Aberrant expression of enhancer of zeste homologue 2, correlated with HIF-1α, refines relapse risk and predicts poor outcome for breast cancer

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Received March 26, 2014; Accepted May 16, 2014

DOI: 10.3892/or.2014.3322

Abstract. Overexpression of enhancer of zeste homologue 2 (EZH2), a key component of polycomb proteins, has been linked to aggressive tumor behavior in a variety of cancers. *In vitro*, hypoxia-inducible factor 1α (HIF-1α) transcriptionally activates EZH2 and promotes the progression of breast tumor initiating cells. Here, we characterized the clinicopathological effect of EZH2 and HIF-1α in 410 breast cancer patients. We examined EZH2 and HIF-1α expression using immunohistochemistry and western blotting. We found that EZH2 and HIF-1α were highly expressed in 99 (24.1%) and 272 (70.6%) patients, respectively. EZH2 overexpression was associated with lymphatic invasion (P=0.025), HER2 expression (P=0.005) and hypoxia (P<0.001). Overexpression of EZH2 predicted a poor 5-year overall survival (OS, 74.8 vs. 93.4%, P=0.001), disease-free survival (DFS, 72.2 vs. 88.6%, P=0.031), local failure-free survival (LFFS, 95.7 vs. 97.9%, P=0.045) and distant metastasis-free survival (DMFS, 75.4 vs. 90.5%, P=0.039). Multivariate analysis confirmed that EZH2 is an independent prognostic factor for OS, DFS and LFFS. Moreover, a positive correlation was identified between EZH2 and HIF-1α (r=0.299, P<0.001). Importantly, tumors coexpressing HIF-1α and EZH2 had a poorer OS (P=0.007). In conclusion, our study demonstrated that EZH2 is an independent negative prognostic biomarker for breast cancer. Tumors overexpressing HIF-1α and EZH2 are more prone to disease progression.

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Key words: EZH2, HIF-1α, prognosis, breast cancer

Introduction

Enhancer of zeste homologue 2 (EZH2), a catalytic subunit in the polycomb repressive complex 2 (PRC2), is involved in methylating histone H3 at lysine 27 (H3K27) and silencing tumor-suppressor genes (1-3). Accumulating evidence suggests that EZH2 is associated with increased tumor cell proliferation, local invasiveness and distant metastasis (3-6). In breast cancer cells, EZH2 was found to promote tumor invasion by transcriptionally repressing the metastasis suppressor RKIP (7). Ectopic expression of EZH2 maintained the differentiation state of basal-like breast cancer cells and promoted the expression of progenitor-associated genes, leading to reduced luminal differentiation, which is a hallmark of the aggressive phenotype in breast cancer (8). Indeed, aberrant expression of EZH2 was identified in a variety of solid tumors and might be correlated with the aggressive features of breast cancer such as higher histological grade, increased tumor cell proliferation, lymph node invasion and larger tumor size (4,9-15). Although the aggressive effects of EZH2 have been confirmed (4,16,17), the prognostic value of EZH2 in breast cancer remains unclear (9,16-18). Kleer *et al* found that high levels of EZH2 mRNA were correlated with poor distant metastasis-free survival (DMFS) and that overexpression of EZH2 protein predicted inferior overall survival (OS) and disease-free survival (DFS) (9). In a nested case-control study, a close correlation between EZH2 and Ki67 was identified, but there was no significant prognostic value after the final multivariate analysis (16).

The activation of hypoxia-inducible factor α (HIF-1α), the main regulator of the cellular response to hypoxia (19), is linked with tumor angiogenesis and metastasis (20). Its aberrant expression was also found to predict an unfavorable prognosis in lymph node-positive breast cancer (21). Importantly, the aggressive clinicopathological effects of HIF-1α may be attributed to its interaction with several important cellular proteins, such as growth factor β (20), Beclin 1 (22,23), EZH2 (24), Aurora-A (25) and AKT (26). For example, HIF-1α transcriptionally upregulates EZH2 by binding to the hypoxia reaction element (HRE) in the EZH2 promoter region, enhancing the activation of RAF1-ERK-β-catenin signaling to promote cancer progression in $CD44^{+}CD24^{-/low}$ breast cancer initiating cells (27). Nevertheless, the clinicopathological effects of EZH2 and HIF-1α in breast cancer have not yet been characterized.

This study was conducted to assess the clinicopathological value of EZH2 and its relationship with HIF-1α in breast cancer patients.

Materials and methods

Patients and eligibility. Tumor samples were harvested from 410 breast cancer patients who underwent mastectomy (n=398) or lumpectomy (n=12) with axillary lymph node dissection from April 1999 to October 2008. The patient clinicopathological characteristics were obtained from archived records (Table I). Patients who met the following inclusion criteria were enrolled in the study: pathologically confirmed breast cancer; no history of ontological surgery, chemotherapy, or radiotherapy; and complete follow-up information and paraffin-embedded specimens were available. Patients were excluded if they previously received any anticancer therapy, had a prior malignancy, or were pregnant and lactating. Histological grade was classified according to the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system (28). Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor type 2 (HER2) status was determined by immunohistochemistry (IHC). The Human Ethics Committee of the Third Affiliated Hospital, Sun Yat-sen University approved this study. Written informed consent was obtained from all patients prior to treatment.

Adjuvant therapy. All the patients received standard postoperative adjuvant therapy according to the National Comprehensive Cancer Network (NCCN) Guidelines. Briefly, patients with tumors >1 cm and/or lymph node metastasis, were administered postoperative adjuvant anthracycline-containing chemotherapy, such as FAC (5-fluorouracil/doxorubicin/cyclophosphamide), TAC (docetaxel/doxorubicin/cyclophosphamide), or AC (doxorubicin/cyclophosphamide) followed by paclitaxel/docetaxel. Adjuvant radiotherapy was performed in patients with four or more positive axillary lymph node metastases and/or with tumors >5 cm. Patients with tumors positive for ER or PR received adjuvant endocrine therapy (tamoxifen or aromatase inhibitors) for 5 years.

Tissue microarray (TMA) construction. Prior to TMA construction, all hematoxylin and eosin-stained tissues were reviewed anew. A tissue array machine (Beecher Instruments, Silver Spring, MD, USA) was then used to harvest three malignant cores and two normal adjacent cores per case to construct the TMAs. Briefly, a hollow needle was used to pinch and remove bipartite cylinder tissue cores (1.0 mm in diameter) from selected regions of donor tissues. The pinched tissue cores were then inserted into a paraffin block in a precisely spaced array pattern (29).

Semi-quantitative assessment of IHC staining. TMAs were sectioned at a 4-μm thickness, dewaxed with xylene, rehydrated with graded ethanol and immersed in sodium citrate (pH 6.0) for antigen retrieval using a microwave. After blocking in hydrogen peroxide and goat serum albumin, sections were incubated with primary rabbit polyclonal antibodies for EZH2 (1:200; BD Pharmingen, 612666) and mouse polyclonal antibodies for HIF-1α (1:100; Millipore, MAB5382) at 4˚C overnight, followed by the appropriate secondary antibody for 30 min at room temperature. Slides were then processed further with diaminobenzidine and counterstained with hematoxylin. Slides with known high expression of EZH2 and HIF-1α were used as the positive control. Replacing the specific primary antibody with phosphate-buffered saline served as a negative control.

Two pathologists, who were blinded to the clinicopathological and follow-up information, evaluated IHC staining independently. Visible brown nuclear staining was considered to indicate positive staining for EZH2 and HIF-1α. Immunoreactivity was assessed based on both the intensity and extent of the staining as we previously described (22). The staining intensity was scored as 0 (negative), 1 (bordering), 2 (weak), 3 (moderate) and 4 (strong). The staining extent was categorized as 0 (negative), 1 (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%) according to the percentage of positive staining cells in the field. The final score was obtained by multiplying the intensity and extent scores.

Cell cultures. The human breast cancer cell lines MCF-7, MDA-MB-231 and BT-474 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The human mammary epithelial cell line MCF-10A was grown in DMEM/F-12 medium supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 10 μg/ml human insulin, 0.5 μg/ml hydrocortisone, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were maintained in a humidified incubator at 37˚C with 5% CO_2.

Western blotting. Proteins were extracted from the cultured cells and liquid nitrogen-preserved tissues using RIPA buffer (Takara Bio) on ice. After determining the protein concentrations using the Bradford method with bovine serum albumin (Sigma-Aldrich) as a standard, 50 μg of protein was loaded onto each lane of 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were then blocked and incubated with mouse anti-EZH2 (1:1,000; BD Pharmingen, 612666), mouse anti-HIF-1α (1:1,000; Millipore, MAB5382), or mouse anti-β-actin (1:1,000; Santa Cruz, sc-81178) antibody.

Receiver operating characteristic (ROC) analysis. ROC curve analysis was used to identify the IHC cutoff score for EZH2 expression as we previously reported (30). Briefly, the sensitivity

and specificity for the prognosis being studied at each IHC score were plotted to generate a ROC curve. The score closest to the point of maximum sensitivity and specificity, the point (0.0, 1.0) on the curve, was fixed as the cutoff score to classify the patients as having or not having the outcome. Prior to ROC analysis, the survival status was dichotomized: survival [death from cancer vs. others (censored, alive, or death from other causes)], local failure (with vs. without) and distant metastasis (with vs. without).

Clinical outcome assessment. All patients were followed up using a strict protocol. After the completion of surgery, patients were observed every 4-6 months for 5 years and every 12 months thereafter. OS was defined as the time from the date of surgery to the date of death due to breast cancer, or the date of last follow-up if patients were still alive. DFS was measured from the date of surgery to the date of local recurrence/distant metastasis, date of death, or the latest date when censored. LFFS was calculated from the date of surgery to the date of local failure, date of death, or the latest date when censored. DMFS was defined as the time from the date of surgery to the date of distant metastases, the date of death, or the latest date when censored (22).

Statistical analysis. The relationship between EZH2 expression and clinicopathological variables was assessed using the Chi-square test. The probability of survival was estimated using the Kaplan-Meier method and the difference between curves was assessed using the log-rank test. The prognostic value of multiple factors on survival was evaluated in a Cox proportional hazards model. Statistical analyses were performed using SPSS v. 17.0 (SPSS, Inc., Chicago, IL, USA). A two-tailed $P<0.05$ was considered to indicate a statistically significant result.

Results

Patient characteristics. A total of 410 breast cancer patients were included in the study. The median age was 49 years (range, 26-84 years). The clinicopathological characteristics of the 410 patients are shown in Table I. After a median follow-up of 78.8 months (range, 36.6-144.7 months), 76 of the 410 patients (18.5%) suffered tumor relapse (10 locoregional recurrences and 66 distant metastases) and 55 patients (13.4%) ultimately died of tumor progression. The 5-year OS, DFS, LFFS and DMFS were 93.1, 88.7, 97.0, and 89.9%, respectively.

EZH2 and HIF-1α expression and ROC analysis. IHC staining showed that both EZH2 and HIF-1α displayed strong nuclear staining in the tumors (Fig. 1A and C), but were weakly or negatively expressed in normal breast glandular epithelia (Fig. 1B and D). Western blotting further confirmed that, compared with adjacent tissues and normal breast cells (MCF-10A), EZH2 was overexpressed in tumors and breast cancer cell lines (MCF-7, MDA-MB-231 and BT-474) (Fig. 1E and F).

For EZH2, the ROC analysis-generated IHC cutoff scores for predicting OS, DFS, LFFS and DMFS were 8.0, 8.0, 4.0, and 8.0, respectively. Therefore, we defined an EZH2 IHC score ≤8.0 as low expression and >8.0 as high expression. For

Table I. EZH2 status in relation to the clinicopathological characteristics of 410 breast cancer patients.

		EZH2 expression		
Characteristics	n	High	Low	P-value
N	410	99	311	
Age (years)				
<50	236	56	180	0.818
≥50	174	43	131	
Menopausal status				
Post-menopausal	164	41	123	0.742
Pre-menopausal	246	58	188	
Histological type				
Ductal	391	97	294	0.359
Lobular	11	1	10	
Other	8	1	7	
Tumor size (cm)				
<2	173	35	138	0.138
2-5	184	53	131	
>5	53	11	42	
Histological grade				
1-2	155	35	120	0.419
3	65	18	47	
Unknown	190			
Lymph node metastasis				
Negative	186	45	141	0.984
Positive	224	54	170	
Lymphatic invasion				
No	390	90	300	0.025
Yes	20	9	11	
ER status				
Negative	3	1	2	0.565
Positive	407	98	309	
PR status				
Negative	18	3	15	0.581
Positive	392	96	296	
HER2 status				
Negative	379	85	293	0.005
Positive	31	14	17	
HIF-1α expression				
Low	113	13	100	<0.001
High	272	81	191	
Unknown	25			

EZH2, enhancer of zeste homologue 2; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor type 2; HIF-1α, hypoxia-inducible factor 1α.

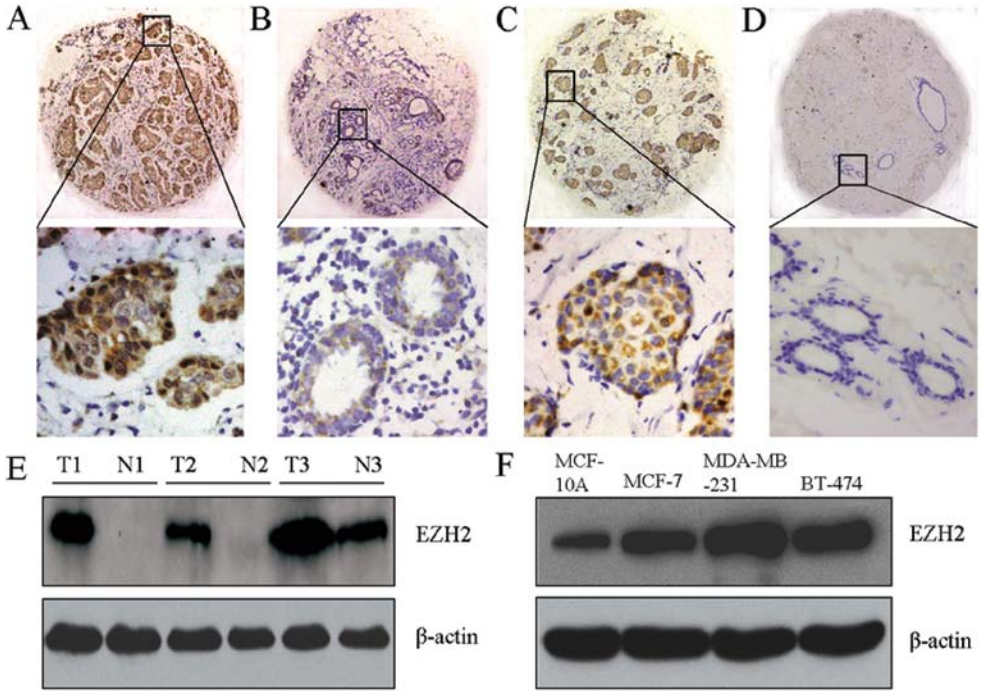

Figure 1. EZH2 and HIF-1α expression in breast cancer, normal breast tissues, and cell lines. EZH2 exhibited nuclear overexpression in (A) breast cancer tissues, and was expressed weakly in (B) normal breast gland duct epithelia. HIF-1α was overexpressed in (C) tumor tissues and not expressed in the (D) normal gland ducts. Upper panels, x50 magnification; lower panels, x400 magnification. (E) Western blotting of EZH2 expression in breast cancer tissues (T) and paired normal gland duct epithelia (N). (F) Western blotting of EZH2 expression in MCF-10A, MCF-7, MDA-MB-231 and BT-474 cells. Equal protein loading was confirmed using β-actin.

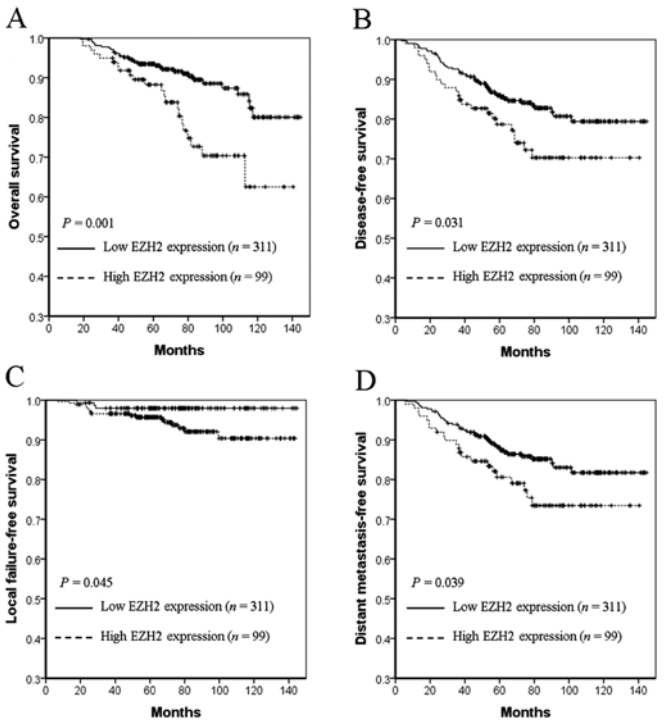

Figure 2. Kaplan-Meier survival curves according to EZH2 expression in 410 breast cancer patients. Higher EZH2 expression was associated with poor (A) overall survival, (B) disease-free survival, (C) local failure-free survival and (D) distant metastasis-free survival.

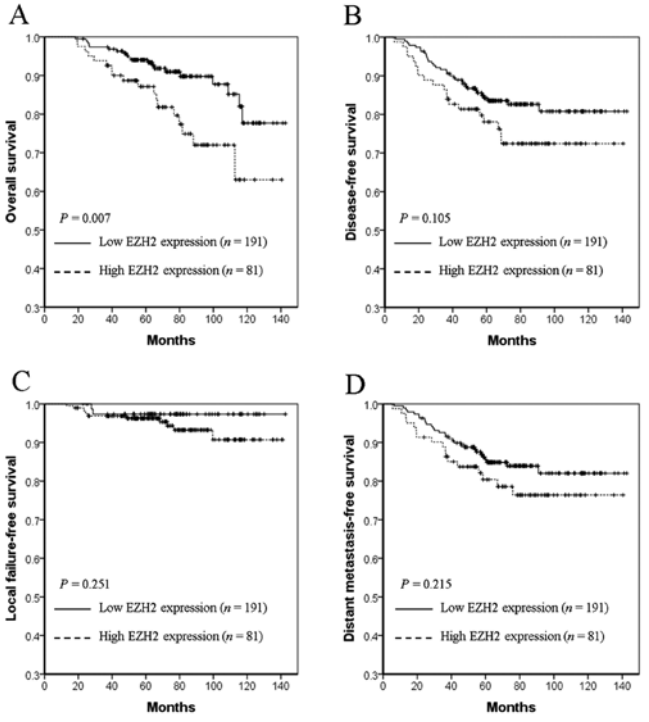

Figure 3. Kaplan-Meier estimated survival according to EZH2 expression in 272 HIF-1α-positive breast cancer patients. EZH2 overexpression correlated with worse (A) overall survival, but not (B) disease-free survival, (C) local failure-free survival and (D) distant metastasis-free survival in HIF-1α-overexpressing patients.

HIF-1α, a cutoff point of 3.0 separated patients into high and low expression subgroups.

According to the cutoff scores generated from ROC analyses, EZH2 had nuclear overexpression in 24.1% (99/410)

Table II. Univariate analysis of OS, DFS, LFFS, and DMFS in 410 breast cancers

Variables	P-values			
	OS	DFS	LFFS	DMFS
Age	0.953	0.902	0.823	0.550
Menopause status	0.449	0.207	0.315	0.181
Tumor size	<0.001	<0.001	0.211	<0.001
Histological grade	0.789	0.160	0.057	0.608
Lymph node metastasis	<0.001	<0.001	0.001	<0.001
Lymphatic invasion	0.090	0.093	0.819	0.156
ER status	0.321	0.425	0.692	0.458
PR status	0.768	0.888	0.962	0.666
HER2 status	0.041	0.083	0.663	0.019
HIF-1α expression	0.363	0.416	0.815	0.224
EZH2 expression	0.001	0.031	0.045	0.039

OS, overall survival; DFS, disease-free survival; LFFS, local failure-free survival; DMFS, distant metastasis-free survival; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor type 2; HIF-1α, hypoxia-inducible factor 1α; EZH2, enhancer of zeste homologue 2.

of patients, whereas HIF-1α was aberrantly expressed in 70.7% (272/385) of patients. Moreover, there was a positive correlation between EZH2 and HIF-1α levels (r=0.299, P<0.001).

EZH2 and HIF-1α expression and clinicopathological characteristics. As shown in Table I, EZH2 overexpression was significantly correlated with aggressive clinicopathological features, including lymphatic invasion (P=0.025), HER2 expression (P=0.005) and hypoxia (defined by HIF-1α expression) (P<0.001). Other potential factors including age, menopausal status, histological type, tumor size, histological grade, lymph node metastasis and ER and PR status were not significantly correlated with EZH2 expression (all P>0.05).

EZH2 and HIF-1α expression and patient outcome. As shown in Fig. 2, EZH2 overexpression was associated with an inferior 5-year OS (74.8 vs. 93.4%, P=0.001, Fig. 2A), DFS (72.2 vs. 88.6%, P=0.031, Fig. 2B), LFFS (95.7 vs. 97.9%, P=0.045, Fig. 2C) and DMFS (75.4 vs. 90.5%, P=0.039, Fig. 2D) in breast cancer. In contrast, there was no predictive value of HIF-1α in OS (93.1 vs. 90.8%, P=0.858), DFS (85.2 vs. 86.5%, P=0.633), LFFS (96.0 vs. 97.3%, P=0.490) and DMFS (87.5 vs. 88.4%, P=0.578). However, in the HIF-1α overexpressing subgroup, high EZH2 expression significantly worsened 5-year OS (72.0 vs. 93.4%, P=0.007, Fig. 3A), but not DFS (72.4 vs. 84.9%, P=0.105, Fig. 3B), LFFS (96.3 vs. 97.3%, P=0.251, Fig. 3C), or DMFS (76.4 vs. 86.3%, P=0.215, Fig. 3D) compared with low EZH2 expression.

Univariate and multivariate analysis. As shown in Table II, poor OS and DMFS were associated with tumor size (P<0.001 and P<0.001, respectively), lymph node metastasis (P<0.001 and P<0.001, respectively), HER2 status (P=0.041 and

Table III. Multivariate Cox proportional-hazards analysis in 410 breast cancers.

Variables	OS			DFS			LFFS			DMFS		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.288	0.741-2.238	0.369	1.227	0.770-1.957	0.389	1.337	0.531-3.362	0.537	1.099	0.663-1.821	0.714
Tumor size	1.777	1.178-2.680	0.006	1.557	1.109-2.188	0.011	1.610	0.804-3.227	0.179	1.584	1.099-2.283	0.014
Lymph node metastasis	3.499	1.720-7.117	0.001	3.395	1.899-6.070	<0.001	8.372	1.904-36.817	0.005	2.944	1.604-5.405	<0.001
Lymphatic invasion	1.343	0.471-3.832	0.581	1.212	0.515-2.850	0.660	0.680	0.087-5.296	0.713	1.167	0.460-2.963	0.745
HER2 status	1.277	0.590-2.765	0.535	1.252	0.613-2.560	0.537	0.662	0.145-3.026	0.594	1.601	0.776-3.304	0.202
EZH2 expression	2.250	1.297-3.903	0.004	1.635	1.002-2.667	0.049	4.165	1.181-14.689	0.027	1.572	0.930-2.659	0.091

OS, overall survival; DFS, disease-free survival; LFFS, local failure-free survival; DMFS, distant metastasis-free survival; HR, hazard ratio; CI, confidence interval; HER2, human epidermal growth factor receptor type 2; EZH2, enhancer of zeste homologue 2.

P=0.019, respectively) and EZH2 expression (P=0.001 and P=0.039, respectively). Moreover, tumor size (P<0.001), lymph node metastasis (P<0.001) and EZH2 expression (P=0.031) might predict poor DFS. For LFFS, only lymph node metastasis (P=0.001) and EZH2 expression (P=0.045) were potential prognostic factors. Multivariate analysis confirmed that tumor size, lymph node metastasis status and EZH2 levels were independent prognostic factors for patient outcome (Table III). Specifically, EZH2 was an independent poor prognostic biomarker for OS [hazard ratio (HR), 2.250; 95% confidence interval (CI): 1.297-3.903, P=0.004)], DFS (HR, 1.635; 95% CI: 1.002-2.667, P=0.049) and LFFS (HR, 4.165; 95% CI: 1.181-14.689, P=0.027), but not for DMFS (HR, 1.572; 95% CI: 0.930-2.659, P=0.091). As expected, tumor size was an independent predictor for poor OS (HR, 1.777; 95% CI: 1.178-2.680, P=0.006), DFS (HR, 1.557, 95% CI: 1.109-2.188, P=0.011) and DMFS (HR, 1.584, 95% CI: 1.099-2.283, P=0.014). Lymph node metastasis status was also a negative independent prognostic factor for OS (HR, 3.499; 95% CI: 1.720-7.117, P=0.001), DFS (HR, 3.395; 95% CI: 1.899-6.070, P<0.001), LFFS (HR, 8.372; 95% CI: 1.904-36.817, P=0.005) and DMFS (HR, 2.944; 95% CI: 1.604-5.405, P<0.001).

Discussion

EZH2 is a core subunit of PRC2 that plays an essential role in catalyzing the trimethylation of H3K27 and mediating transcriptional repression. Therefore, it is involved in cell cycle regulation, deciding cell fate, senescence and cancer (31). Although EZH2 has been linked with an aggressive phenotype in breast cancer, its clinicopathological value remains unclear. In the present study, we examined the expression of EZH2 in 410 breast cancer patients and found that EZH2 levels were closely associated with lymphatic invasion status, HER2 expression and tumor hypoxia (Table I). EZH2 overexpression predicted a poor 5-year OS, DFS, LFFS and DMFS (Fig. 2). Importantly, although no prognostic value of HIF-1α was detected, the overexpression of both HIF-1α and EZH2 significantly worsened OS in the HIF-1α overexpression subgroup of patients (Fig. 3). Moreover, univariate and multivariate analyses demonstrated that EZH2 is an independent prognostic marker for breast cancer (Tables II and III).

A number of studies have reported that EZH2 expression levels might vary in different breast cancer subtypes. In early-stage breast cancer, EZH2 was found to be overexpressed in 57.6% of patients (18). An inflammatory breast cancer cohort study revealed that EZH2 was positively expressed in 75.7% of patients (32). In a subgroup of triple-negative patients, EZH2-positive staining was detected in 85.7% of patients (33). When all breast cancer subtypes were combined, the EZH2 expression rate was 47.4-64.0% (16,17). The corresponding mean mRNA levels of EZH2 in luminal A, luminal B, HER2$^+$, basal-like and normal-like subtypes were -0.476, 0.145, 0.186, 0.778 and -0.853, respectively (34). These results suggest that the EZH2 level might be expressed in a subtype-dependent manner. However, the EZH2 expression levels were not characterized in luminal subtypes. In the present study, most patients were ER-positive (99.3%), PR-positive (95.6%) and HER2-negative (92.4%), suggesting that the subtypes in the present cohort were mainly luminal A and luminal B. The EZH2 expression rate was 24.1%, which is similar to the 33.2% mRNA expression rate identified in 235 ER-positive breast cancer patients (35). Therefore, the current and previous studies demonstrated that EZH2 might have a relatively low expression level in luminal subtype breast cancer.

It was reported that high levels of EZH2 are associated with ER-negative, PR-negative and HER2-overexpressing breast cancer (9,16-18). Consistent with this, we found that enhanced EZH2 was closely correlated with HER2, rather than ER and PR status (Table I). The underlying mechanism for this could be that EZH2 can repress or activate nuclear factor-κB (NF-κB) signaling in different breast cancer subtypes. In ER-negative basal-like breast cancer, EZH2 functioned by transactivating NF-κB signaling molecules such as interleukin 6 and tumor necrosis factor. Conversely, EZH2 might repress other NF-κB signaling molecules such as GATA3 and FOXA1 in ER-positive luminal-like breast cancer (36). Importantly, NF-κB signaling played an essential role in regulating ER, PR and HER2 expression in breast cancer (37). Consistent with this, the present study found that EZH2 levels were ranked from low to high in an ER/PR/HER2-dependent manner: MCF-10A (human mammary epithelial cell line) < MCF-7 (ER-positive) < BT-474 (HER2-positive) < MDA-MB-231 (ER-negative). This suggests that EZH2 might be a determining factor in establishing breast cancer subtypes.

Although luminal breast cancer is associated with a relatively favorable clinical outcome, ~15% of patients ultimately develop cancer-related mortality (38). Therefore, developing additional prognostic biomarkers will greatly benefit the subgroup of patients at high risk of disease progression in luminal subtypes. The present study showed that EZH2 could predict the outcome of luminal subtypes. More importantly, we found that EZH2 had a comparable hazard ratio to tumor size (1.777 and 1.557, respectively) and lymph node metastasis (3.499 and 3.395, respectively) for predicting the risk of death and relapse (Table III). This suggests that combining EZH2 and the TNM staging system would lead to a more accurate prognosis prediction and risk definition for breast cancer. In addition, we also found that the expression of EZH2 and HIF-1α were positively correlated in luminal breast cancer subtypes (r=0.299, P=0.039). Moreover, HIF-1α and EZH2 co-overexpression was a predictor of poorer OS (Fig. 3A). These findings suggest that EZH2 could be a useful negative prognostic predictor and that hypoxia might lead to a more worsened OS in luminal breast cancer patients.

HIF-1α activation is an aggressive clinicopathological biomarker and might be a poor prognostic factor in breast cancer (21). However, the clinicopathological value of HIF-1α in luminal-subtype breast cancer remains unclear. Similar to EZH2, HIF-1α expression was tumor subtype-dependent: luminal-type tumors expressed lower levels of HIF-1α than basal-like and HER2-positive tumors (39). Therefore, HIF-1α was reported to be a negative prognostic biomarker in non-luminal subtype breast cancer, but was not an independent prognostic factor for the luminal subtype in the present study.

In conclusion, this study demonstrated that high levels of EZH2 expression predicted poor OS, PFS, LFFS and DMFS in luminal-subtype breast cancer patients. Importantly, EZH2 and HIF-1α co-overexpression predicted poorer OS, leading to refined risk stratification for luminal breast cancer subtypes.

Acknowledgements

The National Natural Science Foundation of China (no. 81370060 to X-B.W., no. 81372374 to X-Y.W.) and the Science and Technology Foundation of Guangdong Province (no. 2012B091100460 to X-Y.W., no. 2011B031800014 to M.D.) supported this work.

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