Expression of Enhancer of Zeste Homologue 2 Is Significantly Associated with Increased Tumor Cell Proliferation and Is a Marker of Aggressive Breast Cancer

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Abstract The polycomb group protein enhancer of zeste homologue 2 (EZH2) has been linked to invasive properties of aggressive breast cancer. In this report, tissue microarray analysis of 190 breast carcinomas from a nested case-control study shows that EZH2 is significantly associated with interval breast cancers. Further, a strong relationship was found with tumor cell proliferation (by Ki-67 expression), locally advanced disease, metastasis at presentation, markers of the basal epithelial phenotype (positivity for cytokeratin 5/6 or P-cadherin), and p53 status. EZH2 expression was also significantly associated with glomeruloid microvascular proliferation, an aggressive angiogenic phenotype. For prediction of aggressive disease (any event of locally advanced disease, lymph node spread, or distant spread), EZH2 was the only variable of significance in multivariate analysis, whereas no additional information was given by Ki-67. Although EZH2 expression was significant in univariate survival analysis, only tumor cell proliferation and lymph node status were significant in the final multivariate model. In conclusion, our findings indicate an important relationship not only between EZH2 and markers of tumor cell proliferation but also with aggressive disease. These findings might be practically important and relevant because the polycomb group proteins have recently been suggested as candidates for targeted therapy.

Progression of breast cancer is a significant clinical problem and there is a need for novel treatment strategies. Although multiple prognostic factors have been reported, few markers, like c-erbB-2 status (Her2; ref. 1), have practical value as response predictors for targeted therapy (2). The enhancer of zeste homologue 2 (*EZH2*) is a member of the polycomb group of genes and has been involved in cell cycle regulation, and polycomb group proteins were recently suggested as candidates for targeted treatment (3). In human tumors, expression of EZH2 was first associated with hormone refractory and aggressive prostate cancer (4). In breast cancer, Kleer et al. (5) showed that EZH2 expression was increased in malignant tumors and promoted anchorage-independent and invasive growth *in vitro*, whereas proliferation was not significantly enhanced. EZH2 expression was further associated with increased tumor diameter, negative estrogen receptor (ER) and progesterone receptor (PR) status, and advanced stage of disease. Reduced patient survival was significantly predicted by EZH2 positivity in a subgroup of 194 cases. In a different study, Raaphorst et al. (6) found that EZH2 expression was associated with poorly differentiated and invasive breast cancers, whereas no clear relationship with proliferation was found. In this study, there was no prognostic effect of EZH2 expression.

EZH2 is regulated by E2F transcription factors, which are liberated by retinoblastoma protein phosphorylation (7, 8). Activated *p53* down-regulates the *EZH2* gene through repression of its promoter (9). Recently published cDNA microarray data indicate that *EZH2* is specifically down-regulated in senescent fibroblasts and that disruption of EZH2 expression retards cell proliferation and induces cell cycle arrest at the G_2-M transition (9). Expression of EZH2 in cultured mouse embryonic fibroblasts was found to be critical for S-phase entry and G_2-M transition (7), and EZH2 tranfection increased proliferation in lymphoma cells (10).

Thus, whereas several basic studies have indicated that EZH2 expression is involved in the regulation of cell cycle progression, tumor cell proliferation rate has not been examined in previous studies of human breast cancer. The aim of our study was, therefore, to examine EZH2 expression as a marker of aggressive subgroups in breast cancer, with special focus on tumor cell proliferation. We also wanted to evaluate whether EZH2 was related to the recently described molecular subtypes of malignant breast tumors (11), especially the basal-like category, or to the vascular phenotype of glomeruloid microvascular proliferation,

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an important feature of some poor prognosis breast cancers (12). In this first population-based validation study of breast cancer and EZH2, our findings indicate a strong association between EZH2 expression levels and gradually increased tumor cell proliferation. Expression of EZH2 also captures multiple associations with features of aggressive breast cancer, including p53 status, c-erbB-2 staining, and glomeruloid microvascular proliferation, and we found significantly decreased survival in univariate analysis. These novel findings are practically important and especially relevant because the polycomb group proteins have recently been suggested as candidates for targeted therapy (3, 9).

Materials and Methods

The present series of breast cancers was selected from the population-based Norwegian Breast Cancer Screening Program (Hordaland County), which started in 1996 with two-view mammography done every 24 months (13). Briefly, 95 invasive interval cancers occurred during the first two screening intervals (1996-2001), and these were matched by size ($\pm$2.0 mm) with 95 screen-detected tumors from a total of 317 invasive tumors during the first two rounds (median diameter 15.6 and 15.7 mm, respectively; ref. 14). After matching, the mean tumor size for screen detected and interval cases was 25.1 and 23.1 mm, respectively, and the corresponding mean age in these groups was 62 and 59 years. In addition to age and tumor diameter (by pathologic examination), basic characteristics, such as breast density (13), histologic type, histologic grade (15), lymph node metastases, and distant metastases at diagnosis were recorded. All interval cases were reviewed by one experienced radiologist (A. Braaten); 44 cases (46.3%) were true cases (i.e., the screening mammogram was negative but the tumor was visible at time of diagnosis). The median time from the last mammogram to the diagnosis of interval cancer was 17.1 months. Last date of follow-up was November 31, 2004, and median follow-up time (of survivors) was 72 months. During the follow-up period, 31 patients died of breast cancer.

Immunohistochemistry. Tissue microarray slides were used in the present study. The tissue microarray technique is tissue conserving and has been validated in several studies (16, 17). The method has been found reliable and reproducible in studies of breast cancer for markers such as ER, PR, and c-erbB-2 (18, 19). Tissue microarray slides were used for Ki-67, p53, c-erbB-2, cytokeratin 5/6 (CK5/6), E-cadherin, P-cadherin, and EZH2, whereas ER and PR were obtained from the routine pathology reports (based on immunohistochemistry).

Staining of EZH2 was done on a DAKO Autostainer using the EnVision chain-polymer method. Tissue microarray sections were incubated for 1 hour with a monoclonal antibody (clone M18) against EZH2 (20–22). A secondary antibody (antimouse) was added. For Ki-67, p53, and CK5/6, the staining was done on a DAKO TechMate 500 slide processing equipment (DakoCytomation, Copenhagen, Denmark) using the standard avidin-biotin method, whereas for E-cadherin and P-cadherin, staining was done on the DAKO Autostainer. The DAKO HercepTest was used to determine c-erbB-2 protein expression.

For all markers with the exception of Ki-67, c-erbB-2, and glomeruloid microvascular proliferation, a staining index (values 0-9), obtained as a product of staining intensity (0-3) and proportion of immunopositive cells (<10% = 1, 10-50% = 2, >50% = 3), was calculated (23–26). Nuclear EZH2 expression was considered positive for scores >3 (median staining index 3.0), also in agreement with Kleer at al. (positive score $\geq$3; ref. 5). Cutpoints for p53, E-cadherin, and P-cadherin were identified as previously reported from our group (14, 24, 27). Ki-67 staining was assessed according to the approach of Weidner et al. (28), and the upper quartile (22.0 %) was chosen as cutpoint. This is in accordance with Gilliland et al. (29), defining tumors with a high proportion of proliferating cells to >20%. C-erbB-2 immunostaining was scored according to the HercepTest criteria (30). Staining of endothelial cells by factor VIII (A-0082, DakoCytomation) was done on formalin-fixed and paraffin-embedded archival material as previously published (31), and glomeruloid microvascular proliferation was recorded according to Straume and Akslen (17) as either absent or present. For most markers, staining was recorded independently by two observers (K. Collett/I.M. Stefansson or K. Collett/J. Arnes). The interobserver agreement (κ coefficient) between negative and positive cases was 0.74 (c-erbB-2), 0.76 (CK5/6), 0.70 (P-cadherin), 0.90 (p53), and 0.64 (glomeruloid microvascular proliferation), respectively. For EZH2, an intraobserver κ coefficient was recorded (0.85) and the interobserver κ value was 0.73. In cases recorded with different values, each case was discussed with another observer.

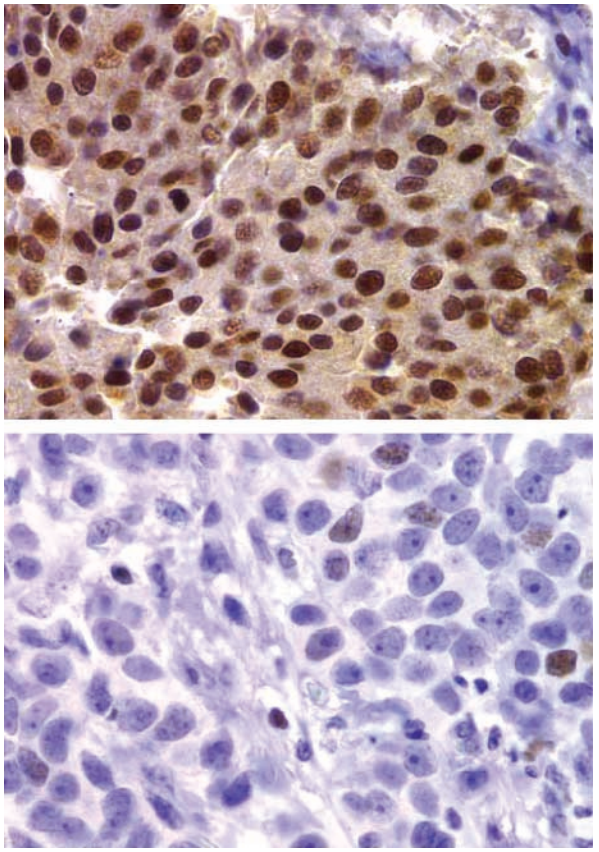

Fig. 1. Positive (*top*) and negative (*bottom*) nuclear staining for EZH2.

Statistical analysis. Associations between categorical variables were assessed by Pearson's χ^2 test. When comparing interval cases to screen detected tumors, the statistical analyses were adapted to matched pair data using McNemar's test for hypothesis testing of the dichotomous variables. Univariate survival analysis (using death from breast cancer as end-point) was done by the product-limit procedure (Kaplan-Meier method) with the log-rank test. Covariates included in multiple logistic regression analysis were ER status, p53 status, histologic grade (1 versus 2/3), and Ki-67 and EZH2 status. For prediction of aggressive disease, defined as any event of locally advanced disease, lymph node spread or distant metastases, CK5/6 and P-cadherin were also included. In Cox proportional hazards regression analysis, tumor size, histologic grade, lymph node status, Ki-67 status, and EZH2 status were entered. Variables were examined by log-log plot to determine whether they could be incorporated in Cox proportional hazards regression models (32). The statistical calculations were done using SPSS 12.0 with the exception of conditional logistic regression analysis for which LogXact 5.0 was used, and the confidence limits for odds ratios (OR) for which special programming in Maple 8 was applied (33).

Results

Positive and negative staining of EZH2 is illustrated in Fig. 1. Tumors positive for EZH2 were more likely to be ductal carcinomas than negative tumors [OR, 4.9; 95% confidence interval (95% CI), 1.6-15.2; Table 1]. A positive staining for EZH2 was associated with higher histologic grade (OR, 5.8; 95% CI, 2.5-13.4), and tumors expressing EZH2 were more

Table 1. The number and proportion of cases (%) for different variables and molecular markers according to EZH2 protein expression, with the corresponding OR and 95% CI

Variable	EZH2 neg (*n* = 100), *n* (%)	EZH2 pos (*n* = 90), *n* (%)	OR (95% CI)	*P*
Breast density				
Low (<30%)	21 (21)	20 (22)	1.5 (1.4-1.6)*	0.550
Mod (30-70%)	65 (65)	51 (57)	1.0	
High (>70%)	14 (14)	19 (21)		
Histologic† type				
Ductal	78 (80)	81 (95)	4.9 (1.6-15.2)	0.003
Lobular	19 (20)	4 (5)	1.0	
Histologic grade				
1-2	92 (92)	60 (67)	1.0	<0.001
3	8 (8)	30 (33)	5.8 (2.5-13.4)	
Nodal‡ status				
Neg	69 (70)	46 (58)	1.0	0.08
Pos	29 (30)	34 (42)	1.8 (0.9-3.3)	
Locally advanced disease				
No	94 (94)	73 (82)	1.0	0.012
Yes	6 (6)	16 (18)	3.4 (1.3-9.2)	
Metastasis at time of diagnosis				
No	100 (100)	84 (93)	1.0	0.01
Yes	0 (0)	6 (7)	∞; (1.4-∞)	
ER				
Neg	12 (12)	30 (33)	3.7 (1.7-7.8)	<0.001
Pos	88 (88)	60 (67)	1.0	
PR				
Neg	23 (23)	40 (44)	2.7 (1.4-5.0)	0.002
Pos	77 (77)	50 (56)	1.0	
Ki-67				
Low	94 (94)	46 (51)	1.0	<0.001
High	6 (6)	44 (49)	15.0 (6.0-37.7)	
P53				
Low, score ≤ 3	96 (96)	61 (68)	1.0	<0.001
High, score > 3	4 (4)	29 (32)	11.4 (3.8-34.0)	
C-erbB-2				
Neg, score 0/1	91 (91)	68 (76)	1.0	0.005
Pos, score 2/3	9 (9)	22 (24)	3.3 (1.4-7.5)	
CK5/6				
Neg, score = 0	94 (94)	66 (73)	1.0	<0.001
Pos, score > 0	6 (6)	24 (27)	5.7 (2.2-14.7)	
P-cadherin				
Neg, score ≤ 3	90 (90)	67 (74)	1.0	0.007
Pos, score > 3	10 (10)	23 (26)	3.1 (1.4-6.9)	
E-cadherin				
Neg, score ≤ 3	51 (51)	33 (37)	1.8 (1.0-3.2)	0.06
Pos, score > 3	49 (49)	57 (63)	1.0	
GMP§				
None	81 (88)	54 (61)	1.0	0.001
>0	11 (12)	34 (39)	4.6 (2.2-9.9)	

*The mean difference between EZH2-negative and EZH2-positive cases with 95% CI.
†Eight cases with unknown or histologic type other than ductal or lobular were excluded.
‡Twelve cases with unknown nodal status were excluded.
§Ten cases with unknown GMP status were excluded.

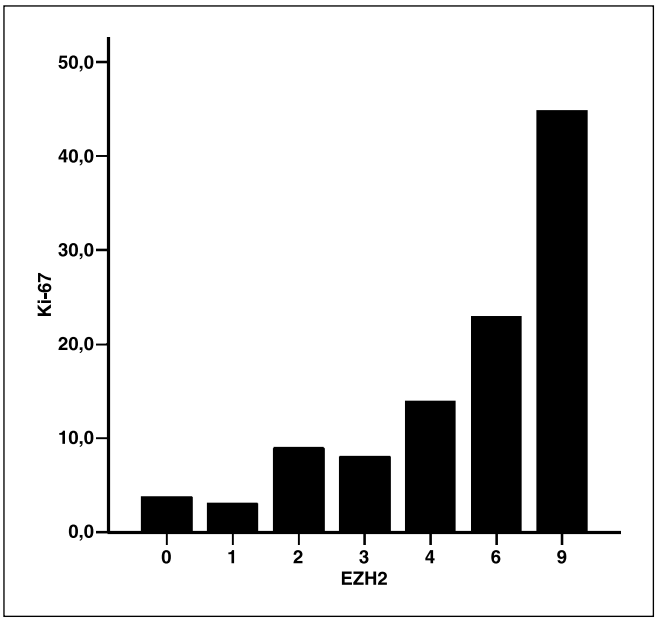

Fig. 2. Tumor cell proliferation as estimated by median Ki-67 staining (%) according to EZH2 expression levels by staining index (groups 0-9).

likely to be ER negative (OR, 3.7; 95% CI, 1.7-7.8) and PR negative (OR, 2.7; 95% CI, 1.4-5.0). A strong association was found between EZH2 and tumor cell proliferation, as patients with EZH2-positive tumors (staining index > 3) were 15 times more likely to have a high proliferation index (by upper quartile) than those with EZH2-negative tumors. The median proliferation rate was 21% in positive tumors compared with 6% in negative cases. The median percentage of Ki-67 staining tumor cells increased gradually with increasing levels of EZH2 staining index (Fig. 2; Pearson correlation coefficient 0.58, $P < 0.0001$). Tumors coexpressing EZH2 and p53 had an especially high median proliferation rate of 36%, compared with 10% for the rest of the cases.

Table 2. The OR, 95% CI, and *P* values for prediction of the basal epithelial phenotype (expression of CK5/6 and/or P-cadherin)

Variable	OR (95% CI)	*P*
ER status		0.001
Positive	1.0	
Negative	4.6 (1.9-11.4)	
P53		0.046
≤3	1.0	
>4	2.8 (1.0-7.5)	
Histologic grade		0.33
1-2	1.0	
3	0.6 (0.2-1.8)	
EZH2		0.49
SI ≤ 3	1.0	
SI > 3	1.4 (0.5-3.5)	
Ki-67		0.049
≤22%	1.0	
>22%	2.9 (1.0-8.4)	

Abbreviation: SI, staining index.

Table 3. The OR, 95% CI, and *P* values for predicting tumor cell proliferation as estimated by Ki-67

Variable	OR (95% CI)	*P*
ER status		0.02
Positive	1.0	
Negative	3.4 (1.2-9.6)	
P53		0.11
≤3	1.0	
>4	2.5 (0.8-7.6)	
Histologic grade		<0.001
1-2	1.0	
3	9.1 (3.3-25.5)	
EZH2		<0.001
SI ≤ 3	1.0	
SI > 3	8.5 (2.9-24.3)	

A significant correlation was seen between EZH2 positivity and locally advanced disease or metastatic disease at time of diagnosis. Patients with EZH2-positive tumors were more likely to present with locally advanced disease at time of diagnosis (OR, 3.4; 95% CI, 1.3-9.2), and all six patients with metastasis at time of diagnosis had EZH2-positive tumors. The relationship with lymph node spread was not significant ($P = 0.08$; Table 1).

A highly significant relationship was found between tumors expressing EZH2 and p53 staining (OR, 11.4; 95% CI, 3.8-34.0), and with markers of the basal-like phenotype. Thus, EZH2 expression was more likely to occur in CK5/6-positive cases (OR, 5.7; 95% CI, 2.2-14.7), and in tumors expressing P-cadherin (OR, 3.1; 95% CI, 1.4-6.9), compared with no expression of these markers. A significant association was also found between c-erbB-2 positivity and EZH2 expression (OR, 3.3; 95% CI, 1.4-7.5). Another marker closely related to the basal epithelial like phenotype is glomeruloid microvascular proliferation (34). Our data showed a highly significant relationship between EZH2 expression and the presence of glomeruloid microvascular proliferation (OR, 4.6; 95% CI, 2.2-9.9). No association was present between EZH2 and E-cadherin ($P = 0.06$).

Combining markers of the basal epithelial phenotype (one or both basal markers positive versus negative cases) in a multiple logistic regression model, ER status significantly predicted the basal epithelial phenotype (OR, 4.6; 95% CI, 1.9-11.4; $P = 0.01$), whereas Ki-67 (OR, 2.9; 95% CI, 1.0-8.4; $P = 0.049$) and p53 gave additional information (OR, 2.8; 95% CI, 1.0-7.5; $P = 0.046$). EZH2 and histologic grade were not significant in this model (Table 2). When predicting tumor cell proliferation as estimated by Ki-67, EZH2 was highly significant (OR, 8.5; 95% CI, 2.9-24.3; $P < 0.001$), along with histologic grade (OR, 9.1; 95% CI, 3.3-25.5; $P < 0.001$). Additional information was given by ER status (OR, 3.4; 95% CI, 1.2-9.6; $P = 0.02$), whereas p53 status was not significant (Table 3). When predicting aggressive disease (any event of locally advanced disease, lymph node spread, or distant spread), EZH2 was significant (OR, 2.4; 95% CI, 1.2-4.8; $P = 0.02$), whereas

Table 4. The OR, 95% CI, and *P* values for predicting aggressive disease (any event of locally advanced disease, lymph node spread, or distant metastases)

Variable	OR (95% CI)	*P*
ER status		0.49
Positive	1.0	
Negative	1.4 (0.6-3.3)	
P53		0.87
≤3	1.0	
>4	1.1 (0.4-2.8)	
Histologic grade		0.11
1-2	1.0	
3	0.5 (0.2-1.2)	
EZH2		0.02
SI ≤ 3	1.0	
SI > 3	2.4 (1.2-4.8)	
CK5/6		0.30
0	1.0	
>0	0.6 (0.2-1.6)	
P-cadherin		0.73
≤3	1.0	
>4	0.8 (0.3-2.1)	
Ki-67		0.27
≤22%	1.0	
>22%	1.7 (0.7-4.3)	

ER, Ki-67, p53, CK5/6, and P-cadherin did not reach significance in this model (Table 4). No significant association was found between aggressive disease and Ki-67 status ($P = 0.088$).

Interval cancers were twice more likely to express EZH2 than their size-matched screen detected counterparts (OR, 2.0; 95% CI, 1.1-3.9; Table 5). Including true interval cancers only (44 cases), the difference was even stronger ($P = 0.03$), because these were 3.2 times more likely to express EZH2 than their screen detected counterparts (OR, 3.2; 95% CI, 1.1-11.2).

In a multiple conditional logistic regression analysis with cancer presentation (interval/screening) as a response variable and EZH2, CK5/6, P-cadherin, and p53 expression as dichotomous explanatory variables, EZH2 gave no significant additional information ($P = 0.33$) to p53 status. However, when other variables were excluded, EZH2 did predict screen or interval cases significantly (OR, 2.9; 95% CI, 1.1-3.9; $P = 0.03$).

In univariate survival analysis, EZH2 was significantly associated with patient survival (Fig. 3), in addition to tumor size ($P = 0.006$), lymph node status ($P = 0.002$), and tumor cell proliferation by Ki-67 ($P = 0.0003$), whereas histologic grade ($P = 0.11$) and ER status ($P = 0.18$) were not significant. At 5 years, 91% of patients with EZH2-negative tumors were living, compared with 81% of patients with EZH2-positive tumors. After stratification on interval/screen-detected cases, EZH2 still gave significant information in univariate analysis ($P = 0.04$). In multivariate analysis, only tumor cell proliferation (Ki-67) and lymph node status remained as independent predictors of survival (Table 6). When tumor cell proliferation (Ki-67) was not included, lymph node status gave significant information (hazard ratio, 3.3; 95% CI, 1.2-9.2; $P = 0.02$), whereas no additional information was given by EZH2 or histologic grade (data not shown).

Discussion

In our present study, we found strong and consistent associations between EZH2 protein expression and features of aggressive breast cancer, such as high tumor cell proliferation, locally advanced disease, metastatic spread at diagnosis, interval presentation between screening mammograms, and reduced patient survival. Especially, all cases with metastases at diagnosis were EZH2 positive. Further, there were highly significant associations with p53 positivity, c-erbB-2 expression, markers of the basal-like phenotype and glomeruloid microvascular proliferation, which are characteristics of poor prognosis breast cancer.

Whereas previous studies have mainly focused on the connection between EZH2 expression and invasive properties in poorly differentiated tumors, proliferation rate has not been examined in human tumors. Basic studies have shown that EZH2 is a cell cycle regulator, and increased EZH2 expression is critical for S-phase entry and G_2-M transition (7, 35). The importance of EZH2 for tumor cell proliferation is supported by our study of human breast cancer, showing a strong association between EZH2 and proliferation rate as estimated by Ki-67 staining. In EZH2-positive tumors, the median proliferation rate was 21%, compared with 6% in negative cases, and this is similar to findings for prostate cancer (36). When high tumor cell proliferation was predicted in a multiple logistic regression analysis, expression of EZH2 was highly significant, whereas p53 was of borderline importance. However, for prediction of aggressive breast cancer, EZH2 was the only variable of significance.

Table 5. The number and proportion of screen-detected and interval cases with low and high expression of EZH2, the number of matched discordant pairs, and the corresponding OR with 95% CI

EZH2	Screen cases (%), *n* = 95	Interval cases (%), *n* = 95	*i*/*s*	OR (95% CI)	*P**
SI ≤ 3	58 (61)	42 (44)	32/16	2.0 (1.1-3.9)	0.03
SI > 3	37 (39)	53 (56)			

Abbreviations: *i*, number of matched pairs with high expression in the interval case, low in the screen detected case; *s*, number of matched pairs with low expression in the interval case, high in the screen-detected case.
*Exact two-tailed McNemars's test.

EZH2 expression was strongly associated with markers of the basal epithelial phenotype of breast cancer, such as expression of CK5/6 and P-cadherin. Also, a highly significant association between EZH2 expression and p53 status was found, and 88% of p53-positive cases were EZH2 positive. A high frequency of p53 alterations has been reported in breast cancers of the basal subtype (37, 38), indicating an important pathogenetic role. The relationship between EZH2 and p53 is consistent with recent experimental findings because p53 has been implicated in the regulation of EZH2 via its promoter (9). Notably, tumors coexpressing p53 and EZH2 had an especially high median proliferation rate of 36% in our study, compared with 10% for the rest of the cases. Still, whereas EZH2 expression was significantly associated with markers of the basal phenotype, positivity was also found in 41% of the cases that were negative for the basal marker CK5/6. When predicting the basal phenotype in a multivariate model, p53 expression turned out to be the strongest factor and EZH2 was no longer significant.

Increased c-erbB-2 (Her2) has previously not been associated with EZH2 expression (5). In contrast, our study showed a significant association between EZH2 and c-erbB-2, further supporting a role of EZH2 in the regulation of tumor cell proliferation. No associations were found between c-erbB-2 and p53, or between c-erbB-2 and the basal markers CK5/6 and P-cadherin in our series, supporting the current view that tumors expressing c-erbB-2 and basal-like cancers should be considered as different molecular subtypes (11). Taken together, our findings indicate that EZH2 expression is a marker of tumor cell proliferation and other features of aggressive breast cancer. Possibly due to its association with proliferation, EZH2 positivity is frequent in the basal-like subtype of breast cancer, but it is also associated with the c-erbB-2-positive subgroup.

Glomeruloid microvascular proliferation is considered an aggressive angiogenic phenotype (39, 40) and has been associated with p53 expression, germ line *BRCA1* mutations (12), and a basal epithelial phenotype of breast cancer by CK5/6 expression (34). In addition, our present data show glomeruloid microvascular proliferation to be strongly associated with EZH2 expression, thus extending previous observations. The detailed pathogenesis of this vascular subtype is not clear.

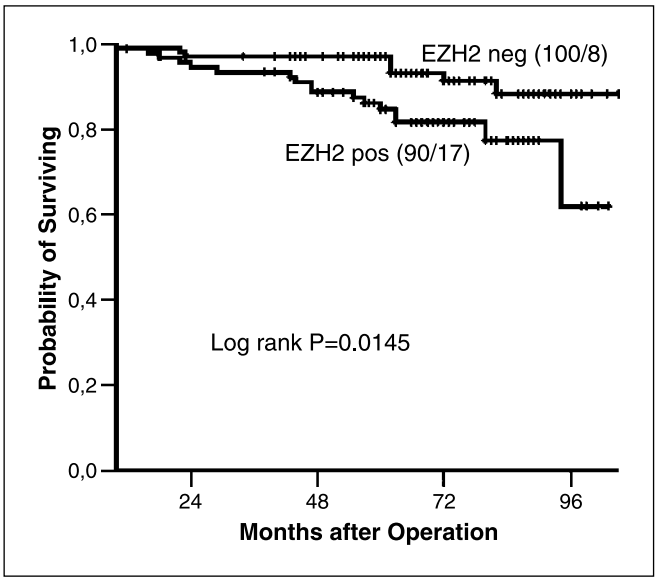

Fig. 3. Estimated survival according to expression of EZH2 (Kaplan-Meier method).

Table 6. Multivariate survival analysis based on the Cox proportional hazards regression method

Variable	No. patients	HR (95% CI)	*P*
Tumor size (mm)			
≤20	102	1	
>20	88	1.5 (0.5-4.1)	0.5
Histologic grade			
1-2	152	1	
3	38	1.4 (0.5-1.4)	0.5
Lymph nodes			
Negative	115	1	
Positive	63	3.7 (1.4-10.1)	0.01
Ki-67 (%)			
≤22.0	140	1	
>22.0	50	4.8 (1.3-18.0)	0.02
EZH2			
SI ≤ 3	190	1.0	
SI > 3	90	0.5 (0.15-1.8)	0.30

Increased EZH2 expression has been associated with recurrent breast cancer (5) similar to what has been found for p53 mutations (41). These results indicate that both markers may have an important role in tumor progression. In our study, EZH2 was significantly associated with locally advanced disease and metastases at the time of diagnosis. When aggressive disease was predicted by multiple logistic regression, EZH2 expression was the strongest predictive factor, whereas tumor cell proliferation was of borderline importance only. Histologic grade, CK5/6, and P-cadherin expression were not significant in the model. These findings suggest that EZH2 expression might also be related to invasion and the metastatic process. Further, EZH2 expression was significantly associated with reduced patient survival in univariate analysis, although it was not an independent prognostic factor. It has previously been controversial whether EZH2 expression is related to survival (5, 6).

Our findings revealed that interval cancers express EZH2 significantly more frequent than screen-detected cases. These results support the hypothesis that detection of breast cancer by mammography screening selects categories with phenotypic differences, in line with what has been reported (14, 42, 43). Further efforts should be made to characterize risk factors for women developing aggressive breast cancer between two regular mammograms.

In conclusion, our study indicates that EZH2 expression is strongly associated with increased tumor cell proliferation and it also captures associations with multiple features of aggressive breast cancer, such as p53 alterations, c-erbB-2 expression, markers of the basal-like subtype, glomeruloid microvascular proliferation, locally advanced disease with metastatic spread at diagnosis, interval presentation between screening mammograms, and reduced patient survival in univariate analysis. Thus,

EZH2 expression seems to be a broad marker of poor prognosis breast cancer. These novel findings are important because the polycomb group proteins have recently been suggested as candidates for targeted therapy (3, 9).

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