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# Relationship between the expression of Notch1 and EZH2 and the prognosis of breast invasive ductal carcinoma

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**ABSTRACT.** We determined whether the coexpression of Notch1 and EZH2 influences the progression and prognosis of breast invasive ductal carcinoma. Using the $\chi^2$ test, a significant difference was found between high and low expression of Notch1 in terms of lymph node, hormone receptor, and p53 expression ($P < 0.05$). Moreover, a significant difference was found between high and low expression of EZH2 in terms of tumor size, histologic grade, hormone receptor, and expression of Ki67 ($P < 0.05$). Using Pearson correlation analysis, we found a significant positive correlation between Notch1 and EZH2 expression in the tissue samples of breast invasive ductal carcinoma ($P = 0.038$). High Notch1 and EZH2 expression was associated with poor progression-free survival compared with low expression ($P_{Notch1} = 0.000$, 40.3 *vs* 48.9 months; $P_{EZH2} = 0.000$, 40.2 *vs* 49.9 months). Moreover, we found that high Notch1 and EZH2 expression was associated with poor overall survival compared with low expression ($P_{Notch1} = 0.000$, 51.2 *vs* 56.2 months; $P_{EZH2} = 0.002$, 51.7 *vs* 56.4 months). In conclusion, Notch1 and EZH2 coexpression contributes to the progression and prognosis of breast invasive ductal carcinoma.

**Key words:** Notch1; EZH2; Invasive ductal carcinoma; Prognosis

 

## INTRODUCTION

Breast cancer is one of the most common malignancies in women (Siegel et al., 2012). In Chinese women, it is the most common cancer and the third most common cause of cancer-related death (Smith et al., 2015). The number of women suffering from breast cancer in China is increasing, while the number of deaths is expected to decrease (Wong et al., 2015). Although improvements in early detection and treatment have reduced breast cancer mortality rates in recent years, prevention and therapy remain a major public health concern (Lang et al., 2015). Thus, identification and determination of new genes/pathways involved in breast cancer carcinogenesis will help in the development of better disease prognoses following treatment of breast cancer.

The Notch gene family encodes a group of evolutionarily conserved transmembrane receptors (Notch1-4) that are expressed on the cell surface as heterodimers. Notch signaling has been implicated in the self-renewal of stem cells or progenitor cells isolated from mammary glands (Dontu et al., 2003, 2004). Notch1 plays a role in human mammary tumorigenesis (Zardawi et al., 2010; Wang et al., 2011); it is reported that Notch1 is highly expressed in human primary breast ductal carcinoma and is activated by the Ras signaling pathway (Stier et al., 2002).

Enhancer of zeste homolog 2 (EZH2) is involved in controlling embryonic development and cell proliferation (Jacobs and van Lohuizen, 2002; Cao and Zhang 2004). EZH2 is a highly conserved histone methyltransferase that targets lysine 27 of histone H3 and methylated H3-K27, and is correlated with the silencing of genes for differentiation (Kirmizis et al., 2004). The expression of EZH2 is associated with proliferative and more aggressive tumor phenotypes, and thus contributes to the development of carcinogenesis (Varambally et al., 2002; Bachmann et al., 2006). Some researchers have reported that EZH2 plays a critical role in the development and prognosis of breast cancer (Bachmann et al., 2006), but none has reported the association in a Chinese population. Moreover, to date no one has reported an association between the expression of Notch1 and EZH2 and the development and prognosis of breast invasive ductal carcinoma. In this study, we attempted to determine whether the coexpression of Notch1 and EZH2 influences the progression and prognosis of breast invasive ductal carcinoma.

## MATERIAL AND METHODS

### Patients and tissue samples

Between 2003 and 2006, a total of 171 patients with breast invasive ductal carcinoma were recruited from the Third Affiliated Hospital of Harbin Medical University. All patients received postoperative adjuvant therapy. Patients who underwent preoperative radiochemotherapy were excluded from the study.

Thirty-two samples of normal tissue adjacent to the carcinoma were collected and used as controls during the same period. Tissue sections exhibiting - or + Her2 immunohistochemical staining were considered negative for Her2 expression. For cases with ++ staining, further *in situ* fluorescence hybridization was carried out to determine expression levels, as described in a previous study (Galgano et al., 2006).

Overall survival (OS) was calculated at the time of operation until death or last known date alive. Progression-free survival (PFS) was calculated at the time of operation until disease progression or death. All patients provided written informed consent prior to study enrollment. This

study was reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Harbin Medical University.

## Immunohistochemistry

Tissue microarrays were prepared according to standard procedures, and Notch1 and EZH2 were immunostained separately. For the immunohistochemical assays, 4-μm-thick sections cut from paraffin-embedded tissue blocks were dewaxed in xylene and dehydrated in graded ethanol. These sections were then placed in 1X ethylenediaminetetraacetic acid buffer for 20 min and incubated in methanol with 3% hydrogen peroxide for 30 min. After rehydration with phosphate-buffered saline, the sections were incubated with rabbit polyclonal antibody against human Notch1 (1:100 dilution, Abcam, USA) or EZH2 primary antibody (1:100 dilution, Cell Signaling, USA) for 2 h at room temperature. The sections were then washed three times in phosphate-buffered saline and reacted with horseradish peroxidase-polymer anti-rabbit IgG (1:100, Solomon Biotechnology Co., Ltd., China) for 1 h. Reactivity was developed in 3,3'-diaminobenzidine chromogen solution. The digital images of the five representative visual fields from slides that were positive for Notch1 and EZH2 (400X) were analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, USA).

The immunohistochemically stained sections were independently evaluated by two pathologists. Four grades were used to evaluate the staining intensity: negative (0), weak (1), medium (2), and strong (3). The degree of staining and the percentage of positive cells were scored as follows: 0, <10%; 1, 10-25%; 2, 26-50%; 3, 50-75%; and 4, ≥76%. Discrepancies were resolved by consensus between the two pathologists. The grades were multiplied to determine an H-score according to the method described in a previous study (Galgano et al., 2006). Negative or weak expression with scores between 0 and 3 was considered low expression, and strong expression (scores ≥ 4) was considered low or high expression.

## Statistical analysis

Continuous variables are reported as means ± SD, and categorical variables are shown by number (N) of subjects (%). The association between the expression level of Notch1 and EZH2 and the clinicopathological characteristics of the patients was assessed using the $\chi^2$ or Fisher exact tests. Correlation between Notch1 and EZH2 expression was evaluated by Pearson coefficient analyses. The Cox proportional hazards regression model was used to analyze the factors for the PFS and OS. The Kaplan-Meier method was used to plot the PFS and OS curves. The SPSS® statistical package, version 11.0 (SPSS Inc., Chicago, IL, USA) for Windows® was used for statistical analyses. All P values were two-tailed, and a difference was considered statistically significant when $P < 0.05$.

## RESULTS

The correlation between Notch1 and EZH2 expression and clinical and pathological characteristics of patients with breast invasive ductal carcinoma are shown in Table 1. The median patient age of the patients with breast invasive ductal carcinoma was 50 years (range, 29-68 years). Of the 171 tumor samples, Notch1 and EZH2 were expressed at high levels in 85 (50%)

and 93 (54%) samples, respectively. Using the $\chi^2$ test, a significant difference was found between high and low expression of Notch1 in terms of lymph node, hormone receptor, and expression of p53 ($P < 0.05$). Moreover, a significant difference was found between high and low expression of EZH2 in terms of tumor size, histologic grade, hormone receptor, and expression of Ki67 ($P < 0.05$).

**Table 1.** Correlation between Notch1 and EZH2 expression and clinical and pathological characteristics of patients with breast invasive ductal carcinoma.

| Parameters | Notch1 | | | EZH2 | | |
|---|---|---|---|---|---|---|
| | High (%) | Low (%) | P value | High (%) | Low (%) | P value |
| Age | | | | | | |
| ≤49 | 44 (52%) | 34 (40%) | 0.126 | 43 (46%) | 35 (45%) | 0.879 |
| >49 | 41 (48%) | 52 (60%) | | 50 (54%) | 43 (55%) | |
| Tumor size | | | | | | |
| ≤2 cm | 44 (52%) | 41 (48%) | 0.647 | 39 (42%) | 46 (59%) | 0.032 |
| >2 cm | 41 (48%) | 45 (52%) | | 54 (58%) | 32 (41%) | |
| Histologic grade | | | | | | |
| I + II | 46 (54%) | 52 (63%) | 0.441 | 46 (50%) | 52 (67%) | 0.03 |
| III | 39 (46%) | 34 (37%) | | 47 (50%) | 26 (33%) | |
| Lymph node | | | | | | |
| Negative | 30 (35%) | 44 (51%) | 0.045 | 38 (41%) | 36 (46%) | 0.537 |
| Positive | 55 (65%) | 42 (49%) | | 55 (59%) | 42 (54%) | |
| Hormone receptor | | | | | | |
| ER(-) | 47 (51%) | 28 (36%) | 0.038 | 60 (65%) | 33 (42%) | 0.005 |
| ER(+) | 46 (49%) | 50 (64%) | | 33 (35%) | 45 (58%) | |
| PR(-) | 48 (56%) | 46 (53%) | 0.759 | 59 (63%) | 35 (45%) | 0.02 |
| PR(+) | 37 (44%) | 40 (47%) | | 34 (37%) | 43 (55%) | |
| Her2/neu (-) | 49 (58%) | 65 (77%) | 0.015 | 55 (59%) | 59 (76%) | 0.024 |
| Her2/neu (+) | 36 (42%) | 21 (23%) | | 38 (41%) | 19 (24%) | |
| Pathologic stage | | | | | | |
| I + II | 62 (73%) | 65 (70%) | 0.729 | 66 (71%) | 61 (78%) | 0.298 |
| III | 23 (27%) | 21 (30%) | | 27 (29%) | 17 (22%) | |
| P53(-) | 36 (42%) | 50 (58%) | 0.047 | 46 (49%) | 40 (51%) | 0.878 |
| P53(+) | 49 (58%) | 36 (42%) | | 47 (51%) | 38 (49%) | |
| Ki67(-) | 45 (53%) | 44 (51%) | 0.879 | 39 (42%) | 50 (64%) | 0.006 |
| Ki67(+) | 40 (47%) | 42 (49%) | | 54 (58%) | 28 (36%) | |
| Menopausal status | | | | | | |
| Pre-menopausal | 26 (31%) | 35 (41%) | 0.202 | 34 (37%) | 27 (35%) | 0.873 |
| Post-menopausal | 59 (69%) | 51 (59%) | | 59 (63%) | 51 (65%) | |

The correlation between EZH2 and Notch1 expression is shown in Table 2. Using Pearson correlation analysis, we found a significant positive correlation between Notch1 and EZH2 expression and breast invasive ductal carcinoma in the tissue samples ($P = 0.038$).

**Table 2.** Correlation between EZH2 and Notch1 expression.

| Notch1 | EZH2 | | Pearson correlation | P value |
|---|---|---|---|---|
| | Negative (N) | Positive (N) | | |
| Negative (N) | 46 | 40 | 0.159 | 0.038 |
| Positive (N) | 32 | 53 | | |

The role of high Notch1 and EZH2 expression in PFS and OS was evaluated using Kaplan-Meier analysis. High Notch1 and EZH2 expression was associated with poor PFS compared with low expression ($P_{Notch1}$ = 0.000, 40.3 *vs* 48.9 months; $P_{EZH2}$ = 0.000, 40.2 *vs* 49.9 months, Figure 1). Moreover, we found that high Notch1 and EZH2 expression was associated with poor OS compared with low expression ($P_{Notch1}$ = 0.000, 51.2 *vs* 56.2 months; $P_{EZH2}$ =0.002, 51.7 *vs* 56.4 months, Figure 2).

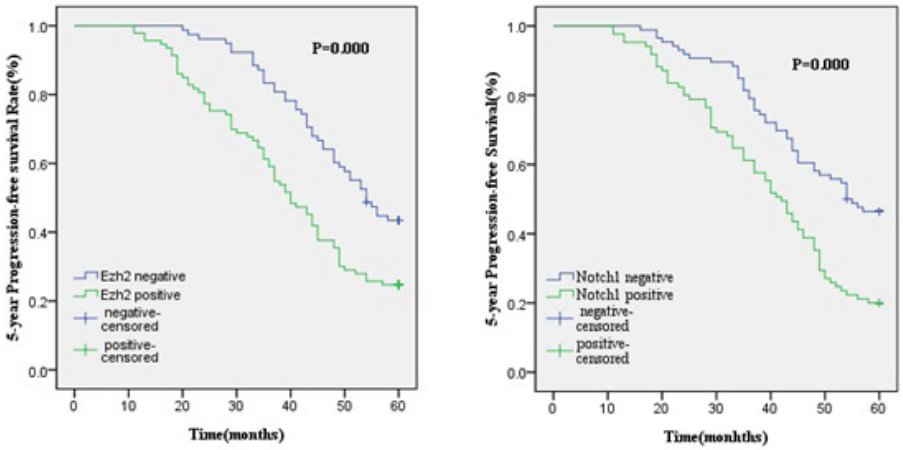


**Figure 1.** Correlation between Notch1 and EZH2 expression and progression-free survival.

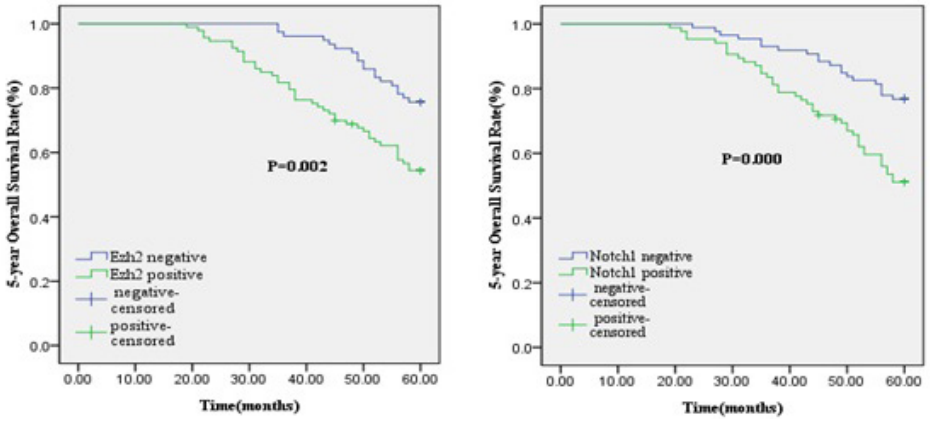


**Figure 2.** Correlation between Notch1 and EZH2 expression and overall survival.

Using the Cox proportional hazards regression model, we found that ER(+) was negatively associated with disease recurrence and metastasis for patients with breast invasive ductal carcinoma (HR = 0.546, 95%CI = 0.361-0.826) (Table 3). The Her-2(+), high Notch1, and EZH2 expression levels were associated with a higher risk of disease recurrence or metastasis for patients with breast invasive ductal carcinoma [for Her-2(+), HR = 2.028, 95%CI = 1.342-3.066; for high Notch1 expression, HR = 1.559, 95%CI = 1.050-2.315; and for high EZH2 expression, HR = 1.514, 95% CI = 1.028-2.229]. Moreover, ER(+) was associated with lower risk of OS compared with ER(-) (HR = 0.229, 95% CI = 0.116-0.450). High Notch1 expression was associated with higher risk of death compared with low Notch1 expression (HR = 1.848, 95% CI = 1.054-3.237).

**Table 3.** Multivariate analysis of prognostic factors for patients with breast invasive ductal carcinoma.

| Parameters | Hazard ratio | P value | 95%CI |
|---|---|---|---|
| PFS | | | |
| ER(+) | 0.546 | 0.004 | 0.361-0.826 |
| Her-2(+) | 2.028 | 0.001 | 1.342-3.066 |
| High Notch1 expression | 1.559 | 0.028 | 1.050-2.315 |
| High EZH2 expression | 1.514 | 0.036 | 1.028-2.229 |
| OS | | | |
| ER(+) | 0.229 | 0.000 | 0.116-0.450 |
| Her-2(+) | 1.059 | 0.830 | 0.626-1.794 |
| High Notch1 expression | 1.848 | 0.032 | 1.054-3.237 |
| High EZH2 expression | 1.638 | 0.084 | 0.936-2.867 |

PFS = progression-free survival; OS = overall survival.

## DISCUSSION

The identification of shared genetic determinants for the early prophylaxis and treatment of breast cancer is the premise emerging from the results of accumulative association studies. In the present study, we found that there was a significant positive correlation between Notch1 and EZH2 expression and breast invasive ductal carcinoma. High Notch1 and EZH2 expression levels were associated with a higher risk of disease recurrence or metastasis in patients with breast invasive ductal carcinoma, and high Notch1 expression was associated with higher risk of death in patients with that affliction. These results indicate that Notch1 and EZH2 coexpression contributes to the progression and prognosis of breast invasive ductal carcinoma.

It has been reported that Notch1 plays a predominantly oncogenic role in tumorigenesis, including that of breast cancer (Reedijk et al., 2005; Klinakis et al., 2006; Alimirah et al., 2007), and Notch1 small hairpin RNA can reduce cellular growth and macro- and micrometastasis (McGowan et al., 2011). Anti-Notch1 monoclonal antibodies can delay tumor recurrence and enhance the anti-tumor efficacy of chemotherapy (Qiu et al., 2013). The authors of previous studies have reported that expression of Notch1 is associated with a prognosis of breast cancer (Ma et al., 2011; Yao et al., 2011; Zhu et al., 2013; Cao et al., 2014; Yuan et al., 2015). Yuan et al. (2015) conducted a study in a Chinese population and reported that Notch inhibitors play a role in blocking the early progression of ductal carcinoma *in situ*; they also reported the outcomes of clinical trials for Notch1-targeting therapeutics. Ma et al. (2011) suggested Notch1 was associated with the clinical parameters of breast cancer patients. Yao et al. (2011) reported that Notch1 is correlated with the prognosis of breast cancer patients. Our study also found an association between the expression of Notch1 and progression and prognosis of breast invasive ductal carcinoma, which corroborates previous studies.

EZH2 is a crucial component of polycomb repressive complex 2. Overexpression of EZH2 is an important driver of tumor development and is often correlated with human cancer progression and poor prognosis (Sauvageau and Sauvageau, 2010). The forced expression of EZH2 in cell lines can increase proliferation and oncogenic capacity (Piunti and Pasini, 2011). Additionally, reducing EZH2 expression and EZH2 degradation inhibits cell growth and reduces tumor formation in cancers (Qiu et al., 2013). Ntziachristos et al. (2012) reported that Notch1 interacts with EZH2 to control gene expression and cell transformation in T-cell acute lymphoblastic leukemia. The authors of a previous meta-analysis reported that EZH2 is a putative factor in the progression of breast cancer, and overexpression of EZH2 is distinctly correlated with poor survival of breast cancer (Wang et al., 2015). In this study, we found that EZH2 is associated with the progression of breast invasive ductal carcinoma, which is correlated to the expression of Notch1.

In conclusion, our study suggests that Notch1 and EZH2 may be promising biomarkers and future therapeutic targets for breast invasive ductal carcinoma. Future studies with larger sample sizes are required to confirm the role of Notch1 and EZH2 in the progression and prognosis of breast invasive ductal carcinoma.

## Conflicts of interest

The authors declare no conflict of interest.

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