VMFS (22 months vs. 50 months, log-rank $p=0.005$) than patients

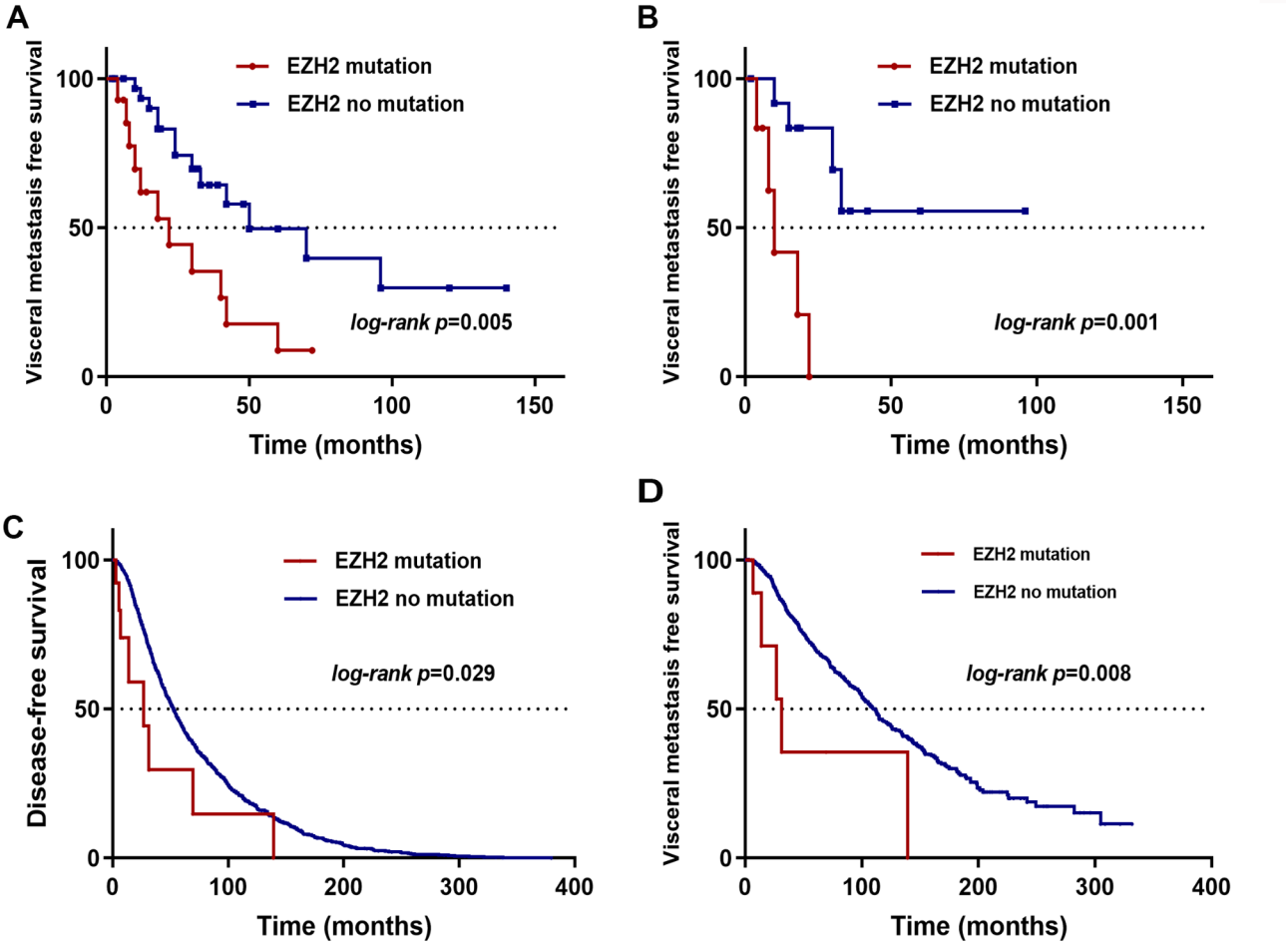


**Fig. 2** EZH2 mutation is associated with the development of visceral metastasis. (**A**) Patients carrying EZH2 mutations had a significantly shorter VMFS (22 months vs. 50 months, $p=0.005$) than patients without EZH2 mutations. (**B**) Patients carrying EZH2 mutations had a significantly shorter VMFS (10 months vs. unreached, $p=0.001$) than patients without EZH2 mutations in the TNBC subtype. (**C**) DFS time of patients with EZH2 mutations was significantly shorter than that of patients without EZH2 mutations (26.9 months vs. 52.7 months, $p=0.029$) in 1,392 patients from cBioPortal. (**D**) VMFS was significantly shorter in the EZH2 mutation group than that of patients without EZH2 mutations (31.5 months vs. 109.7 months, log-rank $p=0.008$) in 1,392 patients from cBioPortal

without EZH2 mutations (Fig. 2A). In the TNBC subtype, we also found that patients with EZH2 mutations had a significantly greater risk of developing VM (HR 6.45, 95% CI 1.1–37.9) and shorter VMFS (10 months vs. unreached, log-rank $p=0.001$) than patients without EZH2 mutations (Fig. 2B). Cox multivariate analysis revealed that EZH2 mutation (HR 2.99, $p=0.009$) were independent prognostic factors for VM after breast cancer surgery (Table 2). In the TNBC subtype, multivariate Cox analysis also revealed that EZH2 mutation (HR 10.1, $p=0.006$) was an independent prognostic factor for VM (Table 3).

#### EZH2 mutation was an independent prognostic factor for VM in a dataset from the cBioPortal

We collected complete follow-up data for 1,392 patients with early breast cancer (stage 1–3) from cBioPortal (dataset MSK, Cancer Cell 2018) to verify our results. The results also showed that the DFS time of patients with EZH2 mutations was significantly shorter than that of patients without EZH2 mutations (26.9 months vs. 52.7 months, log-rank $p=0.029$) (Fig. 2C). Patients with EZH2 mutations had a significantly greater risk of developing visceral metastases (HR 3.1), and the VMFS was significantly shorter in the EZH2 mutation group (31.5 months vs. 109.7 months, log-rank $p=0.008$) (Fig. 2D). With menopausal status, ER status, PR status, HER2 status, TNM stage of the primary disease, and EZH2 mutation status as prognostic indicators, multivariate Cox regression analysis revealed that ER negativity, stage III status, and EZH2 mutation status (HR 2.73, $p=0.026$) were independent factors for the occurrence of VM after breast cancer surgery (Table 4).

**Table 2** Risk factor for VM in 49 MBC

| Factors | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Menstrual status | | | | | | |
| Post vs. pre | 0.94 | 0.45–1.99 | 0.878 | 0.45 | 0.16–1.24 | 0.121 |
| ER | | | | | | |
| Negative vs. Positive | 1.54 | 0.73–3.21 | 0.254 | 1.60 | 0.64–4.02 | 0.32 |
| PR | | | | | | |
| Negative vs. Positive | 1.09 | 0.50–2.38 | 0.837 | 1.93 | 0.68–5.48 | 0.215 |
| HER2 | | | | | | |
| Positive vs. negative | 1.64 | 0.66–4.06 | 0.283 | 1.07 | 0.23-5.00 | 0.934 |
| Subtype | | | | | | |
| Luminal vs. TNBC | 0.70 | 0.30–1.64 | 0.411 | 0.44 | 0.12–1.61 | 0.215 |
| HER2-positive vs. TNBC | 0.81 | 0.10–6.51 | 0.843 | 0.94 | 0.06–13.82 | 0.965 |
| Primary Stage | | | | | | |
| II vs. I | 2.24 | 0.73–6.91 | 0.160 | 2.07 | 0.61–6.95 | 0.241 |
| III vs. I | 1.38 | 0.62–3.07 | 0.427 | 2.25 | 0.90–5.67 | 0.084 |
| EZH2 mutation | | | | | | |
| With vs. without | 2.63 | 1.26–5.48 | 0.010 | 2.99 | 1.31–6.79 | 0.009 |

**Table 3** Risk factor for VM in 20 TNBC subtype

| Factors | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Menstrual status | | | | | | |
| Post vs. pre | 1.27 | 0.31–5.16 | 0.735 | 0.59 | 0.13–2.78 | 0.508 |
| Primary Stage | | | | | | |
| II vs. I | 3.04 | 0.59–15.7 | 0.184 | 1.21 | 0.15–9.52 | 0.857 |
| III vs. I | 1.78 | 0.36–8.86 | 0.480 | 0.33 | 0.04–2.46 | 0.279 |
| EZH2 mutation | | | | | | |
| With vs. without | 15.11 | 2.19-104.14 | 0.006 | 10.1 | 1.92–52.8 | 0.006 |

**Table 4** Risk factor for VM in primary breast cancer from cBioPortal

| Factors | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| Menstrual status | | | | | | |
| Post vs. pre | 0.93 | 0.75–1.15 | 0.482 | 0.84 | 0.67–1.05 | 0.121 |
| peri vs. pre | 0.89 | 0.57–1.39 | 0.597 | 0.95 | 0.60–1.51 | 0.832 |
| ER | | | | | | |
| negative vs. Positive | 1.47 | 1.10–1.97 | 0.010 | 1.71 | 1.24–2.36 | 0.001 |
| PR | | | | | | |
| negative vs. Positive | 1.36 | 1.09–1.70 | 0.008 | 1.18 | 0.89–1.57 | 0.250 |
| HER2 | | | | | | |
| Positive vs. negative | 1.11 | 0.79–1.56 | 0.545 | 0.97 | 0.69–1.38 | 0.881 |
| Primary Stage | | | | | | |
| II vs. I | 1.22 | 0.95–1.96 | 0.119 | 1.26 | 0.96–1.65 | 0.093 |
| III vs. I | 1.39 | 1.06–1.81 | 0.016 | 1.34 | 1.01–1.77 | 0.046 |
| EZH2 mutation | | | | | | |
| With vs. without | 3.09 | 1.28–7.48 | 0.012 | 2.73 | 1.13–6.62 | 0.026 |

#### Relationship of EZH2 mutation and BRCA1/2 mutation

We found that 9 out of 49 patients had BRCA1/2 mutations (3 with only BRCA1 mutations, 4 with only BRCA2 mutations, and 2 with both BRCA1 and 2 mutations). Most BRCA mutations were of the luminal type (8/9). Among the 5 patients with BRCA1 mutations, 4 had the luminal type, and 1 had the HER2-positive type. Among the 6 patients with BRCA2 mutations, 5 had the luminal type, and 1 was HER2 positive. Interestingly, we did not find that TNBC patients in our cohort carried BRCA1 or BRCA2 mutations.

We also analyzed the correlation between EZH2 mutations and BRCA1 and BRCA2 mutations in our cohort, and the results revealed no correlation between EZH2 mutations and BRCA1 mutations (OR=1.78, 95% CI 0.26–11.98, $p=0.616$) and no correlation between EZH2 mutations and BRCA2 mutations (OR=2.91, 95% CI 0.51–16.59, $p=0.334$) (Table S1). We also performed further analysis of the Breast Cancer dataset (MSK, Cancer Cell 2018, http://www.cbioportal.org). We found no correlation between EZH2 mutations and BRCA1 or BRCA2 mutations ($p>0.05$) (Table S2).

There were no differences between the EZH2 mutation rate and the BRCA1/2 mutation rate in any patient cohort ($p=0.233$), luminal subtype ($p=0.758$) or HER2-positive subtype ($p=1.0$), but the rate of EZH2 mutation was higher than that of BRCA1/2 mutation ($p=0.003$). Owing to the presence of both EZH2 and BRCA mutations in some patients in our cohort, we also analyzed the differences in the incidence of EZH2/BRCA comutations among different subtypes. We found that the incidence of EZH2/BRCA comutations was 8.2% in 49 patients. Among these subtypes, the luminal subtype accounts for 12% (3/25), the HER2-positive subtype accounts for 25% (1/4), and the TNBC subtype accounts for 0% (0/20). There was no significant difference in the EZH2/BRCA comutation rate between the luminal- and HER2-positive groups (12% vs. 25%, $p=0.467$). Compared with luminal patients, TNBC patients had a lower EZH2/BRCA comutation rate (0% vs. 12% unilateral $p=0.109$), but the difference was not statistically significant due to sample size. Compared with that in the HER2-positive group, the EZH2/BRCA comutation rate was lower in the TNBC group (0% vs. 25% unilateral $p=0.022$). These results suggest that BRCA/EZH2 comutations occur less frequently in TNBC. Combined with the aforementioned results, there is no correlation between EZH2 mutations and BRCA mutations, suggesting that EZH2 mutations and BRCA mutations in TNBC may be mutually exclusive, which requires further validation with larger sample sizes (Table 5).

**Table 5** Rates of EZH2 mutations, BRCA1/2 mutations, and EZH2 and BRCA1/2 comutations

| | *N* | EZH2 mutation (%) | BRCA1/2 mutation | *P* | EZH2 and BRCA1/2 co-mutations(%) |
|---|---|---|---|---|---|
| All | 49 | 14 (29) | 9 (18) | 0.233 | 4(8.2) |
| Luminal | 25 | 7 (28) | 8 (32) | 0.758 | 3(12) |
| HER2-positive | 4 | 1 (25) | 1 (25) | 1.000 | 1(25) |
| TNBC | 20 | 6 (30) | 0 (0) | 0.003 | 0(0) |

### The EZH2$^{K515R}$ mutation leads to VM by enhancing proliferation and invasion and inhibiting apoptosis

Among the 49 MBC patients, 14 patients carried EZH2 mutations, and 78.6% (11/14) developed VM. Most patients who developed VM (81.8%, 9/11) had "c.1544A>G (p.K515R)" point mutations. In this study, we constructed a lentiviral vector for stably transduced MDA-MB-231 cells (EZH2$^{WT}$ and EZH2$^{K515R}$). The CCK-8 cell proliferation assay showed that the cell proliferation rates of the EZH2$^{K515R}$ and EZH2$^{WT}$ groups were decreased at 48 h and 72 h compared with those of the control group ($p<0.001$), but there was no difference in the cell proliferation rate between the EZH2$^{K515R}$ and EZH2$^{WT}$ groups. A plate colony formation assay showed that the EZH2$^{K515R}$ group had a greater colony formation rate than did the EZH2$^{WT}$ group ($p<0.01$). Transwell chamber migration and invasion experiments showed that the EZH2$^{K515R}$ group had greater migration ($p<0.001$) and invasion ($p<0.001$) than the EZH2$^{WT}$ group. The flow cytometry results showed that the EZH2$^{K515R}$ group had a lower apoptosis rate than the EZH2$^{WT}$ group ($p<0.01$). The EZH2$^{K515R}$ group had a greater proportion of cells in the S phase ($p<0.001$) and G2/M phase ($p<0.01$) and a lower proportion of cells in the G1/G0 phase ($p<0.001$) than did the EZH2$^{WT}$ group (Fig. 3). The proportion of S+G2/M phase cells in the EZH2$^{K515R}$ group (40.86±0.86%) was significantly greater than that in the EZH2$^{WT}$ group (36.63±0.56%) ($p=0.002$).

## Discussion

Since VM is associated with a poor prognosis, previous studies have been conducted to explore the factors and mechanisms associated with VM. Patients with pathological ER-negative, HER2-positive and grade 3 tumors have an increased risk of developing VM [25, 26]. The ESR1 mutation may be associated with VM in estrogen receptor-positive MBC [27]. A specific fourteen-gene expression signature has been identified as an independent prognostic factor for the development of VM in primary breast cancer [28].

In this study, we found that EZH2 mutation was an independent prognostic factor for VM by targeted NGS, and the results were further verified through a dataset from cBioPortal. The function of EZH2 mutations depends on the type of cancer, for example, gain-of-function in lymphomas and loss-of-function in medulloblastoma and bladder and renal cancers [29, 30]. Previous studies on the role of EZH2 mutation in breast cancer are rare. EZH2 rs6950683 and rs3757441 SNPs (TC+CC genotype) have been shown to be related to the tumor size of TNBC patients under 60 years of age [31]. The EZH2$^{K515R}$ mutation is located in exon 13 of the EZH2 gene. This region is an important site for EZH2 to

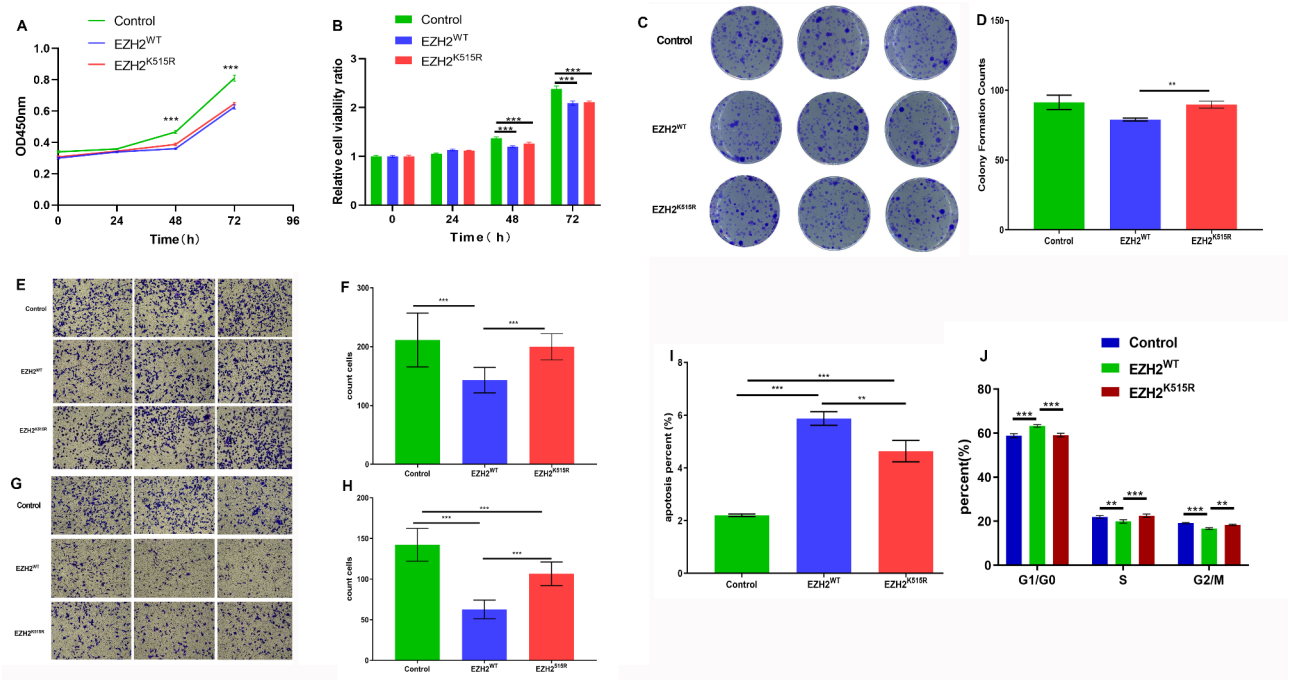


**Fig. 3** *** $p<0.001$; ** $p<0.01$.
The cell functions in stably transduced MDA-MB-231 cells ($EZH2^{WT}$ and $EZH2^{K515R}$).
(A, B.) The cell proliferation rates of the $EZH2^{K515R}$ and $EZH2^{WT}$ groups were decreased at 48 h and 72 h compared with those of the control group ($p<0.001$), but there was no difference in the cell proliferation rate between the $EZH2^{K515R}$ and $EZH2^{WT}$ groups.
(C, D.) The $EZH2^{K515R}$ group had a greater colony formation rate than the $EZH2^{WT}$ group ($p<0.01$).
(E, F.) The $EZH2^{K515R}$ group had greater migration rate than the $EZH2^{WT}$ group ($p<0.001$).
(G, H.) The $EZH2^{K515R}$ group had a greater invasion rate than the $EZH2^{WT}$ group ($p<0.001$).
(I, J.) The $EZH2^{K515R}$ group had a lower apoptosis rate than the $EZH2^{WT}$ group ($p<0.01$). The $EZH2^{K515R}$ group had a greater proportion of cells in the S phase ($p<0.001$) and G2/M phase ($p<0.01$) and a lower proportion of cells in the G1/G0 phase ($p<0.001$) than did the $EZH2^{WT}$ group.

interact with its positive regulatory factor CDYL. Therefore, the $EZH2^{K515R}$ mutation may lead to important functional changes in EZH2. Our results indicate that, compared with $EZH2^{WT}$, the $EZH2^{K515R}$ mutation eventually leads to VM and a poor prognosis in TNBC cells by enhancing proliferation and invasion and inhibiting apoptosis in vitro. The molecular mechanism may be that the $EZH2^{K515R}$ mutation silences the expression of certain genes by inducing H3K27me3, and further study is underway to shed light on the potential of EZH2 as a therapeutic target.

The in vitro results showed that $EZH2^{WT}$ and $EZH2^{K515R}$ TNBC cells appeared to have lower invasion and metastatic abilities than control TNBC cells. In most previous studies, overexpression of EZH2 indicated increased invasiveness and a poor prognosis in patients with breast cancer [8, 32, 33]. However, EZH2 expression seems to play a different role in TNBC subtypes than in other subtypes [31]. We analyzed the correlation between high EZH2 expression and the prognosis of patients with each subtype of breast cancer using a database (https://kmplot.com/analysis/) (Figure S1). The results showed that the RFS (relapse-free survival) ($p=3.4\times10E-7$) and OS (overall survival) ($p=1.4\times10E-5$) rates of high-EZH2 patients were shorter than those of low-EZH2 patients in the whole breast cancer cohort. However, in the TNBC subtype, the RFS ($p=0.0051$) and OS ($p=0.0037$) rates of high-EZH2 patients were greater than those of low-EZH2 patients. In the HER2-positive subtype, the RFS ($p>0.05$) and OS ($p>0.05$) rates of the high-EZH2 subgroup were not significantly different from those of the low-EZH2 subgroup. Only in the Luminal subtype were the RFS ($p=7.6\times10E-6$) and OS ($p=8.9\times10E-9$) rates of high-EZH2 patients shorter than those of low-EZH2 patients. These results revealed that the prognosis of patients with high EZH2 levels was significantly better than that of patients with low EZH2 levels in TNBC. Therefore, the results of our in vitro experiments with the MDA-MA-231 TNBC cell line are consistent with the clinical database results, showing that TNBC cells with high EZH2 expression are less invasive than those with low EZH2 expression. A previous study also showed that EZH2 can inhibit RAS and MAPK in the RAS/RAF/MEK/ERK pathway [34]. In addition, the expression of EZH2 is greater in Brca1/BRCA2-deficient tumors than in Brca1/2 wild-type tumors, and the high-expression EZH2 group shows better platinum therapy sensitivity (regardless of BRAC wild-type or BRACness) and a better

prognosis in TNBC [35]. For now, both the genomic role and the tumor suppressive role of EZH2 have been demonstrated through distinct mechanisms in different cancer types and conditions [36]. Therefore, more research is needed to investigate the relationship between EZH2 expression and TNBC.

(A, B.) the RFS ($p$=3.4×10E-7) and OS ($p$=1.4×10E-5) rates of high-EZH2 patients were shorter than those of low-EZH2 patients in the whole breast cancer cohort. (C, D.) In the TNBC subtype, the RFS ($p$=0.0051) and OS ($p$=0.0037) rates of high-EZH2 patients were greater than those of low-EZH2 patients. (E, F.) In the HER2-positive subtype, the RFS ($p$>0.05) and OS ($p$>0.05) rates of the high-EZH2 subgroup were not significantly different from those of the low-EZH2 subgroup. (G, H.) In the Luminal subtype, the RFS ($p$=7.6×10E-6) and OS ($p$=8.9×10E-9) rates of high-EZH2 patients were shorter than those of low-EZH2 patients.

Interestingly, $EZH2^{K515R}$ seemed to attenuate the tumor suppressor effect of $EZH2^{WT}$ in TNBC. Compared with the $EZH2^{WT}$ strain, the $EZH2^{K515R}$ strain had a greater colony formation rate, greater migration and invasion rate, greater proportion of cells in the S+G2/M phase and a lower apoptosis rate in the TNBC cell line MDA-MB-231. One of the characteristics of triple-negative breast cancer is that it is prone to VM [37]. Combining the above clinical sample and in vitro cytology results, we propose that an EZH2 mutation may promote the occurrence of VM compared to patients without EZH2 mutations in TNBC. Therefore, it may be possible to specifically treat or even prevent the occurrence of VM in TNBC patients with EZH2 mutations if we identify the molecular mechanism by which EZH2 mutations lead to VM, making this a research area that warrants further exploration.

The current study has certain limitations. First, the retrospective and observational nature of the study may have resulted in missing data or possible recall and information bias. Second, the patient sample size was relatively small, and the results may have been unstable during multivariate analysis. Finally, the molecular mechanism by which the $EZH2^{K515R}$ mutation causes VM has not yet been clarified, and we will elucidate this mechanism in subsequent studies.

## Conclusion

Our analysis of patient cohort data and a dataset from cBioPortal revealed that EZH2 mutation is associated with the development of VM in breast cancer through the enhancement of proliferation, invasion, and anti-apoptotic effects in vitro. However, further studies are needed to elucidate the complex mechanism underlying this phenomenon.

### Abbreviations

| | |
|---|---|
| VM | Visceral Metastasis |
| EZH2 | Enhancer of Zeste Homolog 2 |
| MBC | Metastatic Breast Cancer |
| BCSS | Breast Cancer-Specific Survival |
| OS | Overall Survival |
| PRC2 | Polycomb Repression Complex 2 |
| MAPK | Mitogen-Activated Protein Kinase |
| TGFβ | Transforming Growth Factorβ |
| FAK | Focal Adhesion Kinase |
| siRNA | small interfering RNA |
| shRNA | short hairpin RNA |
| IHC | Immunohistochemistry |
| FISH | Fluorescence in situ Hybridization |
| ER | Estrogen Receptor |
| PR | Progesterone Receptor |
| NGS | Next-Generation Sequencing |
| ctDNA | circulating DNA |
| K–M curve | Kaplan-Meier curve |
| TNBC | Triple-Negative Breast Cancer |
| HER2 | Human Epidermal growth factor Receptor 2 |
| TKI | Tyrosine Kinase Inhibit |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-12950-y.

**Supplementary material 1: Fig. S1:** Survival of high-EZH2 or low-EZH2 patients from a database (https://kmplot.com/analysis/)

Supplementary Material 2

Supplementary Material 3

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### Author contributions

FW, NNL and JL designed the study. XFW, WWH, XHC, YH, LLW, KC, LL, and MJY performed data acquisition. MLC conduct cell experiments. FW carried out the data analysis and drafted the manuscript. NNL and JL revised the manuscript for important intellectual content. FW, NNL and JL had full access to all study data and took responsibility for the integrity of the data, accuracy of data analysis, and data interpretation. All authors were responsible for critical revisions and read and approved the final version of this manuscript.

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### Data availability

The data that support the findings of this study are available on request from the corresponding author. Expanded information regarding EZH2 mutations and patient survival time in breast cancer patients was downloaded from cBioPortal ( http://www.cbioportal.org ). Using the Breast Cancer dataset (MSK, Cancer Cell 2018).

## Declarations

### Ethics approval and consent to participate

This retrospective study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), This study was approved by the Ethical Committees of Fujian Cancer Hospital (grant no. K2022-093-01). All participants in this study have provided informed written consent.

**Consent for publication**
Not Applicable.

**Competing interests**
The authors declare no competing interests.

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