Expression of enhancer of zeste homolog 2 correlates with survival outcome in patients with metastatic breast cancer: exploratory study using primary and paired metastatic lesions

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Abstract

Background: In metastatic breast cancer, the status of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), as well as the Ki-67 index sometimes change between primary and metastatic lesions. However, the change in expression levels of enhancer of zeste homolog 2 (EZH2) between primary and metastatic lesions has not been determined in metastatic breast cancer.

Methods: Ninety-six metastatic breast cancer patients had biopsies or resections of metastatic lesions between September 1990 and February 2014 at the Kanagawa Cancer Center. We evaluated ER, PR, HER2, Ki-67, and EZH2 in primary lesions and their corresponding metastatic lesions using immunohistochemistry. We examined the change in expression of EZH2 between primary and metastatic lesions, the correlation between the expression of EZH2 and the expression of other biomarkers, and the relationship between EZH2 expression and patient outcome in metastatic breast cancer.

Results: EZH2 expression was significantly higher in metastatic lesions compared with primary lesions. EZH2 expression was highly correlated with Ki-67 expression in primary and metastatic lesions. High-level expression of EZH2 was associated with poorer disease-free survival (DFS) outcomes in patients with primary lesions (P < 0.001); however, high-level expression of EZH2 was not associated with poorer DFS outcomes in patients with metastatic lesions (P = 0.063). High-level expression of EZH2 was associated with poorer overall survival (OS) postoperatively in patients with primary (P = 0.001) or metastatic lesions (P = 0.005). High-level expression of EZH2 was associated with poorer OS outcomes after recurrence in patients with metastatic lesions (P = 0.014); however, high-level expression of EZH2 was not associated with poorer OS outcomes after recurrence in patients with primary lesions (P = 0.096). High-level expression of EZH2 in metastatic lesions was independently associated with poorer OS outcomes after recurrence.

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Background

Metastatic breast cancer (MBC) is difficult to treat using currently available conventional therapies, and median long-term survival rates in MBC patients have been reported to be as little as 18–24 months or 2–4 years from the time of diagnosis [1, 2]. Following chemotherapy, 10-year survival rates are approximately 5% and 2–3% in patients with MBC and those who survive >20 years, respectively [3, 4].

Management of MBC generally consists of systemic treatment (chemotherapy and targeted therapy, including anti-estrogen and anti-human epidermal growth factor receptor 2 [HER2] therapies). Treatment decisions for patients with MBC are usually based on estrogen receptor (ER), progesterone receptor (PR) and HER2 status of the primary tumor, the disease-free interval (DFI), site(s) of recurrence and performance status [5]. Because performing biopsies of metastatic lesions risks damaging vital organs and tissues, investigation of biomarkers in metastatic lesions is often challenging. Therefore, based on biomarkers in the primary tumor, the systemic treatment is often given to MBC patients. However, previously published reports show that, because biomarker levels change between primary and metastatic lesions, surgical biopsy of metastatic lesions followed by pathological confirmation for the investigation of biomarkers is occasionally proposed as an effective strategy in the treatment of MBC patients [6–12].

Enhancer of zeste homolog 2 (EZH2) is a well-known histone modifier protein that functions as a methyltransferase at lysine 27 of histone H3 [13]. EZH2 is a member of the polycomb group of genes [14] that is important for transcriptional regulation through chromatin remodeling, nucleosome modification and interactions with other transcription factors. It is assumed that EZH2 promotes breast cancer progression by transcriptional repression of tumor suppressors and by maintaining cells in a stem cell-like state [15, 16]. EZH2 has been demonstrated to be overexpressed in many types of malignancies, including breast, prostate and endometrial cancers, and has been suggested as a candidate for targeted treatment [17, 18]. In primary breast cancer (PBC), Kleer et al. [17] showed that EZH2 overexpression was further associated with a larger tumor size, ER- and PR-negative status, an advanced stage of disease, and significantly reduced disease-free survival (DFS) and overall survival (OS). Other investigators have reported that EZH2 promotes neoplastic progression in the breast, and that downregulation in EZH2 expression reduces in vivo tumor growth of breast cancer cells [17, 19, 20]. EZH2 is important for the control of cell proliferation and invasion, and has recently been shown to regulate DNA repair pathways and genomic stability [19, 21–24]. However, few reports have examined EZH2 expression in metastatic lesions, changes in EZH2 expression levels between primary and metastatic lesions, and patient outcome measures in MBC in relation to EZH2 expression.

The purpose of this study was to examine the expression levels of EZH2 in 96 pairs of primary cancer tissues and metastatic lesions obtained from patients with MBC. To evaluate the clinicopathological significance of EZH2 expression in metastatic lesions, we examined the correlations and changes in ER, PR, HER2, Ki-67 and EZH2 expression between primary cancer tissues and metastatic lesions, and DFS and OS outcomes after primary surgery and recurrence in patients with MBC.

Methods

Patients and samples

We retrospectively studied surgical specimens of PBC tumors and their corresponding metastatic lesions from patients who underwent surgery for their PBC tumor at the Kanagawa Cancer Center, Yokohama, Japan, between December 1977 and March 2013. Of those who relapsed after primary surgery between September 1990 and February 2014, there were 96 consecutive patients from whom metastatic lesions were obtained, either by surgery or biopsy, and evaluated using immunohistochemistry (IHC). In all cases, archival hematoxylin and eosin-stained slides of the PBC tumor and its corresponding metastatic lesion were retrieved and reviewed for confirmation of pathological features, as well as to select suitable tissue blocks for IHC analysis. We constructed tissue microarrays (TMAs) using PBC tumors and metastatic lesions. In patients receiving neoadjuvant chemotherapy, we examined the PBC tumor using a core needle biopsy before treatment was commenced in order to avoid potential bias. The Ethics Committees of the Kanagawa Cancer Center, Yokohama, Japan, approved the study protocol.

TMAs

TMAs consisting of cores, each measuring 2 mm in diameter, were assembled from formalin-fixed, paraffin-
embedded blocks of surgically removed tissue from primary tumors and their metastatic lesions in breast cancer patients. We included tissue cores from each primary tumor, metastatic lesion and normal breast tissue, which was used as a control, in the array.

**IHC analysis**

IHC staining for biomarkers ER, PR, HER2, Ki-67 and EZH2 was performed in all cases. TMAs were cut into 4-μm-thick sections and mounted onto pre-coated glass slides. All sections were stained using an autostainer (trade name Histostainer; Nichirei Biosciences Inc., Tokyo, Japan) using primary antibodies to ER (clone 1D5, dilution 1:80; Nichirei Biosciences Inc., Tokyo, Japan), PR (clone A9621A, dilution 1:100; Nichirei Biosciences Inc., Tokyo, Japan), HER2 (clone D8F12, dilution 1:800; Cell Signaling Technology Inc., Danvers, MA, USA), Ki-67 (clone SP-6, dilution 1:200; Nichirei Biosciences Inc., Tokyo, Japan) and EZH2 (clone D2C9, dilution 1:50; Cell Signaling Technology Inc., Danvers, MA, USA).

The results of the IHC analysis were assessed in a blinded fashion by a breast surgeon (H.I.) and pathologist (K.K.) who examined each slide independently. Unclear cases were discussed between the breast surgeon and pathologist. Each tumor was assessed twice and an average was calculated between the two scores. Nuclear immunoreactivity of each hormone receptor was scored independently by evaluating the percentage of positively stained cancer cells. ER and PR were defined as positive if there was staining of ≥1% of tumor cell nuclei. HER2 expression was scored as 0, 1+, 2+ or 3+ in accordance with the guidelines of the American Society of Clinical Oncology/College of American Pathologists [25]. A HER2 score of 3+ was considered positive. IHC 2+ tumors were not analyzed using in situ hybridization techniques. A HER2 score of 2+ was considered negative (see Additional file 1).

Regardless of the staining intensity, nuclear immunoreactivity of EZH2 and Ki-67 expression were scored independently by evaluating the proportion of positively stained cancer cells. Score 1 = ≤1/100 cells stained; Score 2 = ≤1/10 cells stained; Score 3 = ≤1/3 cells stained; Score 4 = ≤2/3 cells stained; and Score 5 = >2/3 cells stained (Fig. 1). EZH2 expression scores of 4 and 5, and Ki-67 expression scores of 3, 4 and 5, were considered high expression. EZH2 expression scores of 1, 2 and 3, and Ki-67 expression scores of 1 and 2 were considered low expression. The median EZH2 score and Ki-67 expression score across all PBC tumors sampled were 4 and 3, respectively (see Additional file 2). ER, PR, HER2 and Ki-67 expression were used to identify distinct molecular subtypes (Table 1). These were defined as follows: luminal A = ER and/or PR+, HER2−, and low Ki-67 expression; luminal B = ER and/or PR+, HER2−, and high Ki-67 expression; luminal HER2 = ER and/or PR+, HER2+; HER2-type = ER and PR−, HER2+; and triple-negative breast cancer (TNBC) = ER and PR−, HER2−.

**Follow-up**

Follow-up was performed using the KCCH Cancer Registry until October 31, 2015. Active follow-up was conducted by accessing hospital visit records, resident registration cards, and permanent domicile data. During the study period, no subject was lost to follow-up. The day of the biopsy of the metastatic lesions was defined as the date of diagnosis of recurrence. DFS was defined as the period from the day of primary surgery until the day of the biopsy of the metastatic lesions. OS after primary surgery was defined as the period from the day of primary surgery until the day of death. OS after recurrence was defined as the period from the day of biopsy of the metastatic lesions until the day of death. Median follow-up time was 96 months (range, 1–299 months) after the primary operation, and median follow-up time was 40 months (range, 0–231) after recurrence.

**Statistical analyses**

Relationships between biomarkers of the primary and metastatic breast cancer lesions and clinicopathological characteristics of the patients were analyzed using chi-square tests. Correlations between EZH2 expression and that of other biomarkers were evaluated using Pearson product-moment correlation coefficients (r). EZH2 and Ki-67 scores between primary and metastatic breast cancer lesions were compared using independent t-tests. DFS, survival rates after primary surgery, and survival rates after recurrence were analyzed using the Kaplan–Meier method, and any differences in survival rates were assessed using log-rank tests according to the expression of EZH2 in the primary and metastatic lesions. Cox proportional hazards models were applied to the multivariate analyses. Since we showed Ki-67 expression and EZH2 expression in primary and metastatic lesions to be strongly correlated (Table 2), we assessed prognostic factors (except for the Ki-67 expression) in multivariate analysis. For Pearson product-moment correlation coefficients (r), a P < 0.01, and for chi-square tests, independent t-tests, log-rank tests and Cox proportional-hazards models, a P < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA).

**Results**

Clinicopathological characteristics of all patients with MBC in this study

Patient characteristics are summarized in Table 3. Among 96 biopsies or resections of metastases, 26 (27.0%) were of
brain, eight (8.3%) of lung, one (1.0%) of liver, two (2.0%) of ovary, 13 (13.5%) of chest wall, 15 (15.6%) of lymph nodes, seven (7.3) of distant skin and 24 (25%) of bone.

**Changes in ER, PR, HER2, Ki-67 and EZH2 expression between primary and metastatic lesions**

Compared with primary lesions, metastatic lesions exhibited significantly higher levels of expression of Ki-67 (75.0% vs. 57.3% \( P = 0.010 \)) and EZH2 (82.3% vs. 56.3% \( P < 0.0001 \)). Conversely, no statistical differences in ER (42.7% vs. 53.1%, \( P = 0.149 \)), PR (40.6% vs. 49.0%, \( P = 0.246 \)) or HER2 status (14.6% vs. 16.7%, \( P = 0.691 \)) were observed between primary and metastatic lesions (Table 1). We subsequently analyzed the scores of expression levels of Ki-67 and EZH2, which demonstrated a significant difference between the two groups (i.e., high vs. low expression) (Fig. 2). The means and standard deviations of the Ki-67 scores were 2.74 ± 0.92 and 3.10 ± 0.97 for primary and metastatic lesions, respectively (\( P = 0.009 \)) (Fig. 2a), while the means and standard deviations of the EZH2 expression scores were 3.56 ± 1.34 and 4.26 ± 1.08 for primary and metastatic lesions, respectively (\( P < 0.001 \)) (Fig. 2b). Ki-67 and EZH2 expression scores were significantly higher in metastatic lesions compared with PBC lesions.

**Correlation coefficients of ER, PR, HER2, Ki-67 and EZH2 expression scores in primary and metastatic lesions**

In PBC lesions, ER (\( r = -0.103, P = 0.318 \)) and PR (\( r = -0.111, P = 0.282 \)) status were not significantly correlated with EZH2 expression, HER2 status exhibited a significant low correlation with EZH2 expression \( (r = 0.361, P < 0.001) \), and Ki-67 expression exhibited a significant high correlation with EZH2 expression \( (r = 0.722, P < 0.0001) \) (Table 2). Similarly, in metastatic lesions, ER \( (r = -0.099, P = 0.339) \) and PR \( (r = -0.190, P = 0.064) \) status were not significantly correlated with EZH2 expression, HER2 status exhibited a significant low correlation
### Table 1
Comparison of estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, Ki-67, and enhancer of zeste homolog 2 biomarkers between primary lesions and metastatic lesions in breast cancer patients \((n = 96)\)

| Biomarker                   | Primary lesions          | Metastatic lesions | \(P\)-value |
|-----------------------------|--------------------------|--------------------|-------------|
| ER status, \(n\) (%)        | 51 (53.1)                | 41 (42.7)          | 0.149       |
| positive                    |                          |                    |             |
| negative                    | 45 (46.9)                | 55 (57.3)          |             |
| PR status, \(n\) (%)        | 47 (49.0)                | 39 (40.6)          | 0.246       |
| positive                    |                          |                    |             |
| negative                    | 49 (51.0)                | 57 (59.4)          |             |
| HER2 status, \(n\) (%)      | 16 (16.7)                | 14 (14.6)          | 0.691       |
| positive                    |                          |                    |             |
| negative                    | 80 (83.3)                | 82 (85.4)          |             |
| Ki-67 expression, \(n\) (%) | 55 (57.3)                | 72 (75.0)          | 0.010*      |
| high                        |                          |                    |             |
| low                         | 41 (42.7)                | 24 (25.0)          |             |
| EZH2 expression, \(n\) (%)  | 42 (43.7)                | 17 (17.7)          |             |
| high                        | 54 (56.3)                | 79 (82.3)          | <0.0001*    |
| low                         |                          |                    |             |
| Molecular subtype, \(n\) (%)| 31 (32.3)                | 19 (19.8)          | 0.304       |
| luminal A\(^a\)             |                          |                    |             |
| luminal B\(^b\)             | 22 (22.9)                | 29 (30.2)          |             |
| luminal HER2\(^c\)          | 5 (5.2)                  | 5 (5.2)            |             |
| HER2-type\(^d\)             | 11 (11.5)                | 9 (9.4)            |             |
| TNBC\(^e\)                  | 27 (28.1)                | 34 (35.4)          |             |

**Abbreviations:** ER estrogen receptor, EZH2 enhancer of zeste homolog 2, HER2 human epidermal growth factor receptor 2, PR progesterone receptor, TNBC triple-negative breast cancer

\(^a\) Luminal A = ER and/or PR+, HER2−, and low Ki-67 expression

\(^b\) Luminal B = ER and/or PR+, HER2−, and high Ki-67 expression

\(^c\) Luminal HER2 = ER and/or PR+, HER2+

\(^d\) HER2-type = ER and PR−, HER2+

\(^e\) TNBC = ER and PR−, HER2−

*Indicates values that are statistically significant \((P < 0.05)\)

### Table 2
Correlation coefficient of biomarkers and EZH2 scores

| Biomarker                   | \(r\)        | \(P\)-value |
|-----------------------------|-------------|-------------|
| Primary ER status           | -0.103      | 0.318       |
| Primary PR status           | -0.111      | 0.282       |
| Primary HER2 status         | 0.361       | <0.001*     |
| Primary Ki-67 expression    | 0.722       | <0.0001*    |
| Metastatic ER status        | -0.099      | 0.339       |
| Metastatic PR status        | -0.190      | 0.064       |
| Metastatic HER2 status      | 0.306       | 0.002*      |
| Metastatic Ki-67 expression | 0.685       | <0.0001*    |

**Abbreviations:** ER estrogen receptor, EZH2 enhancer of zeste homolog 2, HER2 human epidermal growth factor receptor 2, PR progesterone receptor

*Indicates values that are statistically significant \((P < 0.01)\)

### Table 3
Clinicopathological Characteristics of all metastatic patients in this study

| Characteristic                  | Patients \((n = 96)\) | Percent |
|---------------------------------|------------------------|---------|
| Age, years \((\text{mean} ± \text{SD})\) | 51 ± 7.5               |         |
| Menopausal status               |                        |         |
| pre-                            | 44                     | 45.8    |
| post-                           | 52                     | 54.2    |
| Tumor size                      |                        |         |
| \(\leq 20\) mm                  | 17                     | 17.7    |
| > 20 mm                         | 77                     | 80.2    |
| unknown                         | 2                      | 2.1     |
| LN status                       |                        |         |
| positive                        | 63                     | 65.6    |
| negative                        | 33                     | 34.4    |
| Histological type               |                        |         |
| ductal                          | 83                     | 86.5    |
| special\(^a\)                   | 13                     | 13.5    |
| LVI status                      |                        |         |
| positive                        | 63                     | 65.6    |
| negative                        | 25                     | 26.1    |
| unknown                         | 8                      | 8.3     |
| Operation status                |                        |         |
| partial mastectomy              | 17                     | 17.8    |
| mastectomy                      | 79                     | 82.2    |
| Stage at the primary diagnosis  |                        |         |
| 1                               | 11                     | 11.5    |
| 2                               | 48                     | 50      |
| 3                               | 34                     | 35.4    |
| 4                               | 1                      | 1.0     |
| unknown                         | 2                      | 2.0     |
| Chemotherapy                    |                        |         |
| adjuvant                        | 83                     | 86.5    |
| none                            | 13                     | 13.5    |
| Hormone therapy                 |                        |         |
| adjuvant                        | 50                     | 52.1    |
| none                            | 46                     | 47.9    |
| Site of recurrence              |                        |         |
| brain                           | 26                     | 27.0    |
| lung                            | 8                      | 8.3     |
| liver                           | 1                      | 1.0     |
| ovary                           | 2                      | 2.1     |
| chest wall                      | 13                     | 13.5    |
| lymph node                      | 15                     | 15.6    |
| distant skin                    | 7                      | 7.3     |
| bone                            | 24                     | 25      |

**Abbreviations:** LN lymph node, LVI lymphovascular invasion, SD standard deviation

*Special type is invasive breast carcinoma except invasive ductal carcinoma
with EZH2 expression \( (r = 0.306, P = 0.002) \), and Ki-67 expression scores exhibited a high correlation with EZH2 expression \( (r = 0.685, P < 0.001) \) (Table 2).

**Relationship between EZH2 expression and patient clinicopathological characteristics**

Patient clinicopathological characteristics and their correlation with EZH2 expression in primary and metastatic lesions are summarized in Table 4. Relationships between the expression level status of EZH2 and patient age, menopausal status, tumor size, lymph node status, histological type (i.e., ductal vs. special), lymphovascular invasion status, operation status (i.e., partial vs. full mastectomy), adjuvant chemotherapy and hormone therapy status, primary ER status, primary PR status, primary HER2 status, primary Ki-67 expression, metastatic ER status, metastatic PR status, metastatic HER2 status, metastatic Ki-67 expression, the site(s) of recurrence (i.e., viscera, soft tissue or bone) and DFI (disease-free interval) (i.e., ≤2 years, >2 years, ≤10 years, >10 years) were evaluated. Factors significantly associated with PBC lesions included lymph node status, histological type, primary ER status, primary PR status, primary HER2 status, primary Ki-67 expression, metastatic ER status, metastatic PR status, metastatic HER2 status, metastatic Ki-67 expression, the site(s) of recurrence (i.e., viscera, soft tissue or bone) and DFI (disease-free interval) (i.e., ≤2 years, >2 years, ≤10 years, >10 years) were evaluated. Factors significantly associated with PBC lesions included lymph node status, histological type, primary ER status, primary PR status, primary HER2 status, primary Ki-67 expression, metastatic ER status, metastatic PR status, metastatic HER2 status, metastatic Ki-67 expression, and DFI, whereas factors significantly associated with metastatic lesions included histological type, adjuvant chemotherapy status, primary PR status, primary HER2 status, primary and metastatic Ki-67 expression, and the site(s) of recurrence (Table 4).

**Relationship between EZH2 expression and patient outcome**

We examined DFS and OS outcomes after primary surgery and recurrence in patients with MBC according to primary EZH2 expression (Fig. 3a, b, c), and DFS and OS outcomes after primary surgery and recurrence in patients with MBC according to metastatic EZH2 expression (Fig. 3d, e, f). Patient clinicopathological characteristics and their correlation with EZH2 expression in primary and metastatic lesions are summarized in Table 4. First, DFS, survival rates after primary surgery, and survival rates after recurrence were analyzed according to the expression of EZH2 in PBC lesions. Low EZH2 expression in PBC lesions occurred in 42 patients and high EZH2 expression in PBC lesions in 54 patients. Median DFS time in patients with high expression levels of EZH2 in PBC lesions was 30 compared with 74 months in patients with low expression levels of EZH2 in PBC lesions (Fig. 3a). Median survival time after primary surgery in patients with high expression levels of EZH2 in PBC lesions was 55 compared with 133 months in patients with low expression levels of EZH2 in PBC lesions (Fig. 3b). Patients expressing high levels of EZH2 in PBC lesions had significantly poorer DFS and OS outcomes than patients expressing low levels of EZH2 in PBC lesions \( (P < 0.001, P = 0.001) \). Second, DFS, survival rates after primary surgery, and survival rates after recurrence were analyzed according to the expression of EZH2 in the metastatic lesions. Low EZH2 expression in metastatic lesions occurred in 17 patients and high EZH2 expression in metastatic lesions occurred in 79 patients. Median survival time after primary surgery in patients with high expression levels of EZH2 in metastatic lesions was 66 compared with 161 months in patients with low expression levels of EZH2 in metastatic lesions (Fig. 3e). Median survival time after recurrence in patients with high expression levels of EZH2 in metastatic lesions was 25 compared with 50 months in patients with low expression levels of EZH2 in metastatic lesions (Fig. 3f). Patients expressing high levels of EZH2 in metastatic lesions had significantly poorer OS outcomes after primary surgery and recurrence than patients expressing low levels of EZH2 in metastatic lesions \( (P = 0.005, P = 0.014) \).
| Characteristic               | Primary EZH2 | Metastatic EZH2 | P-value | Primary EZH2 | Metastatic EZH2 | P-value |
|-----------------------------|--------------|-----------------|---------|--------------|----------------|---------|
|                            | low (n = 42) | high (n = 54)   |         | low (n = 17) | high (n = 79)  |         |
| **Age, years (mean ± SD)**  | 52 ± 8.5     | 50 ± 9.8        | 0.198   | 50 ± 7.8     | 51 ± 9.7       | 0.241   |
| **Menopausal status, n (%)**|              |                 |         |              |                 |         |
| pre-                        | 17 (38.6)    | 27 (61.4)       | 0.353   | 10 (22.7)    | 34 (77.3)      | 0.236   |
| post-                       | 25 (48.1)    | 27 (51.9)       |         | 7 (13.5)     | 45 (86.5)      |         |
| **Tumor size, n (%)**       |              |                 |         |              |                 |         |
| ≤ 20 mm                     | 8 (47.1)     | 9 (52.9)        |         | 2 (11.8)     | 15 (88.2)      |         |
| > 20 mm                     | 33 (42.9)    | 44 (57.1)       | 0.936   | 14 (18.2)    | 63 (81.8)      | 0.396   |
| unknown                     | 1 (50.0)     | 1 (50.0)        |         | 1 (50.0)     | 1 (50.0)       |         |
| **LN status, n (%)**        |              |                 |         |              |                 |         |
| positive                    | 23 (36.5)    | 40 (63.5)       | 0.048*  | 11 (17.5)    | 52 (82.5)      | 0.930   |
| negative                    | 19 (57.6)    | 14 (42.4)       |         | 6 (18.2)     | 27 (81.8)      |         |
| **Histological type, n (%)**|              |                 |         |              |                 |         |
| ductal                      | 32 (38.6)    | 51 (61.4)       | 0.010*  | 12 (14.5)    | 71 (85.5)      | 0.035*  |
| special                     | 10 (76.9)    | 3 (23.1)        | 5 (38.5) | 8 (61.5)     |                 |         |
| **LVI status, n (%)**       |              |                 |         |              |                 |         |
| positive                    | 32 (50.8)    | 31 (49.2)       |         | 11 (17.5)    | 52 (82.5)      |         |
| negative                    | 8 (32.0)     | 17 (68.0)       | 0.148   | 4 (16.0)     | 21 (84.0)      | 0.842   |
| unknown                     | 2 (25.0)     | 6 (75.0)        |         | 2 (25.0)     | 6 (75.0)       |         |
| **Operation status, n (%)** |              |                 |         |              |                 |         |
| partial mastectomy          | 4 (23.5)     | 13 (76.5)       | 0.064   | 2 (11.8)     | 15 (88.2)      | 0.479   |
| mastectomy                  | 38 (48.1)    | 41 (51.9)       |         | 15 (19.0)    | 64 (81.0)      |         |
| **Chemotherapy, n (%)**     |              |                 |         |              |                 |         |
| adjuvant                    | 34 (41.0)    | 49 (59.0)       | 0.164   | 12 (14.5)    | 71 (85.5)      | 0.035*  |
| none                        | 8 (61.5)     | 5 (38.5)        |         | 5 (38.5)     | 8 (61.5)       |         |
| **Hormone therapy, n (%)**  |              |                 |         |              |                 |         |
| adjuvant                    | 26 (52.0)    | 24 (48.0)       | 0.089   | 12 (24.0)    | 38 (76.0)      | 0.092   |
| none                        | 16 (34.8)    | 30 (65.2)       |         | 5 (10.9)     | 41 (89.1)      |         |
| **Primary ER status, n (%)**|              |                 |         |              |                 |         |
| positive                    | 28 23        | 0.019*          | 12 39   | 0.112        |                 |         |
| negative                    | 14 31        | 5 40            |         |              |                 |         |
| **Primary PR status, n (%)**|              |                 |         |              |                 |         |
| positive                    | 24 23        | 0.157           | 12 35   | 0.049*       |                 |         |
| negative                    | 18 31        | 5 44            |         |              |                 |         |
| **Primary HER2 status, n (%)**| 1 15        | 0.001*          | 0 16    | 0.042*       |                 |         |
| positive                    | 41 39        | 17 63           |         |              |                 |         |
| negative                    |              |                 |         |              |                 |         |
| **Primary Ki-67 expression, n (%)** | 8 47     | <0.001*         | 2 53    | <0.001*      |                 |         |
| high                        | 34 7         |                 | 15 26   |              |                 |         |
| low                         |              |                 |         |              |                 |         |
In the univariate analysis, compared with low EZH2 expression, high EZH2 expression was not a poor prognostic indicator of OS after recurrence outcome in PBC (hazard ratio [HR] 1.449; 95% confidence interval [CI] 0.930–2.258, \( P = 0.101 \)) (Table 5), whereas in metastatic lesions, high EZH2 expression was a poor prognostic indicator of OS outcome after recurrence (HR 2.116; 95% CI 1.143–3.916, \( P = 0.017 \)) (Table 5). Other poor prognostic indicators of OS outcome after recurrence in PBC and metastatic lesions from the univariate analysis included primary ER or primary PR status, primary and metastatic high Ki-67 expression, and ≤2 years of DFI (Table 5). A Cox proportional-hazards model using multivariate analysis but not including Ki-67 expression demonstrated that high EZH2 expression was independently associated with poorer OS outcomes after recurrence in patients with metastatic lesions (HR 2.047; 95% CI 1.074–3.902, \( P = 0.029 \)) (Table 5). Multivariate analysis of prognostic factors related to OS after recurrence including Ki-67 expression is shown in Additional file 3: Table S1.

**Discussion**

In this study, using primary and paired metastatic lesions from patients with MBC, EZH2 expression scores correlated significantly with Ki-67 expression scores in both primary and metastatic lesions, and Ki-67 expression and EZH2 expression scores were significantly higher in metastatic lesions compared with PBC lesions. Because Ki-67 expression scores in metastatic lesions increased more than in PBC lesions, we considered that proliferation in metastatic lesions increased more than in PBC lesions. We expected that EZH2 expression in metastatic lesions would be higher than that in PBC lesions. We showed that EZH2 expression correlated significantly with the Ki-67 expression in both PBC and metastatic lesions. We thought that EZH2 promotes breast cancer progression by transcriptional repression of tumor suppressors; consequently, Ki-67 expression increased in breast cancer cells with high EZH2 expression. We showed that EZH2 expression levels in bone metastatic lesions were significantly lower than in viscera and soft tissue metastatic lesions. Expression of

| Table 4 | Relationship between EZH2 expression in primary and metastatic breast cancer lesions and the clinicopathological characteristics of patients (n = 96) (Continued) |
|---|---|---|---|---|---|---|
| Metastatic ER status, n (%) | positive | 21 | 20 | 0.203 | 8 | 33 | 0.689 |
| negative | 21 | 34 | 9 | 46 |  
| Metastatic PR status, n (%) | positive | 22 | 17 | 0.039* | 9 | 30 | 0.254 |
| negative | 20 | 37 | 8 | 49 |  
| Metastatic HER2 status, n (%) | positive | 1 | 13 | 0.003* | 0 | 14 | 0.060 |
| negative | 41 | 41 | 17 | 65 |  
| Metastatic Ki-67 expression, n (%) | high | 23 | 49 | <0.001* | 2 | 70 | <0.001* |
| low | 19 | 5 | 15 | 9 |  
| Site of recurrence, n (%) | viscera b | 14 (37.8) | 23 (62.2) | 0.102 | 5 (13.5) | 32 (86.5) | 0.012* |
| soft tissue c | 13 (37.1) | 22 (62.9) | 3 (8.6) | 32 (91.4) |  
| bone | 15 (62.5) | 9 (37.5) | 9 (37.5) | 15 (62.5) |  
| Disease free interval | ≤2 years | 7 (23.5) | 24 (77.4) | 0.04* | 4 (12.9) | 27 (87.1) | 0.394 |
| >2 years | 35 (53.8) | 30 (46.1) | 13 (20) | 52 (80) |  
| ≤10 years | 32 (38.6) | 51 (61.4) | 0.010* | 13 (15.7) | 70 (84.3) | 0.185 |
| >10 years | 10 (76.9) | 3 (23.1) | 4 (30.8) | 9 (69.2) |  

**Abbreviations**: ER estrogen receptor, EZH2 enhancer of zeste homolog 2, HER2 human epidermal growth factor receptor 2, LN lymph node, LVI lymphovascular invasion, PR progesterone receptor, SD standard deviation

*Special type is invasive breast carcinoma except invasive ductal carcinoma

bViscera includes brain, lung, liver and ovary

Soft tissues includes chest wall, lymph node, distant skin

*Indicates values that are statistically significant (\( P < 0.05 \))
EZH2 in primary tumors of patients with DFIs ≤2 years was higher than in those with DFIs >2 years, whereas expression of EZH2 in primary tumors of patients with DFIs >10 years was lower than in those with DFIs ≤10 years. Given that bone metastasis occurs more frequently in ER-positive than ER-negative breast cancer [26] and in MBC patients with DFIs >10 years [27], we expected that EZH2 expression would be low in bone metastasis.

Fig. 3 Kaplan–Meier survival curves for breast cancer patients (n = 96) with high and low enhancer of zeste homolog 2 (EZH2) expression. Kaplan–Meier survival curves for breast cancer patients with high and low enhancer of zeste homolog 2 (EZH2) expression in (a) disease-free survival in patients with primary lesions, (b) overall survival in patients after primary surgery with primary lesions, (c) overall survival in patients after recurrence with primary lesions, (d) disease-free survival in patients with metastatic lesions, (e) overall survival in patients after primary surgery with metastatic lesions, and (f) overall survival in patients after recurrence with metastatic lesions.

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Table 5 Univariate and multivariate analysis of prognostic factors related to overall survival after recurrence \((n = 96)\)

| Prognostic factor                        | Patients \((n = 96)\) | Univariate analysis | Multivariate analysis |
|-----------------------------------------|-----------------------|---------------------|-----------------------|
|                                         |                       | HR  | 95% CI       | \(P\)-value | HR  | 95% CI       | \(P\)-value |
| Menopausal status, \(n\) (\%)           |                       |     |              |             |     |              |             |
| pre-                                    | 44 (45.8)             | 1   |              |             |     |              |             |
| post-                                   | 52 (54.2)             | 1.396 | 0.891–2.186 | 0.145      |     |              |             |
| Tumor size, \(n\) (\%)                  |                       |     |              |             |     |              |             |
| \(\leq 20\) mm                         | 17 (17.7)             | 1   |              |             |     |              |             |
| \(> 20\) mm                            | 77 (80.2)             | 1.317 | 0.726–2.388 | 0.364      |     |              |             |
| unknown                                 | 2 (2.1)               |     |              |             |     |              |             |
| LN status, \(n\) (\%)                   |                       |     |              |             |     |              |             |
| positive                                | 63 (65.6)             | 1.309 | 0.829–2.066 | 0.248      |     |              |             |
| negative                                | 33 (34.4)             | 1   |              |             |     |              |             |
| Histological type, \(n\) (\%)          |                       |     |              |             |     |              |             |
| ductal                                  | 83 (86.5)             | 1   |              |             |     |              |             |
| special\(^a\)                           | 13 (13.5)             | 1.028 | 0.543–1.945 | 0.932      |     |              |             |
| LVI status, \(n\) (\%)                  |                       |     |              |             |     |              |             |
| positive                                | 63 (65.6)             | 1.287 | 0.769–2.156 | 0.337      |     |              |             |
| negative                                | 25 (26.1)             | 1   |              |             |     |              |             |
| unknown                                 | 8 (8.3)               |     |              |             |     |              |             |
| Operation status, \(n\) (\%)            |                       |     |              |             |     |              |             |
| partial mastectomy                      | 17 (17.7)             | 1   |              |             |     |              |             |
| mastectomy                              | 79 (82.3)             | 0.894 | 0.500–1.596 | 0.704      |     |              |             |
| Chemotherapy, \(n\) (\%)                |                       |     |              |             |     |              |             |
| adjuvant                                | 83 (86.5)             | 1.320 | 0.707–2.461 | 0.383      |     |              |             |
| none                                    | 13 (13.5)             | 1   |              |             |     |              |             |
| Hormone therapy, \(n\) (\%)             |                       |     |              |             |     |              |             |
| adjuvant                                | 50 (52.1)             | 0.719 | 0.460–1.122 | 0.146      |     |              |             |
| none                                    | 46 (47.9)             | 1   |              |             |     |              |             |
| Primary ER status, \(n\) (\%)           |                       |     |              |             |     |              |             |
| positive                                | 51 (53.1)             | 0.541 | 0.344–0.850 | 0.008\(^*\) | 0.724 | 0.382–1.372 | 0.322      |
| negative                                | 45 (46.9)             | 1   |              |             |     |              |             |
| Primary PR status, \(n\) (\%)           |                       |     |              |             |     |              |             |
| positive                                | 47 (49.0)             | 0.595 | 0.380–0.931 | 0.023\(^*\) | 0.911 | 0.484–1.714 | 0.773      |
| negative                                | 49 (51.0)             | 1   |              |             |     |              |             |
| Primary HER2 status, \(n\) (\%)         |                       |     |              |             |     |              |             |
| positive                                | 16 (16.7)             | 1.122 | 0.616–2.046 | 0.706      |     |              |             |
| negative                                | 80 (83.3)             | 1   |              |             |     |              |             |
| Primary Ki-67 expression, \(n\) (\%)\(^b\) |                       |     |              |             |     |              |             |
| high                                    | 55 (57.3)             | 1.848 | 1.170–2.917 | 0.008\(^*\) |     |              |             |
| low                                     | 41 (42.7)             | 1   |              |             |     |              |             |
| Primary EZH2 expression, \(n\) (%)       |                       |     |              |             |     |              |             |
| high                                    | 54 (56.3)             | 1.449 | 0.930–2.258 | 0.101      |     |              |             |
| low                                     | 42 (43.7)             | 1   |              |             |     |              |             |
metastasis and in MBC patients with DFIs >10 years. However, because MBC patients with DFIs ≤2 years had a poor prognosis, we expected that EZH2 expression levels would be higher. We showed that high EZH2 expression in primary lesions was shown to be independently associated with poorer DFS and OS outcomes after primary surgery in MBC, whether or not high EZH2 expression in primary lesions was shown to be associated with OS after recurrence. High EZH2 expression in metastatic lesions was not associated with DFS after primary surgery, even if high EZH2 expression in metastatic lesions was shown to be independently associated with poorer OS outcomes after primary surgery and recurrence. We had considered the proliferation of primary lesions associated with DFS and OS after primary surgery until recurrence; on the other hand, proliferation of metastatic lesions was more associated with OS after recurrence than PBC lesions.

Previous reports have demonstrated that in PBC, EZH2 expression was significantly increased in malignant tumors, and was associated with a larger tumor size, ER- and PR-negative status, TNBC, advanced stage disease and reduced progression-free survival and OS [17, 28, 29]. In colon cancer and poorly differentiated synovial sarcomas, EZH2 expression was significantly related to increased tumor cell proliferation, as assessed using the Ki-67 expression [30, 31]. Nishimura et al. [12] reported that, in comparison to primary lesions, the Ki-67 expression score increased significantly in metastatic lesions. We found that in breast cancer patients, EZH2 expression scores correlated significantly with Ki-67 expression scores in both primary and metastatic breast cancer lesions.

### Table 5

| Metastatic ER status, n (%) | n (%) | 95% CI | HR         |
|---------------------------|-------|--------|------------|
| positive                  | 41 (42.7) | 0.972 | 0.625–1.513 | 0.901 |
| negative                  | 55 (57.3) | 1      |            |
| Metastatic PR status, n (%) | n (%) | 95% CI | HR         |
| positive                  | 39 (40.6) | 0.692 | 0.440–1.089 | 0.112 |
| negative                  | 57 (59.4) | 1      |            |
| Metastatic HER2 status, n (%) | n (%) | 95% CI | HR         |
| positive                  | 14 (14.6) | 1.282 | 0.673–2.440 | 0.450 |
| negative                  | 82 (85.4) | 1      |            |
| Metastatic Ki-67 expression, n (%) | n (%) | 95% CI | HR         |
| high                      | 72 (75.0) | 2.422 | 1.394–4.206 | 0.002* |
| low                       | 24 (25.0) | 1      |            |
| Metastatic EZH2 expression, n (%) | n (%) | 95% CI | HR         |
| high                      | 59 (77.6) | 2.116 | 1.143–3.916 | 0.017* |
| low                       | 17 (22.4) | 1      |            |
| Metastatic sites, n (%)    | n (%) | 95% CI | HR         |
| bones                     | 24 (25.0) | 0.899 | 0.548–1.476 | 0.674 |
| others                    | 72 (75.0) | 1      |            |
| Disease free interval      | n (%) | 95% CI | HR         |
| ≤ 2 years                  | 28 (29.0) | 1.713 | 1.086–2.702 | 0.021* |
| > 2 years                  | 68 (71.0) | 1      |            |
| ≤ 10 years                 | 83 (87.0) | 1      |            |
| > 10 years                 | 13 (13.0) | 0.861 | 0.442–1.679 | 0.661 |

**Abbreviations:** CI confidence interval, ER estrogen receptor, EZH2 enhancer of zeste homolog 2, HER2 human epidermal growth factor receptor 2, HR hazard ratio, LN lymph node, LVI lymphovascular invasion, PR progesterone receptor

*Surgical type is invasive breast carcinoma except invasive ductal carcinoma

*Ki-67 expression was excluded from the multivariate analysis because of an association with EZH2 expression in primary and metastatic breast cancer lesions

*Indicates values that are statistically significant (P < 0.05)
new therapeutic approaches is needed. Considering the downstream effects of EZH2, silencing of the EZH2 gene in the ER+ MCF-7 cell line resulted in higher expression of ER and increased sensitivity to anti-estrogen therapy [32]. EZH2 gene silencing has also been reported to result in a significant reduction in tumor growth in the MB-231 TNBC orthotopic mouse model of breast carcinomas. High EZH2 expression was shown to be significantly associated with TNBC and reduced OS outcomes [28]. In our cohort of MBC patients, we demonstrated a significant correlation between EZH2 expression and Ki-67 expression scores in primary and metastatic lesions. Therefore, EZH2 may represent a potential therapeutic target for this aggressive breast cancer that exhibits high expression levels of Ki-67, thus warranting further investigation. Using data obtained in this study as a reference of expression of EZH2 status in metastatic lesions and the correlation between EZH2 and other biomarkers in MBC, biopsy of metastatic lesions may become less necessary, thereby avoiding risk of vital organ damage because of the biopsy procedure.

Recently, several EZH2 inhibitors have been developed and tested in multiple types of cell lines and xenografts [33, 34]. Both EPZ-6438 (E7438) and GSK126, selective small-molecule inhibitors of histone methyltransferase activity, have yielded promising results in small cell lung cancer cell lines and malignant rhabdoid tumors [33, 34]. In the second quarter of 2015, Epizyme Inc., Cambridge, MA, USA initiated a phase 2 monotherapy trial of EPZ-6438 in patients with relapsed or refractory non-Hodgkin lymphomas [35].

This study has some limitations. First, the retrospective nature of the study design was prone to selection bias. Patients in this study tended to have a more aggressive breast cancer with poorer prognosis in comparison with a group from the general breast cancer patient population, since all of those recruited were diagnosed with MBC. Second, this study could not consider the effects of adjuvant therapy, which differed according to each patient, owing to the fact that patients were recruited over a long period, from 1977 to 2013. Previous reports have demonstrated that examining change due to treatment based on Ki-67 expression, the number of responders to endocrine therapy as the neoadjuvant therapy and hormonal therapy should be considered.

Conclusion
Our results suggest that EZH2 expression levels correlate significantly with the Ki-67 expression score. Therefore, EZH2 may represent a potential therapeutic target for this aggressive breast cancer, especially for those with a high Ki-67 expression score, which warrants further investigation. EZH2 expression scores were significantly higher in metastatic lesions compared with PBC lesions. We also showed that high EZH2 expression levels in primary lesions were independently associated with poorer DFS and OS after primary surgery, and that high EZH2 expression levels in metastatic lesions were independently associated with poorer OS outcomes after primary surgery and recurrence. A part of the present work was previously reported at the 2016 ASCO Annual Meeting [39].

Additional files

Additional file 1: Reasons for assessing cases scored as HER2 2+ as negative. (DOC 23 kb)

Additional file 2: Evaluation of immunostaining of EZH2 and Ki67. (DOC 52 kb)

Additional file 3: Table S1. Univariate and multivariate analysis of prognostic factors related to overall survival after recurrence including Ki-67 expression. (DOC 111 kb)

Additional file 4: Dataset of this study. (XLSX 26 kb)

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Availability of data and materials
The dataset supporting the conclusions of this article is included within the article (and its Additional file 4).

Authors’ contributions
HI, NS, TO, and YM designed the study. HI performed clinical, pathological and statistical investigation, and drafted the manuscript. KM participated in the histological and immunohistochemical evaluation. HI, TY and MY constructed TMAs. HI, TY and YN performed the immunohistochemical staining. TY, HN, AY, and KM assisted the clinical investigation. TY, YR, SS, and MM participated in preparing and drafting the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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