ORIGINAL ARTICLE

Prognostic value of EZH2, paxillin expression and DNA ploidy of breast adenocarcinoma: Correlation to pathologic predictors

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Summary

Purpose: *The objective of this study was to examine the association of EZH2 and paxillin expression and DNA ploidy status with pathological parameters of breast cancer, aiming to correlate tumor phenotype with its malignant behavior.*

Methods: *EZH2 and paxillin expression and DNA ploidy were evaluated in imprint smear samples obtained from 105 breast tumors after surgical removal.*

Results: *Increased expression of paxillin was associated with p53 expression (p=0.005), Ki-67 expression (p=0.018) and EZH2 expression (p<0.0001). EZH2 expression correlated with estrogen receptor (ER) and progesterone receptor (PR) status (p=0.01 and p=0.035, respectively), and expression of p53 and Ki-67 (p=0.007 and p<0.0001, respectively).*

Aneuploid tumors were significantly correlated with poor differentiation (p=0.000), stage of disease (p=0.000), size of the primary tumor (p=0.015), presence of nodal metastasis (p=0.001), ER status (p=0.008), cerbB2 status (p=0.012), and expression of Ki-67 (p=0.001) and EGFR (p=0.018). Multivariate analysis of ploidy results using paxillin and EZH2 expression as dependent variables revealed that aneuploid tumors were associated with disease stage and grade of differentiation, cerbB2 expression and EZH2 expression.

Conclusion: *Our results show that aneuploid tumors, EZH2 expression and paxillin expression correlate with more aggressive phenotype of breast cancer.*

Key words: *breast cancer biomarkers, EZH2, DNA ploidy, paxillin*

Introduction

The accuracy of conventional prognostic markers of breast carcinoma is not as precise as desired, leading to inefficient application of chemotherapy. So, there is a need for novel biological predictors of tumor behavior at the time of diagnosis, that will help guide clinical therapeutic decisions [1-3]. Established biomarkers such as ER, PR and HER-2 have significant importance in the selection of the appropriate therapy. However, identification of new prognostic factors that are more precise and reliable is required [4-6].

EZH2 is a polycomb group protein homologous to Drosophila Enhancer of Zest and involved in gene silencing [7]. EZH2 gene amplification has recently been characterized in a variety of human cancers and was found to be predictive of poor outcome for prostate and breast cancer [8,9].

Paxillin , a multi domain focal adhesion adaptor protein , localizes to the cytoplasmic face of integrin-mediated adhesion sites where functions as molecular scaffold for the coordination of RhoGTPase signaling during cell migration on planar 2D surfaces. Paxillin has been identified as a marker of aggressive breast cancer and a promoter of neoplastic migration and invasion through three-dimensional extracellular matrices [10-14].

Table 1. Pathological characteristics of the studied cases

| Characteristics	N	%
Histological type		
Ductal carcinoma	106	91.5
Lobular carcinoma	9	8.5
Histologic grade		
1	8	7.6
2	43	41.0
3	54	51.4
Tumor size (cm)		
< 2	53	50.5
> 2	52	49.5
Stage		
1	35	33.3
2	46	43.8
3, 4	24	22.9
Metastatic lymph nodes		
Negative	56	53.3
Positive	49	46.7
Estrogen receptor		
Negative	26	24.8
Positive	79	75.2
Progesterone receptor		
Negative	46	43.8
Positive	59	56.2
c-erbB2		
Negative	60	57.1
Positive	45	42.9
p53		
Negative	31	29.5
Positive	74	70.5
Ki-67		
Negative	52	49.5
Positive	53	50.5
EGFR		
Negative	89	84.8
Positive	16	16.2

The aim of this study was to investigate the role of the expression of EZH2 and paxillin in the determination of the biological behavior of breast cancer based on its associations to established clinicopathologic factors.

Methods

One hundred and five imprint smears were obtained from patients with breast cancer immediately after tumor removal in the operation room. Female patients with histologically proven invasive breast carcinomas, in whom axillary lymph node dissection had been performed and who had had all their selected ma-
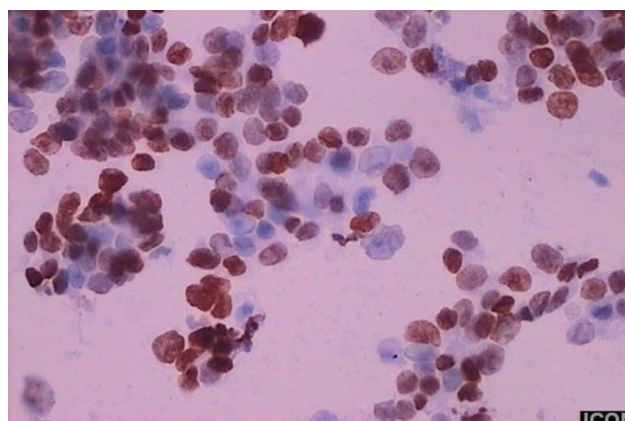
Figure 1. Photomicrograph showing a smear positive for EZH-2 with >10% of the tumor cells showing distinct nuclear staining (x40).
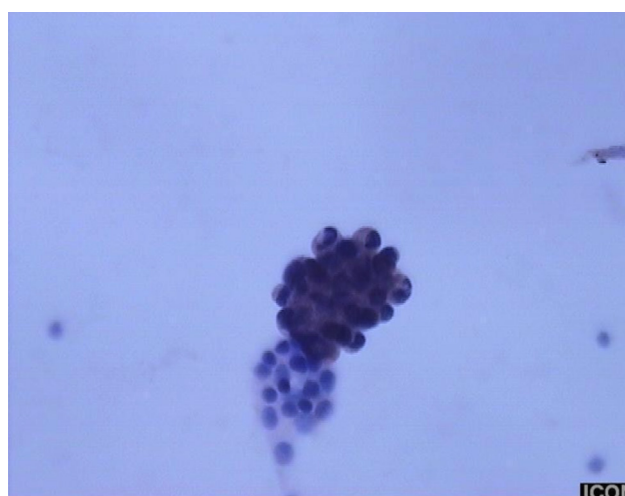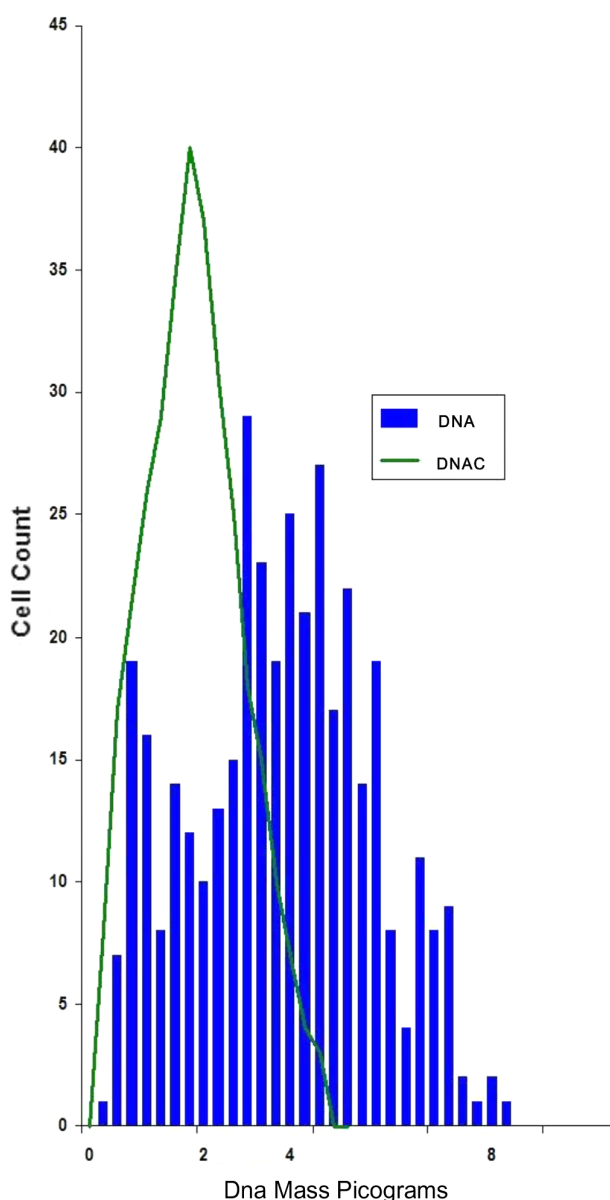

Figure 2. Photomicrograph showing a smear positive for paxillin with >10% of the tumor cells showing distinct cytoplasmic staining (x40).

terial studied histologically were enrolled in this study. Table 1 summarizes the patient data.

The classification of the breast cancer was made according to World Health Organization criteria [15] and were recorded as invasive ductal or invasive lobular. All invasive ductal carcinomas were of the "not otherwise specified" type and so they were graded according to a modified Scarff-Bloom-Richardson histological grading system, with guidelines as suggested by the Nottingham City Hospital pathologists [16]. Staging at the time of diagnosis was based on the TNM System [17]. Tumor size (<2 vs >2cm) and lymph node status data were extracted from the final pathology report. The patient ER and PR status and HER-2 expression as well as p53, Ki-67 and EGFR status were determined immunohistochemically on tissue sections.

Immunostaining was performed in tumor imprint smears fixed in 5% buffered formalin solution for 20 min. A standard avidin-biotin-peroxidase complex technique (Vectastain Elite, Vector Laboratories, Burlingame, California, USA) was used. The smears were incubated at 40 $^{\circ}$C in a humidity chamber with mon-

For each protein examined, positive/negative immunoreaction was determined using the following cut offs: for p53, EZH2, paxillin and EGFR staining, the smear was interpreted as positive when >10% of the tumor cells showed distinct nuclear (EZH2,p53) or cytoplasmic (paxillin, EGFR) staining and Ki-67 expression was considered positive when >25% had distinct nuclear staining (Figures 1,2).

DNA analysis was performed in imprint smears stained by the Thionin Feulgen procedure. The measurements of DNA were performed with image Pro Plus Software (V 5.1, Media Cybernetics Inc. Maryland, USA). A light microscope (Nicon Eclipse 80i, Nikon Corp. Tokyo, Japan) and magnification of x400 were used in order to identify the heterogeneity of the nucleus and measure the optical density accurately. A Nikon color camera (Nikon DS-2MW, Nikon Corp., Tokyo, Japan) adapted to a microscope was used for image capture. The data of the measurements were automatically exported to Microsoft Excel spreadsheets and DNA index (D.I.) and ploidy histograms were subsequently produced. The procedure was performed for all nuclei in the examined fields and the overall mean represented the DNA content or D.I. The mean measured from examining 100 control cells served as the diploid standard (2c) and reference for the D.I. calculation for the targeted cells. DNA ploidy classification was based on D.I. and histograms according to the 4th updated ESACP consensus report on diagnosis of D.I. The lesions were categorized as diploid if the D.I. ranged from 0.9 to 1.1 and the relevant DNA histogram revealed only one peak at 2c, and aneuploid if anyone from the previous two criteria was absent (Figure 3).

Statistics

The statistical analysis was performed with PASW Statistics 18 (SPSS Inc., Chicago, Illinois, USA). The significance level was set at 0.05. Pearson's x^2 test (with continuity correction for 2x2 tables) and logistic regression were used to compare DNA ploidy, paxillin and EZH2 expression with clinicopathological parameters such as stage, grade, lymph node metastasis, tumor size, Ki-67 expression, p53 expression and ER, PR and cerbB2 status.

Results

Regarding the three main studied parameters, 57.1% of the tumors were EZH2 positive, 26.7% paxillin positive and 44.8% were classified as aneuploid.

The results of univariate analysis (Pearson's x^2 test) for the pathological factors examined are shown in Table 2. In summary, increased expression of paxillin was associated with positive expression of p53 (p=0.005), positive expression of Ki-67 (p=0.018) and positive expression of EZH2 (p<0.0001). Moreover, positive expression of

Figure 3. Histogram showing increased DNA content of the cells of the cancerous smears (DNAT) relative to the control sample (DNAC).

oclonal antibodies to EZH2, paxillin, Ki-67, p53 (clone DO7), EGFR monoclonal antibodies (Menarini Diagnostics, Florence, Italy) at dilutions of 1:40, 1:100, 1:50, 1:50 and 1:100, respectively.

Visualization was achieved by incubating the smears in diaminobenzidine (DAB) used as chromogen. Smears were counterstained with Mayer's hematoxylin. The processing of the controls was the same as in the experimental cases with the deletion of the use of the primary antibodies. Smears of known positive reactivity for the antibodies used were included as positive controls.

Results were interpreted by two independent cytopathologists. In cases with heterogeneous staining in the examined fields of the slide, those with the highest and lowest percentages of cells stained were included.

Table 2. Univariate analysis (Pearson's x^2 test) of the clinical and pathological factors in relation to paxillin and EZH2 expression

Factors	Paxillin					EZH2				
	(-)		(+)		p-value	(-)		(+)		p-value
	N	%	N	%		N	%	N	%	
cerbB2										
Negative	44	73.3	16	26.7	1.000	28	46.7	32	53.3	0.477
Positive	33	73.3	12	26.7		17	37.8	28	62.2	
p53										
Negative	29	93.5	2	6.5	0.005	20	64.5	11	35.5	0.007
Positive	48	64.9	26	35.1		25	33.8	49	66.2	
Ki-67										
Negative	44	84.6	10	15.4	0.018	34	65.4	18	34.6	<0.0001
Positive	33	62.3	20	37.7		11	20.8	42	79.2	
EGFR										
Negative	66	74.2	23	25.8	0.886	42	47.2	47	52.8	0.065
Positive	11	68.75	5	31.25		3	18.8	13	81.3	
EZH2										
Negative	44	97.8	1	2.2	<0.0001					
Positive	33	55.0	27	45.0						
Grade										
1	8	100	0	0	0.000					
2	39	90.7	4	9.3						
3	30	56.6	24	43.4						
Lymph nodes										
Negative	49	87.5	7	12.5	0.001					
Positive	28	57.1	21	43.9						
Stage										
1	31	88.6	4	11.4	0.000					
2	36	78.3	10	21.7						
3	10	41.7	14	58.3						
ER										
Negative						5	19.2	21	80.8	0.01
Positive						40	50.6	39	49.4	
PR										
Negative						14	30.4	32	69.6	0.035
Positive						31	52.5	28	47.5	

ER: estrogen receptor, PR: progesterone receptor

EZH2 correlated with negative ER and PR status (p=0.01 and p=0.035, respectively) (Table 2), and positive expression of p53 and Ki67 (p=0.007 and p<0.0001, respectively) (Table 2).

In regard to the clinical factors examined, univariate analysis (Pearson's x^2 test) showed the expression of paxillin correlated with disease stage (p=0.000), lymph node metastasis (p=0.001) and grade of differentiation (p=0.000) (Table 2).

The results related to DNA ploidy showed that aneuploid tumors were significantly correlated with poorly differentiated tumors (p=0.000, tumor stage (p=0.000), tumor size (p=0.015), presence of nodal metastasis (p=0.000), positive ER

Table 3. Correlation of DNA ploidy with pathologic factors

	Value (Continuity correlation, x^2)	DF	p-value (2-sided)
Tumor size	5.969	1	0.15
Stage	28.558	1	0.000
Grade	26.852	1	0.000
Lymph node metastasis	17.280	1	0.000
ER+	7.104	1	0.008
PR+	1.325	1	0.25
c-erbB2	6.36	1	0.012
p53	2.110	1	0.146
Ki-67	11.868	1	0.001
EGFR	5.612	1	0.018

For abbreviations, see text

status (p=0.008), cerbB2 expression (p=0.012), positive expression of Ki-67 (p=0.001) and positive EGFR (p=0.018) (Table 3). Multivariate analysis of ploidy revealed that aneuploid tumors were associated with stage, poor differentiation, cerbB2 expression and EZH2 expression (Table 4). Logistic regression analysis with paxillin as dependent variable revealed that only grade was marginally associated with paxillin expression (p=0.06; Table 5). Logistic regression analysis using EZH2 as

dependent variable showed that EZH2 correlated significantly with grade and DNA ploidy, whereas it was only marginally associated with paxillin expression (Table 6).

Discussion

The enhancer of zeste homologue 2 (EZH2) is a member of the polycomb group of genes mainly acting as a chromatin modifying enzyme regulating cell cycle. The common finding is that EZH2 levels are abnormally elevated in cancer tissues compared with normal tissues with higher EZH2 levels correlating with advanced disease stages and poor prognosis [18,19].

In this study we investigated the expression of EZH2 in breast cancer imprint smears. EZH2 expression was found significantly increased in our cases and correlated with poorly differentiated tumors and nodal metastasis, suggesting that EZH2 may play a role in invasion and breast cancer metastasis. Furthermore EZH2 expression correlated with disease stage, ER/PR status and positive expression of p53 and Ki-67 and DNA aneuploidy.

Our findings are in agreement with other studies documenting that EZH2 expression is significantly up-regulated in invasive carcinomas and that overexpression of EZH2 is associ-

Table 4. Multivariate analysis of ploidy in relation to EZH2, stage, grade and cerbB2

Variables	B	S.E.	Wald	DF	Sig.	Exp(B)	95% C.I. for Exp(B)	
							Lower	Upper
Stage	-1.790	0.572	9.793	1	0.002	0.167	0.54	0.512
Grade	-1.875	0.782	5.742	1	0.017	0.153	0.33	0.711
cerbB2	-1.561	0.792	3.888	1	0.049	0.210	0.44	0.991
EZH2	-2.050	0.922	4.947	1	0.026	0.129	0.21	0.784
Constant	-11,386	2.836	16.119	1	0.000	88058.909		

Table 5. Logistic regression analysis for paxillin

	B	S.E.	Wald	DF	Sig.	Exp(B)	95% C.I. for Exp(B)	
							Lower	Upper
Grade	1.773	.641	7.648	1	0.06	5.887	1.676	20.680
Constant	-6.939	1.827	14.429	1	0.000	0.001		

Table 6. Logistic regression analysis for EZH2

	B	S.E.	Wald	DF	Sig.	Exp(B)	95% C.I. for Exp(B)	
							Lower	Upper
Grade	1.354	0.502	7.286	1	0.007	3.873	1.449	10.353
Paxillin	2.074	1.136	3.333	1	0.068	7.954	0.858	73.699
Ploidy	-2.218	0.653	11.523	1	0.001	0.109	0.030	0.392
Constant	-1.846	1.372	1.809	1	0.179	0.158		

ated with poor outcome in breast cancer patients [20-23]. Kleer et al. found increased EZH2 expression in invasive and metastatic carcinoma of the breast and an association with poorly differentiated tumors and adverse prognosis [24]. In a study of Zeidler and Kleer it was observed that EZH2 overexpression in breast epithelial cells resulted in aneuploidization and chromosomal instability [25].

Biological evidence has shown that overexpression of EZH2 induced type 1 histone deacetylation (HDAC) enzymatic activity in breast epithelial cells. Inhibition of HDAC activity blocked the transcriptional repressor functions of EZH2 [18,25,26]. This finding may have therapeutic implications in that inhibitors of HDAC may be useful therapeutic compounds in EZH2 overexpressing tumors [27]. Furthermore, the HDAC activity induced by EZH2 may explain the strong association between EZH2 protein expression and negative ER, documented in our study, showing that EZH2 may transcriptionally repress ER [27].

Paxillin is an adaptor protein and has been implicated in the regulation of several cellular events such as adhesion and metastasis. Furthermore, it is one of a few cytoskeletal proteins that have been recently shown to interact with certain oncogenes and growth factors receptors. There are two isoforms of paxillin, a and b. The b isoform of paxillin has been implicated with malignancy [14,27-30]. The cytoskeleton plays an important role in abnormal growth, invasion and metastasis which are characteristics of malignant tumors. Paxillin is one of the key components of cellular adhesion, contributing to the formation of a structural link between the extracellular matrix and the actin in the cytoskeleton. Cellular adhesions between tumor and normal cells and between adjacent tumor cells are essential for the progression of cancer [30,31].

Our results showed that the negative expression of paxillin was correlated with negative expression of EZH2 expression (p<0.0001). According to Turner [11] paxillin binds to many proteins that are involved in effecting changes in the organization of the actin cytoskeleton which are necessary for cell motility events associated with tumor metastasis [12]. In particular, paxillin plays a central role in coordinating the action of the Rho family of small GTPases, which regulate the actin cytoskeleton, by recruiting an array of GTPase activator, suppressor and effector proteins to cell adhesions [12]. Moreover, EZH2 is also de-

tected in the cytoplasm of human cells and seems to be implicated in controlling actin polymerization in response to cell signaling. Thus nuclear and cytoplasmic functions could both contribute to EZH2 mediated alterations in cancer cells [32].

In the present study, increased expression of paxillin was associated with disease stage, grade of differentiation, nodal metastasis, positivity of p53 and Ki-67 and DNA aneuploidy. These data extend some of the recent observations in the literature [13,33]. In the relevant literature, paxillin upregulation correlated with lymph node metastasis in breast tumors [34]. On the contrary, another report stated that paxillin overexpression is a marker of a less invasive tumor phenotype in breast cancer [12].

Paxillin has been shown to be transcriptionally upregulated and phosphorylated by human epidermal growth factor receptor 2 (HER2) signaling *in vitro*. Paxillin expression may also correlate with HER-2 amplification in breast cancer patients [32,35]. Because cell motility is dependent on the dynamic disassembly and subsequent reassembly of local adhesions, adhesion mobility induction by HRG pathway may provide an advantage and contribute to increased metastatic potential of cells with activated HER2 signaling. However, our results showed that paxillin expression did not correlate with HER2 status.

Multivariate analysis of ploidy results using paxillin and EZH2 expression as dependent variables revealed that aneuploid tumors were associated with higher stage, poor differentiation and positive expression of cerbB2 and EZH2. Recently it has been observed that overexpression of EZH2 in breast epithelial cells represses genes that function in the homologous recombination pathway of DNA repair, the dysregulation of which may cause aneuploidy and malignant transformation [8,23]. On the other hand, paxillin's overexpression is thought to unite with tyrosine phosphorylation which is inhibited at mitosis and is crucial to the assembly of intergrin signaling. Perturbing the intergrin function results in the generation of multipolar spindles which has important implications for cancer biology; multipolar spindles and the resulting aneuploidy are thought to contribute to tumorigenesis and metastasis [35,36].

Our results showed that the expression of EZH2 and paxillin may be a valuable marker in the process of predicting the malignant behavior of breast adenocarcinoma.

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