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Increased Risk for Distant Metastasis in Patients with Familial Early-Stage Breast Cancer and High EZH2 Expression

Sharon Hensley Alford[1], **Katherine Toy**[2,3], **Sofia D. Merajver**[3,4], and **Celina G. Kleer**[2,3]

[1]Department of Public Health Sciences, Henry Ford Hospital, Detroit, MI

[2]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI

[3]Department of Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI

[4]Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI

Abstract

Identification of women with early stage breast cancer who will develop distant metastasis may improve clinical management. The transcriptional regulator Enhancer of Zeste-2 (EZH2) is over expressed in invasive breast carcinoma compared to benign breast tissues, with maximal expression in breast cancer metastasis. Our purpose was to investigate the performance of EZH2 protein detection as a predictor of metastasis in women with early stage breast cancer, which is unknown. We developed a cohort of 480 women with stage I-IIA breast cancer diagnosed between 1996 and 2002 and recorded detailed socio demographic, clinical and pathological information. Tumors were histologically characterized and arrayed in tissue microarrays containing 1,443 samples. Nuclear EZH2 expression was investigated by immunohistochemistry and was scored as 1–2 (negative and weak) or 3–4 (moderate and strong) using a validated scoring schema. Scores 1–2 were considered low EZH2; scores 3–4 were considered high EZH2. We found that after a median follow up of 9 years (range 0.04–14.5 years) 46 of 480 patients (9.6%) developed distant metastasis. High EZH2 was associated with larger size, high histological grade, negative hormone receptors, and first degree family history of breast and/or ovarian carcinoma. While EZH2 could not predict survival in the entire cohort, high EZH2 was a predictor of disease-specific survival in patients with early stage disease and first degree family history (log rank p-value 0.05). Importantly, in this group of patients, high EZH2 was an independent predictor of distant metastasis up to 15 years after primary carcinoma diagnosis (hazard ratio 6.58, 95% CI: 1.40–30.89, p=0.016) providing survival information above and beyond currently used prognosticators. In conclusion, EZH2 may bea useful biomarker of long-term metastatic risk in women with familial early stage breast cancer, and warrant further validation studies.

Keywords

EZH2; Enhancer of Zeste-2; prognosis; recurrence; metastasis; breast cancer

Introduction

An increasing number of women are diagnosed with node negative invasive breast carcinomas smaller than 2 cm. Even though the majority of patients with small tumors

Corresponding Author: Dr. Celina G. Kleer, University of Michigan Medical School, Department of Pathology, 4217 Comprehensive Cancer Center, 1500 E. Medical Center Dr., Ann Arbor, MI 48109, Tel: 734-615-3448, kleer@umich.edu.

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treated with breast conservation have a favorable outcome, the 10-year rate of distant metastasis is up to 20% [1, 2]. As metastatic disease is incurable, accurate prognosticators and more efficacious treatments are needed. Clinical management of patients diagnosed with early breast cancer is critical but available data on biomarkers of tumor metastasis are limited. This is in part due to the difficulties in developing a cohort of early stage invasive carcinoma with long term follow-up and comprehensive pathological, clinical and treatment information.

Enhancer of Zeste-2 (EZH2) is a Polycomb Group (PcG) protein involved in control of transcriptional memory and gene silencing which has been shown to exert oncogenic effects in the breast [3–7]. EZH2 methylates histone H3 at lysine 27 (H2K27) thereby recruiting other members of the PcG family to specific genetic target loci [3–7]. It is postulated that EZH2 promotes breast cancer progression by transcriptional repression of tumor suppressors and by maintaining the cells in a stem cell state [8, 9]. We and other investigators have found that EZH2 promotes neoplastic progression in the breast and that EZH2 downregulation reduces *in vivo* tumor growth of breast cancer cells [10–12]. EZH2 controls cell proliferation, invasion, and has recently been shown to regulate DNA repair pathways and genomic stability [10, 13–17]. In breast cancer, EZH2 is associated with estrogen (ER) and progesterone (PR) receptor negative status and available data support the role of EZH2 as a biomarker of breast cancer recurrence [11, 18, 19]. However, whether EZH2 can predict metastasis in the clinically challenging group of early stage invasive carcinomas is unknown.

Here, we evaluated the ability of EZH2 expression to predict the risk of metastasis of invasive carcinomas smaller than 2 cm, either node negative or with limited axillary disease (1–3 positive lymph nodes) (Stages I and IIA T1 N1). We developed a cohort of 480 early stage invasive breast carcinomas with up to 15 years of follow-up, clinical data and treatment information.

Methods

Selection of Patients and Medical Record Abstraction

Using a managed health system tumor registry, we identified early stage breast cancer cases diagnosed and treated at Henry Ford Hospital (HFH) between 1996 and 2002 including Stage I and node positive Stage IIA (T1, N1) primary breast carcinomas. The Henry Ford Health System maintains an electronic medical record for each patient. The medical record captures all patient encounters and test results. For each patient identified through the HFH tumor registry we reviewed the medical record to confirm eligibility. Patients were eligible for the study if they had been diagnosed and treated for a primary, initial invasive breast cancer. Patients were excluded if primary treatment was not received at HFH, the cancer was bilateral, patient was pregnant at the time of diagnosis, or if there was a prior breast cancer. We also excluded patients with any other clinically active malignancy. Medical records were reviewed for eligible cases to collect clinical-pathologic and demographic data. Variables abstracted include age at diagnosis, race, family history, parity, age of first live birth, age at menarche, age at menopause, tumor characteristics, treatment received, and tumor recurrence. All aspects of this study were approved by the Institutional Review Boards at Henry Ford Hospital and University of Michigan.

Microarray Construction and Immunohistochemistry

A total of 480 primary invasive carcinomas T1 (smaller than 2 cm), node negative or having 1–3 positive lymph nodes were used for tissue microarray (TMA) construction in triplicate (n= 1,443 tissue microarray samples). Each H&E slide was reviewed by the study

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pathologist and arrayed in six TMAs as described [11]. Optimally, three 0.4 mm cores were taken from each patient's sample.

Immunohistochemistry was performed on the TMAs by using a standard biotin-avidin complex technique and by using a standard polyclonal antibody against EZH2 that was previously validated by immunoblot analysis [11, 20]. TMAs were immunostained for HER-2/neu as performed in clinical practice [21]. At least two authors scored each tumor core blinded to pathological and clinical characteristics. Nuclear EZH2 expression was scored as negative (score=1, no staining); weak (score 2, <25% of nuclei staining, any intensity); moderate (score=3, 25–75% of nuclei staining, any intensity); and strong (score=4, >75% of nuclei staining, any intensity) following previous studies [11, 20]. High EZH2 expression was defined as scores 3 and 4; low EZH2 was defined as scores 1 and 2. Reporting recommendations for tumor marker prognostic studies (REMARK) were followed [22].

Stastistical Analysis

The *a priori* planned analysis was to explore the relationship between EZH2 expression with each clinicopathologic variable available for the cohort. EZH2 expression was dichotomized into high and low. Since three core samples were obtained for each patient, the highest value of the 3 scores was used for subsequent analysis. For each variable, we assessed the association with EZH2 expression using chi-square and logistic regression. Time to any recurrence and distant metastasis survival curves were constructed by the Kaplan-Meier method. Cox Proportional Hazards models were used to calculated hazard ratios for distant metastasis with 95% confidence intervals. Univariate analyses of time to distant metastasis were performed by using a two-sided log-rank test to evaluate stage, grade, tumor size, nodal status, histology, ER status, PR status, HER-2/neu status, and EZH2. Multivariate associations were modeled using a stepwise modeling approach to identify variables that best fit the model for distant metastasis. Model entrance criteria were p-value of 0.25 and model retention was p=0.15. Hazard ratios and 95% confidence intervals are reported. Analyses were performed for all cases and separately for those with a positive family history of breast and/or ovarian cancer in a first-degree relative.

Results

Patient Characteristics

We identified a total of 906 cases through the HFH tumor registry that met the stage and year of diagnosis criteria for inclusion in the study. Of these, 637 were Stage I (< 2 cm with negative axillary lymph nodes) and 269 were Stage II (T1 N1) cases (< 2 cm with 1–3 positive axillary lymph nodes). After medical record review, 137 (15%) were excluded from the study. Of the 769 eligible cases, tumor specimens were not available for 233 (30%). Of the 536 cases with blocks available we successfully evaluated the histopathology, immunostained and scored 480 (90%). Clinical and pathological characteristics, as well as the breakdown of treatment modalities are summarized in Table 1.

After a median follow up of 9 years (range 0.04–14.5 years), 80 and 46 of the 480 patients developed any recurrence and distant metastasis (17% and 9.6%, respectively). The 5-, 10- and 15-year recurrence free experience for the entire cohort of patients was 92%, 87%, and 85%, respectively. The 5-, 10- and 15-year metastasis free experience for the entire cohort of patients was 94%, 91%, and 90%, respectively.

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Associations of EZH2 Expression and Clinical and Pathological Features of Early Stage Breast Cancer

High EZH2 expression was present in 277 of 480 tumors (57.6%). Figure 1A shows representative cases of EZH2 immunohistochemical staining. EZH2 was expressed in the nuclei of breast cancer cells as previously reported [11]. The association between EZH2 protein levels with socio-demographic and clinicopathological characteristics is shown in Tables 2 and 3, respectively. EZH2 protein levels were not associated with age at cancer diagnosis, race, age at menarche, or parity. We found that high EZH2 expression was significantly associated with a family history of breast and/or ovarian cancer in a first degree relative (odds ratio 1.63, 95% CI: 1.07–2.50, p=0.02).

High EZH2 was associated with tumor size (HR 1.69, CI: 1.13–2.51, p=0.01), one of the strongest known predictors of survival. High EZH2 expression was also associated with high histologic grade (HR 4.94, CI: 3.23–7.69, p<0.0001), a measure of the degree of tumor differentiation and poor prognostic indicator. High EZH2 expression was associated with ductal tumor histology (HR 2.20, CI: 1.22–4.00, p=0.008), negative estrogen receptor status (HR 5.96, CI: 3.71–9.59, p<0.0001) and negative progesterone receptor status (HR 3.28, CI: 2.17–4.95, p<0.0001), and with HER-2/neu overexpression (HR 1.72, CI: 1.11–2.70, p=0.01).

High EZH2 levels independently predict distant metastasis in early stage breast cancer patients with first degree family history

We next tested the hypothesis that EZH2 expression may predict the development of distant metastasis in women with early stage breast cancer and first degree family history. We focused on this group of women because of recent studies from our laboratory and other investigators showing a mechanistic link between EZH2 and the breast and ovarian cancer tumor suppressor protein BRCA1 [10, 15, 23, 24]. In our cohort of 480 patients, 112 (23%) had a first degree family history of breast and/or ovarian cancer while 368 (77%) patients had no family history or family history in a non-first degree family member. The characteristics and associations of clinical and pathological features according to the presence or absence of first degree family history as shown in Table 4. Patients with first degree family history were significantly younger, had tumors with higher histological grade, and more frequently estrogen receptor negative than patients with non-first degree family history or no family history. In our cohort, first degree family history was not associated with tumor size, nodal status, progesterone status, HER-2/neu overexpression, tumor recurrence or metastasis.

We next sought to determine whether EZH2 expression could predict metastasis in women with first degree family history and early stage breast cancer. Higher tumor stage (I vs. IIA), negative estrogen and negative progesterone receptor status, and high EZH2 expression had significant univariate associations with development of distant metastasis for patients with first degree family history at 5- , 10- and up to 15 years after primary invasive carcinoma diagnosis (Table 5 and Figure 1B). The multivariable model indicates that high EZH2 expression was independently associated with the development of distant metastasis at 5 years (HR 13.04; 95% CI 1.42–119.50, p=0.023) and up to 15 years following primary breast carcinoma diagnosis (HR 6.58; 95% CI 1.40–30.9 (p=0.0169) Table 6.

Discussion

In this study we tested the hypothesis that EZH2 expression may be clinically useful in predicting prognosis in a challenging group of patients with invasive carcinomas smaller than 2 cm (T1) with negative lymph nodes (N0) or 1–3 positive nodes (N1) treated by

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standard of care between 1996 and 2002. There are few studies in the literature which focus on biomarker development in early stage breast cancer with limited clinical significance. The identification of patients with early stage biologically aggressive tumors has the potential to assist in patient management.

Based on a cohort of 480 early stage invasive carcinomas of the breast rigorously selected and histopathologically analyzed we made several novel observations. Consistent with its oncogenic function, EZH2 overexpression is associated with high histological grade (Nottingham grade 3) and tumor size, negative estrogen and progesterone receptors and with HER-2/neu overexpression. We discovered that EZH2 expression is higher in early stage breast carcinomas from women with first degree family history of breast and/or ovarian cancer, suggestive of inherited breast cancer susceptibility [25]. Our finding that EZH2 overexpression is associated with familial breast cancers in early stage disease is especially relevant given previous studies supporting a mechanistic connection between EZH2 and hereditary breast cancer genes BRCA1 and TP53. Our laboratory has shown that EZH2 protein is upregulated in benign appearing lobules of prophylactic mastectomies from BRCA1 mutation carriers [23, 26]. In estrogen receptor negative invasive breast carcinomas, EZH2 overexpression is able to regulate BRCA1 expression and intracellular localization [10, 15]. The relationship between EZH2 and BRCA1 is likely complex and reciprocal as EZH2 overexpression was found in breast cancers arising in women with BRCA1 mutations [24]. Pietersen and colleagues have reported that invasive breast carcinomas with high EZH2 protein harbor TP53 mutations and suggested that during tumorigenesis EZH2 overexpression coincides with TP53 activation [27]. Our results highlight the relevance of these basic studies to human breast cancer and suggest the hypothesis that EZH2 overexpression may be associated with other molecular determinants of hereditary breast cancer in addition to BRCA1 and TP53 gene mutations, which warrant investigation.

Even though most women with early stage breast cancer in the sporadic or familial settings do not progress, distant metastases may occur long after the first 5 years following primary breast cancer treatment [1]. A major challenge in the management of women with early stage breast cancer is the identification of those who will develop metastasis from those that will not. We tested the hypothesis that EZH2 overexpression in early stage primary breast carcinoma may predict long-term metastasis up to 15 years following diagnosis of the primary tumor. Indeed, EZH2 expression levels in primary breast carcinoma were associated with metastasis-specific survival in women with first degree family history at 5, 10 and up to 15 years after primary breast carcinoma diagnosis. The 15- year metastasis free survival was 83% and 94% for women with tumors exhibiting high and low EZH2 expression respectively (log rank test $p=0.05$). The best multivariable model predictive of metastasis-specific survival included high EZH2 expression, high histological grade, higher stage, and absence of anti-hormonal treatment, reflecting negative estrogen and progesterone receptor status. High EZH2 expression was a strong independent predictor of metastasis providing 15 year survival information above other independent prognostic features. These data are clinically relevant because EZH2 may identify women with early stage breast cancer and first degree family history at higher risk to develop distant metastasis. Furthermore our data support the potential clinical utility of incorporating EZH2 into clinical nomograms to help determine the risk of cancer progression in this specific group of patients.

A limitation of our analysis is that tumor tissue samples were not available for 30% of patients identified. However, given that these samples would have not been selected on EZH2 expression it is unlikely to have biased our results. Our study focused on very small tumors so the material resource was very limited. Despite this, we successfully utilized 90% of the available tissues in our analysis. Our findings were focused on the very small group of women in our cohort (n=112 out of 480) who had first degree family history of breast and/or

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ovarian cancer. Therefore, some of our results included large confidence intervals. Nonetheless, we were able to demonstrate a significant association of EZH2 with distant metastasis even after controlling for important covariates, which strengthens our results.

Since our initial reports of EZH2 overexpression in breast cancer our findings have been supported by other investigations. EZH2 overexpression has been found in breast and other solid tumors including bladder [28–30], gastric[31], lung [32], melanoma [17], and hepatocellular carcinoma [33]. In these malignancies, EZH2 has been implicated in neoplastic transformation, progression, invasion and metastasis. Taken together, these data suggest that EZH2 may be involved in a global, rather than a tissue type specific mechanism of tumor progression.

In conclusion, our results indicate that in early stage breast cancer, EZH2 overexpression is associated with features of aggressive disease. We discovered a novel association between EZH2 overexpression and first degree family history of breast and/or ovarian cancer, which paves the way to mechanistic and functional studies. Our finding that EZH2 overexpression is an indicator of metastasis and metastasis-related deaths in women with early stage breast cancer and first degree family history may have important clinical implications. Specifically, EZH2 detection at the time of primary tumor diagnosis may aid clinicians in guiding management decisions and lays the foundation for the development of targeted treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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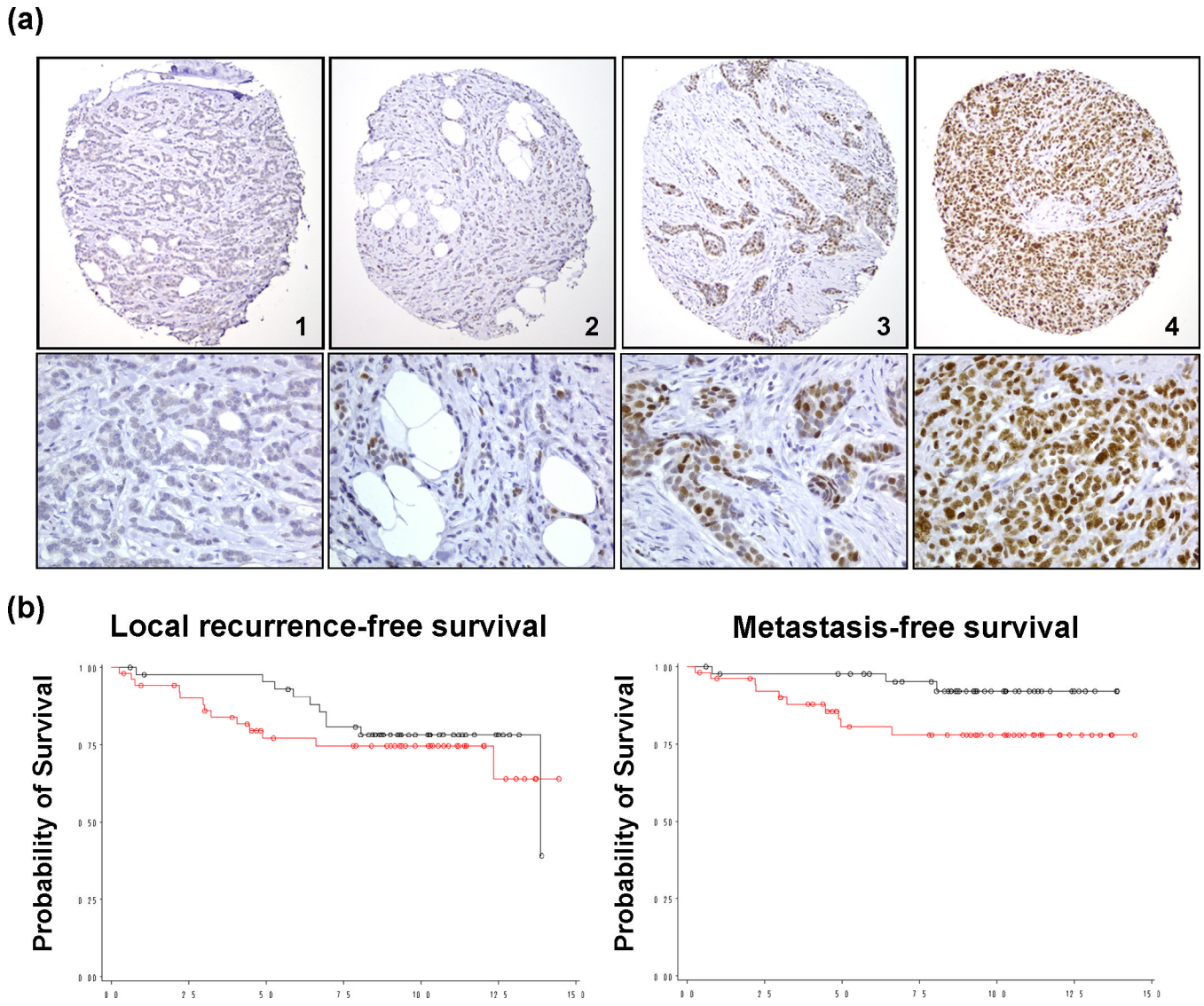

Figure 1.

A. EZH2 expression in early stage invasive breast carcinomas. Tissue microarray elements containing representative invasive carcinomas with negative (1), weak (2), moderate (3), and strong (4) EZH2 staining intensities. Note that EZH2 protein is expressed in the nuclei of cancer cells. Original magnification 40×. **B.** EZH2 protein expression is associated with metastasis-specific survival in patients with early stage breast cancer and first degree family history. Kaplan Meier plot showing that women with tumors expressing high EZH2 have worse metastasis-free survival compared with women with low EZH2 expressing tumors.

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Table 1

Distribution of clinical features and treatment information.

Characteristics	N (%)
Age at Diagnosis	
≤ 50	107 (22)
> 50	373 (78)
Race[1]	
White	319 (69)
Black	152 (32)
Menarche	
≤ 12	232 (48)
> 12	248 (52)
Age of First Birth	
≤ 30	318 (66)
> 30	162 (34)
Parity	
Childless	70 (27)
More than 1 child	193 (73)
Family History[2]	
Any	200 (42)
None	280 (58)
Family History[2]	
Any 1st Degree Relative	112 (23)
None or No 1st Degree	368 (77)
Tumor Stage	
I	413 (86)
II (T1, N1)	67 (14)
Tumor Size (cm)	
≤ 1	153 (32)
> 1	327 (68)
Tumor Grade	
I or II	335 (70)
II	145 (30)
Histologic Type	
Ductal	417 (87)
Lobular, mixed or other	63 (13)
Estrogen Receptor	
Negative	113 (23)
Positive	367 (77)
Progesterone Receptor	
Negative	138 (29)
Positive	342 (71)

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Characteristics	N (%)
Her2 Neu Status	
Negative	350 (77)
Positive	104 (23)
Axillary Lymph Nodes	
Negative	411 (85)
Positive	69 (15)
Recurrence	
Any	73 (15)
None	407 (85)
Distant Metastasis	
Yes	48 (10)
No	432 (90)
Surgery	
Lumpectomy	237 (49)
Partial Mastectomy	156 (33)
Mastectomy	87 (18)
Chemotherapy	
Any	171 (36)
None	309 (74)
Hormonal therapy	
Any*	265 (55)
None	214 (45)
Radiation	
Any	347 (72)
None	133 (28)

[1] 9 women missing because of “other” race

[2] Family history of breast cancer and/or ovarian cancer

* all but 4 patients received Tamoxifen.

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Table 2

Association of EZH2 expression with socio-demographic features

Characteristic	OR (95% CI)	χ^2 p-value	N (%) EZH2 high	N (%) EZH2 low
Age at Diagnosis	1.08 (0.70–1.66)	0.74		
≤ 50			47 (23)	60 (22)
> 50			157 (77)	216 (88)
Race[3]	1.31 (0.89–1.93)	0.18		
White			128 (64)	191 (70)
Black			71 (37)	81 (30)
Menarche	1.16 (0.81–1.67)	0.42	103 (50)	129 (48)
≤ 12			101 (50)	147 (52)
> 12				
Age of First Birth	1.25 (0.85–1.84)	0.25	141 (69)	177 (64)
≤ 30			63 (31)	99 (36)
> 30				
Parity	1.06 (0.61–1.84)	0.84		
Childless			30 (27)	40 (26)
More than 1 child			80 (73)	113 (74)
Family History[1]	1.19 (0.83–1.72)	0.35		
Any			90 (44)	110 (40)
None			114 (56)	166 (60)
Family History[1]	1.63 (1.07–2.50)	0.02		
Any 1st Degree Relative			58 (28)	54 (20)
None or No 1st Degree			146 (72)	222 (80)

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Table 3

Association of EZH2 expression with clinical and pathological features.

Characteristic	OR (95% CI)	χ^2 p-value	N (%) EZH2 high	N (%) EZH2 low
Stage	1.28 (0.76–2.15)	0.35		
II (T1, N1)			32 (16)	35 (13)
I			172 (84)	241 (87)
Grade	4.94 (3.23–7.69)	<0.0001		
High (=3)			100 (49)	45 (16)
Low (<3)			104 (51)	231 (84)
Tumor Size	1.69 (1.13–2.51)	0.01		
> 1 cm			152 (75)	175 (63)
≤ 1 cm			52 (25)	101 (37)
Nodal Status	1.20 (0.72–2.00)	0.48		
Positive			32 (16)	37 (13)
Negative			172 (84)	239 (87)
Histology	2.20 (1.22–4.00)	0.008		
IDC			187 (92)	230 (83)
LDC, other			17 (8)	46 (17)
Estrogen Receptor	5.96 (3.71–9.59)	<0.0001		
Negative			84 (41)	29 (11)
Positive			120(59)	247 (89)
Progesterone Receptor	3.28 (2.17–4.95)	<0.0001		
Negative			87 (43)	51 (18)
Positive			117 (57)	225 (82)
HER-2/neu Status	1.72 (1.11–2.70)	0.01		
Positive			55 (28)	49 (19)
Negative			138 (72)	212 (81)
Recurrence	1.00 (0.61–1.66)	0.62		
Any			31 (15)	42 (15)
None			173 (85)	234 (85)
Distant Metastasis	1.28 (0.70–2.32)	0.44		
Yes			23 (11)	25 (9)
No			181 (89)	251 (91)

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Table 4

Distribution of clinical and pathological features according to the presence or absence of first degree family history.

Characteristic	χ^2 p-value	N (%) First Degree	N (%) Non-First or None
Age at Diagnosis	0.004		
≤ 50		36 (32)	71 (19)
> 50		76 (68)	297 (81)
Race[1]	0.61		
White		73 (65)	246 (67)
Black		38 (34)	114 (31)
Stage	0.84		
I		97 (87)	316 (86)
II (T1, N1)		15 (13)	52 (14)
Grade	0.05		
High (=3)		42 (38)	103 (28)
Low (<3)		70 (63)	265 (72)
Tumor Size	0.39		
≤ 1 cm		32 (29)	121 (33)
> 1 cm		80 (71)	247 (67)
Nodal Status	0.78		
Positive		17 (15)	52 (14)
Negative		95 (85)	316 (86)
Histology	0.39		
IDC		100 (89)	317 (86)
LDC, mixed, other		12 (11)	51 (14)
Estrogen Receptor	0.05		
Positive		78 (70)	289 (79)
Negative		34 (30)	79 (21)
Progesterone Receptor	0.36		
Positive		76 (68)	266 (72)
Negative		36 (32)	102 (28)
HER-2/neu Status	0.80		
Positive		24 (21)	80 (22)
Negative		85 (76)	265 (72)
Distant Metastasis	0.52		
Yes		13 (12)	35 (9)
No		99 (88)	333 (91)

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Table 5

Univariate Hazard Ratios (HR) for distant metastasis in women with first degree family history.

Characteristic	≤ 5-year (n= 9)		≤ 15-year (n= 13)		
	Estimate	95% CI	Estimate	95% CI	Log-rank p-value
Stage					0.03
II (vs I)	3.94	0.98–15.79	3.58	1.10–11.63	
Grade					0.31
High (vs Low)	2.09	7.77	1.98	0.66–5.88	
Tumor Size	---[1]	---			0.12
≤ 1 cm (vs > 1 cm)			4.59	0.60–35.31	
Nodal Status					0.07
Pos (vs Neg)	3.25	0.81–13.01	2.97	0.91–9.66	
Histology					0.64
IDC (vs LDC, etc)	1.13	0.14–9.06	1.78	0.23–13.71	
Estrogen Receptor					0.003
Neg (vs Pos)	21.28	2.66–166.7	4.54	1.47–13.89	
Progesterone Receptor					0.003
Neg (vs Pos)	20.41	2.55–166.7	4.39	1.43–13.51	
HER-2 Neu					0.18
Neg (vs Pos)	2.43	0.30–19.2	3.45	0.44–27.0	
EZH2 Expression					0.05
High (vs Low)	7.99	1.00–63.9	3.57	0.98–12.99	

[1] Calculation was not feasible due to empty cell.

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Table 6

Independent factors predicting metastasis-free survival at 15 years for women with early stage breast cancer and first degree family history (n=112).

Patient/tumor characteristic	Distant Metastasis		
	HR	95% CI	p-value[1]
EZH2 high (vs. low)	6.58	1.40–30.89	0.0169
High Grade (3 vs. 1 and 2)	3.29	0.82–13.16	0.0939
Tumor Stage (IIA vs. I)	17.38	3.35–90.29	0.0007
Hormonal treatment (yes vs. none)	0.06	0.01–0.31	0.0007

[1] Modelling done using a stepwise modeling approach with model entrance criteria of p-value <0.25 and model retention of p-value<0.15.