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Global H3K27 trimethylation and EZH2 abundance in breast tumor subtypes

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ABSTRACT

Polycomb repressive complex 2 (PRC2) and its core member enhancer of zeste homolog 2 (EZH2) mediate the epigenetic gene silencing mark: trimethylation of lysine 27 on histone 3 (H3K27me3). H3K27me3 is characteristic of the chromatin at genes involved in developmental regulation in undifferentiated cells. Overexpression of EZH2 has been found in several cancer types such as breast, prostate, melanoma and bladder cancer. Moreover, overexpression is associated with highly proliferative and aggressive types of breast and prostate tumors. We have analyzed the abundance of EZH2 and H3K27me3 using immunohistochemistry in two large and well-characterized breast tumor data sets encompassing more than 400 tumors. The results have been analyzed in relation to the molecular subtypes of breast tumors (basal-like, luminal A, luminal B, HER2-enriched and normal-like), as well as in subtypes defined by clinical markers (triple negative, ER+/HER2-/Ki67low, ER+/HER2-/Ki67high and HER2+), and were validated in representative breast cancer cell lines by western blot. We found significantly different expression of both EZH2 and H3K27me3 across all subtypes with high abundance of EZH2 in basal-like, triple negative and HER2-enriched tumors, and high H3K27me3 in luminal A, HER2-enriched and normal-like tumors. Intriguingly, the two markers show an inverse correlation, particularly for the basal-like and triple negative tumors. Consequently, high expression of EZH2 was associated with poor distant disease-free survival whereas high expression of H3K27me3

Abbreviations: ANOVA, Analysis of variance; DDFS, Distant disease-free survival; ER, Estrogen receptor; ES, Embryonic stem; GEX, Gene expression; H3K27me3, Trimethylation of lysine 27 on histone 3; IHC, Immunohistochemistry; PRC2, Polycomb repressive complex 2; PR, Progesterone receptor; TMA, Tissue microarray.

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was associated with better survival. Additionally, none of 182 breast tumors was found to carry a previously described EZH2 mutation affecting Tyr641. Our observation that increased expression of EZH2 does not necessarily correlate with increased abundance of H3K27me3 supports the idea that EZH2 can have effects beyond epigenetic silencing of target genes in breast cancer.

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1. Introduction

EZH2 (enhancer of zeste homolog 2) is a histone methyltransferase and the core member of polycomb repressive complex 2 (PRC2) that catalyzes trimethylation of lysine 27 on histone 3 (H3K27me3). Trimethylation of H3K27 is an epigenetic label mediating gene silencing and can function as a mark for *de novo* DNA methylation in cancer cells by recruitment of DNA methyltransferases (DNMTs) (Ohm et al., 2007; Schlesinger et al., 2007; Vire et al., 2006). PRC2 seems to bind to response elements of target genes in a cell-type specific manner (Squazzo et al., 2006) but has a preference for genes involved in developmental regulation such as the Hox genes (Margueron and Reinberg, 2011). PRC2-mediated silencing is essential for retaining stem cells in an undifferentiated state (Bracken et al., 2006). During differentiation, pluripotency genes are repressed and PRC2 target genes in undifferentiated cells are preferentially activated (Sparmann and van Lohuizen, 2006). Furthermore, PRC2 is relocated to other target genes to repress alternative gene expression programs in terminally differentiated cells (Jones and Wang, 2010).

EZH2 has been shown to be overexpressed in several cancer types such as breast, melanoma, bladder, and prostate cancer (Bachmann et al., 2006; Collett et al., 2006; Kleer et al., 2003; Raman et al., 2005; Varambally et al., 2002). Moreover, it has been shown to be a marker of aggressive phenotypes in both breast and prostate cancer (Bachmann et al., 2006; Pietersen et al., 2008). A few studies have identified a recurrent gain-of-function mutation in EZH2 in specific subtypes of lymphomas (Bodor et al., 2011; Morin et al., 2010) resulting in increased levels of H3K27me3 (Yap et al., 2011). However, reports of H3K27me3 levels in cancer samples are more contradictory. Low H3K27me3 levels have been associated with poor outcome in breast, ovarian and pancreatic cancers (Wei et al., 2008). On the other hand, high levels have been associated with poor outcome in hepatocellular carcinomas (Cai et al., 2011) and esophageal squamous cell carcinomas (He et al., 2009). These contradictory findings may in part reflect that H3K27me3 marks different genes for silencing in different cell types.

Breast cancer is a profoundly heterogeneous disease and at least five molecular subtypes have been repeatedly identified: luminal A, luminal B, HER2-enriched, basal-like and normal-like (Hu et al., 2006; Perou et al., 2000; Sorlie et al., 2001). We (Holm et al., 2010) and others (Bediaga et al., 2010; Kamalakaran et al., 2011; Ronneberg et al., 2011; Van der Auwera et al., 2010) have shown that the molecular subtypes of breast tumors display epigenetic differences in terms of specific DNA methylation profiles. In the current study, we aimed to examine if breast cancer subtypes also display

different levels of the H3K27me3 epigenetic mark associated with gene silencing. We investigated global levels of H3K27me3 and EZH2 protein expression in a large panel of breast tumors encompassing all subtypes. We have also analyzed the correlation between EZH2 and H3K27me3. Additionally, we performed a mutation screen of EZH2 to search for mutations that potentially would affect the normal function of EZH2.

2. Material and methods

2.1. Tumor sets

Breast tumor tissues placed on six tissue microarrays (TMAs) originally collected for two different studies were evaluated for EZH2 and H3K27me3 in the present study. The first data set (set I) was originally collected in a prospective study of the prognostic value of the S-phase fraction and includes 237 tumors from node negative patients (Malmström et al., 2001). Later, TMAs were constructed and the material was evaluated for Ki67 and HER2 (Klintman et al., 2010). EZH2 was considered evaluable in 205 and H3K27me3 in 208 cases for this tumor set. The second tumor set (set II) originates from a randomized study of tamoxifen in 564 premenopausal breast tumors in relation to estrogen receptor (ER) and progesterone receptor (PR) status (Rydén et al., 2005). Of the 564 patients, 276 received tamoxifen for two years and 288 patients did not receive any adjuvant treatment (Rydén et al., 2005). TMAs for this set were constructed and have been evaluated for Ki67 (Jirstrom et al., 2005) and HER2 (Rydén et al., 2008) as described previously. In set II, EZH2 was considered evaluable in 223 cases and H3K27me3 in 238 cases. Both studies are well-characterized regarding ER and PR status and have long-term follow-up. Gene expression (GEX) data were available for 165 tumors as part of other studies (Gruvberger-Saal et al., 2007; Honeth et al., 2008; Jönsson et al., in press; Saal et al., 2007). The study was approved by the regional ethics committee at Lund University.

2.2. Subtype classification

For the sample material for which we have GEX data, the tumors have been classified into molecular subtypes (basal-like, luminal A, luminal B, HER2-enriched and normal-like) according to the Hu et al. centroids (Hu et al., 2006) as described (Honeth et al., 2008; Jönsson et al., 2010). This set of 165 tumors, which is a subset of the tumors in sets I and II, is hereafter called the GEX set (Table 1). We used ER, PR, HER2, and Ki67 to group both tumor sets into triple negative,

Table 1 — Subtype classifications of the tumors.

	Type of data	Method of subtype classification	Subtype	No of tumors
Set I	IHC/FISH	ER, PR and HER2 negative	Triple negative	35
		ER and PR positive, Ki67 low (<20%)	ER+/HER2–/Ki67low	96
		ER and PR positive, Ki67 high (≥20%)	ER+/HER2–/Ki67high	16
		HER2 positive	HER2+	21
Set II	IHC/FISH	ER, PR and HER2 negative	Triple negative	52
		ER and PR positive, Ki67 low (1 or 2)	ER+/HER2–/Ki67low	73
		ER and PR positive, Ki67 high (3)	ER+/HER2–/Ki67high	16
		HER2 positive	HER2+	45
GEX set	GEX	Centroids from Hu et al. (2006)	Basal-like	38
			Luminal A	41
			Luminal B	19
			HER2-enriched	18
			Normal-like	23

HER2+, ER+/HER2–/Ki67high and ER+/HER2–/Ki67low subgroups. For set I, a cut-off of 20% for Ki67 was used and patients with HER2-amplified tumors as determined by FISH or with Herceptest 3+ were considered positive for HER2 as described (Klintman et al., 2010) (Table 1). Set II was subgrouped as set I, however for Ki67, samples in groups 1 or 2 were regarded as low, while those in group 3 were considered high as described (Jirstrom et al., 2005) (Table 1). ER and PR were measured using several methods: the enzyme immunoassay, isoelectric focusing in polyacrylamide gels, dextran coated charcoal method and/or immunohistochemistry, and were interpreted as described in detail in the original studies (Malmstrom et al., 2001; Rydén et al., 2005). Comparisons between the different techniques as well as investigations of the reproducibility have yielded satisfactory results (Fernö et al., 1995, 1997, 2000). A total of 168 tumors in set I and 186 tumors in set II could be assigned to the subgroups using these clinical markers. Tumors that could not be classified into a subtype either due to a correlation of <0.2 to any of the subtypes (GEX set) or due to lack of any of the components used for classification (set I and II) were designated non-classified.

2.3. Immunohistochemical staining and evaluation

EZH2 and H3K27me3 protein expression were determined by the labeled polymer method (DAKO, K5007, Copenhagen, Denmark) using the antibodies anti-EZH2 (clone 11, BD Transduction Laboratories, Franklin Lakes, NJ, 1:50 dilution) and anti-H3K27me3 (clone 6002, Abcam, Cambridge, MA, 1:50 dilution). Antigen retrieval was performed in a 2100 Retriever pressure cooker with TRS-buffer pH9 (DAKO S2367). Slides were stained in an Autostainer Plus (DAKO) with incubation times of 30 min (H3K27me3) and 60 min (EZH2) at room temperature. The TMA slides were scanned using a MiraxMidi slidescanner (Zeiss, Oberkochen, Germany) and evaluated using the TMA Module in Mirax Viewer v1.10 (3DHistech, Budapest, Hungary).

The TMAs were evaluated twice by one person (KH) in a blinded fashion under guidance of an experienced pathologist (DG). Unclear cases were discussed with the pathologist. Cases with <20 cancer cells, those with only cancer *in situ*, or lost TMA cores were excluded. Staining was seen in the cell nuclei. The tumors were assessed both by intensity and

prevalence of positive nuclei. Intensity values ranged between 1 and 3, with 1 being weak, and 3 strong. Proportion scores ranged between 1 and 10, with 1 representing 1–10% positive nuclei, and 10 representing 91–100% positive nuclei. Negative staining was scored as –1. A score that ranged from –1 to 30 was calculated as the product of the intensity and proportion scores. Each tumor is represented twice on the TMAs and an average was calculated between the two scores.

2.4. Sequencing of EZH2

For 176 of the 189 tumors included in our previous methylation study (Holm et al., 2010), we performed a mutation screen of EZH2 by sequencing the part of the gene covering the previously described recurrent mutation affecting Tyr641 in the SET domain of EZH2 (Morin et al., 2010). Additionally, we sequenced 4 normal breast tissue samples, 4 tumor-adjacent breast tissue samples, and an additional 6 breast tumor samples, in total giving 190 samples. DNA was isolated as described (Holm et al., 2010). PCR-primers for EZH2 were identical to those used previously (Morin et al., 2010): EZH2_015F: CAGGTTATCAGTGCCTTACCTCTCC and EZH2_015R3: TCTCAGCAGCTTTCACGTTG. PCR conditions were 94 °C for 7 min, then 15 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s, with 0.5 °C decrement in annealing temperature per cycle, followed by 20 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, and a final elongation step of 7 min at 72 °C. The PCR was performed in 25 µl reactions containing 3 µl 10× PCR buffer, 2.5 µl 25 mM MgCl₂, 1.2 µl 10 mM dNTPs, 0.12 µl 2.5U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA), and 0.6 µl 10 µM of each primer. The PCR products were analyzed on polyacrylamide gels, purified by MultiScreen PCR (Millipore, Billerica, MA), and sequenced using BigDye Terminator chemistry and a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Sequence chromatograms were analyzed with Sequencher (Gene Codes, Ann Arbor, MI).

2.5. Immunoblotting

All cell lines were obtained from American Type Culture Collection/LCG Standards except for SUM185PE, which was

obtained from S. Ethier. MDAMB157, MDAMB231, MDAMB468, and MCF7 were maintained in DMEM supplemented with 10% FBS. HCC1937, HCC70, HCC1187, and ZR751 were cultured in RPMI 1640 supplemented with 10% FBS. SUM185PE was maintained in Ham's F12 supplemented with 5% FBS, 5 µg/ml insulin, and 1 µg/ml hydrocortisone. Whole-cell lysates were resolved by SDS-PAGE and were then transferred to 0.45 µm PVDF membranes (Invitrogen, Carlsbad, CA) by electroblotting. The membranes were blocked by 5% milk (EZH2) or 5% BSA (H3K27me3) in TBST and were then probed with primary antibodies for EZH2 (diluted 1:800 in 2.5% milk) and H3K27me3 (diluted 1:800 in 2.5% BSA). The same antibodies were used for western blot as for IHC. The blots were probed with mouse secondary antibodies (Pierce Biotechnology, Rockford, IL) in 5% milk or 5% BSA. The membranes were developed using ECL (GE Healthcare, Chalfont St. Giles, UK).

2.6. Statistical analysis

Gene expression-based Outcome for Breast cancer Online (GOBO; <http://co.bmc.lu.se/gobo>) is a publicly available database including gene expression data from 1881 breast tumors (Ringnér et al., 2011). The database was used to investigate gene expression of EZH2 in an independent set of breast tumors. Gene expression of EZH2 in breast tumor cell lines was also analyzed using data from Neve et al. (2006) processed as described (Ringnér et al., 2011). Analysis of variance (ANOVA), t-tests, Fisher's exact tests, boxplots, and Spearman correlations were generated in R (The R Project for Statistical Computing; <http://cran.r-project.org>). Survival analysis was performed in R using the Survival package with distant disease-free survival (DDFS) censored at 5 years follow-up in log-rank tests and Kaplan–Meier curves. The log-rank test for trend was used to compare survival in more than two ordered groups. P-values smaller than or equal to 0.05 were considered significant.

3. Results

3.1. Subtype classification

For the tumors in the GEX set, we compared the subtype classification based on clinical markers with the GEX-based molecular subtype classification. As expected, the majority of the triple negative tumors were classified as basal-like and the majority of HER2+ as HER2-enriched (Kreike et al., 2007; Staaf et al., 2010a) (Table 2). Moreover, for ER+/HER2–/Ki67low tumors the most frequent molecular subtype was luminal A,

and for ER+/HER2–/Ki67high tumors it was luminal B (Cheang et al., 2009) (Table 2), corresponding to a significant enrichment within ER+/HER2– for Ki67high tumors among luminal B compared to among luminal A tumors (67% vs. 24%; $p = 0.05$, Fisher's exact test).

3.2. EZH2, H3K27me3 and clinical parameters

Representative IHC staining of EZH2 and H3K27me3 are shown in Figure 1. Of the 428 tumors evaluable for EZH2, 50 (12%) showed no staining at all (score –1), and 31 of 446 tumors (7%) were negative for H3K27me3. Seven tumors (2%) showed strong and complete staining for EZH2 (score 30) and 24 tumors (5%) showed strong and complete staining for H3K27me3.

We used all data sets to investigate associations between EZH2 or H3K27me3 and different clinical parameters. The results are presented in Table 3. ER, PR, histological grade and S-phase fraction were significantly associated with both EZH2 and H3K27me3, although with opposite correlations. EZH2 was high in tumors that were of histological grade 3, ER/PR negative, and with high S-phase fraction, while H3K27me3 was low in these tumor groups. Additionally, for tumor size, there was a significant association between high H3K27me3 and small tumor size (Table 3).

3.3. Differential expression of EZH2 in breast cancer subtypes

We and others have found high gene expression of EZH2 in basal-like tumors (Holm et al., 2010; Pietersen et al., 2008). To further validate this finding, we used a publicly available database (GOBO) including gene expression data from a large panel of human breast tumors (Ringnér et al., 2011). We found a pattern identical in this tumor material to our previously published data, with highest EZH2 gene expression in basal-like tumors and lowest in normal-like tumors ($P < 0.00001$, ANOVA; Figure 2). For the tumors in the GEX set, we investigated to what extent the expression of the gene affects the protein abundance. We found a good correlation (Spearman correlation coefficient 0.4, $P = 1 \times 10^{-7}$) between EZH2 mRNA and protein expression, measured by arrays and IHC, respectively, across all tumors for which EZH2 protein levels were considered evaluable ($n = 147$).

Next, we investigated protein expression of EZH2 in the tumors stratified by subtypes. In both sets I and II the expression was highest in triple negative tumors, and in the GEX set, we found the highest expression in basal-like tumors (Figure 3a–c). The EZH2 expression was lowest in ER+/

Table 2 – The distribution of classified samples using GEX data and clinical markers.

	Basal-like	Luminal A	Luminal B	HER2-enriched	Normal-like	Non-classified
Triple negative	24	0	1	0	0	0
ER+/HER2–/Ki67high	2	2	4	0	2	7
ER+/HER2–/Ki67low	0	26	8	1	10	11
HER2+	4	1	2	17	4	2
Non-classified	8	12	4	0	7	6

Figure 1 — Immunohistochemical stainings of breast tumors for EZH2 and H3K27me3. Shown are representative examples of low and high abundance, respectively.

HER2[−]/Ki67low (in both sets I and II), and luminal A and normal-like tumors (GEX set). The differences between subtypes were significant for all subtype comparisons (set I: $P = 2 \times 10^{-9}$, set II: $P = 1 \times 10^{-4}$, GEX set: $P = 2 \times 10^{-8}$, ANOVA) and followed the pattern we found earlier when studying the gene expression (Holm et al., 2010) (Figure 2).

3.4. Mutation analysis of EZH2

We did not find any evidence of mutations affecting Tyr641 or any other amino acids in the sequenced region in any of our 182 breast tumor samples. Neither did we find mutations in the normal breast tissue or tumor-adjacent tissue that were also included in the mutation analysis.

3.5. Differential expression of H3K27me3 in breast cancer subtypes

The presence of H3K27me3 was measured by IHC using the same tumor material as for the EZH2 analysis. In set I, the trimethylation was least abundant in triple negative and HER2⁺ tumors, while in set II, it was least abundant in triple negative and ER⁺/HER2[−]/Ki67high tumors (Figure 3d and e). The differences across the subtypes were significant in sets I and II

($P = 3 \times 10^{-7}$ and $P = 5 \times 10^{-4}$, respectively, ANOVA). In the GEX set, the histone modification was least common in basal-like and luminal B tumors, while it was most frequent in normal-like tumors; the difference between all subtypes was significant ($P = 4 \times 10^{-6}$, ANOVA; Figure 3f).

3.6. Expression of EZH2 and H3K27me3 in breast cancer cell lines

Western blot analysis was performed on nine breast cancer cell lines to validate the expression patterns of EZH2 and H3K27me3. Breast cancer cell lines are generally classified as 3 molecular subtypes: luminal, basal-like, and a subtype called basal B or claudin-low (Neve et al., 2006; Prat et al., 2010). The cell lines were selected to represent these subtypes: basal-like (HCC1187, MDAMB468, HCC70, and HCC1937), claudin-low (MDAMB231 and MDAMB157) and luminal (MCF7, ZR751, and SUM185PE) (Prat et al., 2010). Gene expression of EZH2 obtained from Neve et al. (Neve et al., 2006) for the cell lines displayed generally higher expression in basal-like and claudin-low cell lines (Figure 4a). The western blot analysis of EZH2 corresponds well with the mRNA expression in the cell lines (Figure 4a and b). H3K27me3 shows relatively lower expression in the basal-like cell lines except for HCC1937, which

Table 3 – Associations between clinical parameters and EZH2 or H3K27me3.

	EZH2			H3K27me3		
	No (%)	Average score	P-value ^c	No (%)	Average score	P-value ^c
ER ^a			3×10^{-8}			5×10^{-7}
Positive	251 (62)	7.5		259 (62)	12.2	
Negative	154 (38)	12.9		161 (38)	7.6	
PR ^b			2×10^{-10}			2×10^{-7}
Positive	266 (66)	7.4		278 (66)	12.1	
Negative	139 (34)	13.8		143 (34)	7.3	
Node status			0.07			0.4
Positive	18 (8)	7.3		18 (8)	12.3	
Negative	215 (92)	10.3		217 (92)	10.5	
Histological grade			2×10^{-12}			1×10^{-9}
1	63 (29)	6.8		63 (28)	14.3	
2	77 (35)	6.9		80 (36)	12.9	
3	81 (37)	15.6		81 (36)	5.8	
S-phase fraction			1×10^{-6}			0.0005
<12%	124 (68)	8.0		126 (70)	11.7	
≥12%	58 (32)	15.7		55 (30)	7.2	
Size			0.06			1×10^{-5}
≤20 mm	156 (69)	9.2		158 (70)	12.0	
>20 mm	71 (31)	11.8		69 (30)	6.8	

a ER = Estrogen receptor.

b PR = Progesterone receptor.

c t-test (two categories) or ANOVA (more than two categories) based on EZH2 or H3K27me3 scores. Significant P-values in bold.

interestingly has an inverted pattern with low EZH2 and high H3K27me3. Of the two claudin-low cell lines, MDAMB231 has a moderate expression of both EZH2 and H3K27me3 while MDAMB157 has low expression of EZH2 and high H3K27me3.

Figure 2 – Gene expression of EZH2. Gene expression of EZH2 is significantly different across the molecular subtypes for tumors compiled in GOBO. Highest expression is found in basal-like and HER2-enriched tumors. P-value was calculated using ANOVA. The number of tumors in each subtype is shown at top.

The luminal cell lines generally have a low expression of both EZH2 and H3K27me3. Due to their ability to proliferate in cell culture conditions luminal cell lines most often resemble luminal B tumors (Charafe-Jauffret et al., 2006; Neve et al., 2006). Therefore, it is not surprising that the expression in the luminal cell lines (Figure 4) resembles that of luminal B tumors (Figure 3). In all, the western blot analysis validates our findings of an inverse-correlation expression pattern for EZH2 and H3K27me3.

3.7. Survival analysis based on abundance of EZH2 and H3K27me3

Survival analysis was performed separately for both markers in the two tumor sets. For EZH2, we stratified tumors as EZH2 low (score −1–14) or high (score 15–30). In both sets I and II we found significantly shorter DDFS for patients with high EZH2 ($P = 0.02$ and $P = 0.04$, respectively, log-rank; Figure 5a and b). For H3K27me3, we divided the tumors into three groups: H3K27me3 low, intermediate and high, respectively, based on tertiles of the H3K27me3 scores for each tumor set separately. For H3K27me3 the differences in DDFS were significant between the three groups for both tumor sets, with patients with low abundance having a shorter survival than those with intermediate or high levels ($P = 0.01$ and $P = 0.004$, respectively, log-rank; Figure 5c and d). Approximately half of the patients in set II had received tamoxifen for two years while the remaining half had not received any adjuvant treatment. Survival analysis performed in the treated and untreated patient groups separately, showed similar

Figure 3 – Expression of EZH2 and H3K27me3 across subtypes. Protein expression of EZH2 stratified by subtype in (a) set I, (b) set II, and (c) the GEX set. Abundance of H3K27me3 in the breast tumor subtypes in (d) set I, (e) set II, and (f) the GEX set. *P*-values were calculated using ANOVA and excluding non-classified cases. The number of tumors in each subtype is shown at top.

results to when all patients were analyzed together (Supplementary Figure 1).

3.8. Correlations between EZH2 and H3K27me3

The correlation between the protein expression of EZH2 and the trimethylation of H3K27 was investigated within each subtype for each of the three subtype classifications, as well as across all samples. Both markers were measurable in 404 samples, and we observed no correlation between the markers across all samples (Spearman correlation 0.03). Spearman correlations for all subtypes can be found in Table 4. Significant positive correlations were only found for HER2+ tumors in sets I and II ($P = 0.007$ and $P = 0.05$, respectively, Spearman), and ER+/HER2-/Ki67low in set I ($P = 0.05$, Spearman). Interestingly, the correlations are higher for 12 of 13 tested subgroups (excluding non-classified tumors) than for all tumors (Table 4).

4. Discussion

We have analyzed EZH2 and H3K27me3 expression in relation to different breast cancer subtypes and validated our findings

in breast cancer cell lines. Subtype classifications have been carried out using both clinical markers and GEX data, and a comparison of the subset of tumors classified using both types of data showed an association between the two methods comparable to what has been demonstrated by others (Cheang et al., 2009; Nielsen et al., 2010; Parker et al., 2009). A perfect association between subtypes defined by GEX data and these clinical markers is not expected since they capture somewhat different aspects of breast tumor heterogeneity. In our material triple-negative tumors were almost exclusively classified as basal-like. Although the majority of HER2+ tumors typically are classified to the HER2-enriched subtype, HER2+ tumors have repeatedly been found in all GEX subtypes in agreement with our findings (Parker et al., 2009; Staaf et al., 2011, 2010a), and HER2+ tumors are heterogeneous with respect to gene expression patterns (Staaf et al., 2010b). On the other hand HER2+ tumors can be identified from GEX data using sets of HER2-associated genes (Roepman et al., 2009). For ER+/HER2- tumors we found a significant enrichment for Ki67high tumors among luminal B tumors compared to among luminal A tumors. Nevertheless, Ki67-based classification of ER+/HER2- tumors is where the discrepancy between GEX subtypes and clinical subtypes is

Figure 4 – Validation of EZH2 and H3K27me3 expression in nine breast cancer cell lines using western blot. (a) mRNA expression of *EZH2* in nine breast cancer cell lines. Publicly available microarray expression data were used (Neve et al., 2006). Red bars correspond to basal-like, grey bars to claudin-low and blue bars to luminal cell lines. (b) Western blot analysis of EZH2 and H3K27me3 for the same cell lines as in (a).

largest in our material. The reason for this discrepancy could depend on many factors. For example, Ki67 cut-points are often assigned against clinical outcome, which could result in an optimization to identify ER-negative tumors as Ki67high since ER-negative tumors generally have poor outcome and the highest proliferation. However, even when Ki67 cut points are optimized for separation of luminal A and B tumors the correspondence is far from perfect (Cheang et al., 2009). Moreover, the separation of luminal tumors into A and B using GEX data is also where different versions of GEX subtype classifiers differ the most (Weigelt et al., 2010), likely reflecting that there is heterogeneity also within the luminal subtypes.

EZH2 is involved in PRC2-directed gene silencing through mediation of the epigenetic mark H3K27me3. Both the gene and its encoded protein have been shown to be overexpressed in breast cancer and correlate with invasiveness, increased proliferation and poor outcome (Bachmann et al., 2006; Bracken et al., 2003; Collett et al., 2006; Pietersen et al., 2008). In the current study we found high expression of EZH2 in basal-like, triple negative, as well as in the HER2-positive subtypes. We also showed that the expression pattern across the subtypes is the same with regards to both mRNA and protein

levels. Corroborating earlier findings, we found highest expression of EZH2 in two GEX subtypes generally connected with poor outcome: basal-like and HER2-enriched. Mutations of EZH2 have been found in lymphomas and myeloid malignancies (Bodor et al., 2011; Makishima et al., 2010; Morin et al., 2010; Nikoloski et al., 2010). However, we did not find any mutations in the corresponding region of EZH2 in breast tumors and to our knowledge very few mutations have been found in epithelial malignancies.

For H3K27me3, we observed a significant difference between the subtypes, with particularly low expression in basal-like, triple negative, luminal B, and ER-positive tumors with high proliferation. In accordance, loss of H3K27me3 has earlier been shown to be a predictor of poor outcome in breast cancer (Wei et al., 2008). The epigenetic characteristics we have observed in this and our previous study (Holm et al., 2010) for the different molecular subtypes of breast cancer are summarized in Table 5. Polycomb target genes that are marked by H3K27me3 in stem and undifferentiated cells showed reduced expression in both basal-like and luminal B tumors in our previous study (Holm et al., 2010). The silencing of these polycomb target genes in the luminal B tumors could be explained by DNA methylation but this was not the case in the basal-like tumors (Table 5). This suggests the possibility that silencing of these genes in basal-like cancer may be due to polycomb-mediated H3K27me3 marking similar to what is observed in stem/progenitor and undifferentiated cells. This is consistent with the hypothesis of basal-like cancers having a more progenitor-like cell origin than luminal tumors (Lim et al., 2009). Although we observed low global abundance of H3K27me3 in basal-like tumors (Table 5) it is still possible that these polycomb targets are silenced through H3K27me3. We propose that the low H3K27me3 abundance is simply indicative of there being relatively fewer polycomb gene targets in the basal-like cancer subtypes compared to the number of genes under polycomb-control in more differentiated breast cancer subtypes. In contrast, the low abundance of H3K27me3 that we observed in luminal B tumors perhaps reflects the fact that the polycomb targets in both undifferentiated and differentiated cells are silenced instead by DNA methylation in luminal B tumors (Holm et al., 2010). Consistent with this observation, it has been proposed that the genes susceptible to DNA methylation in a given cancer type are a combination of genes that include both genes marked by H3K27me3 in undifferentiated cells, and those marked by H3K27me3 in corresponding differentiated normal cells (Rada-Iglesias et al., 2009).

To investigate the epigenomic landscape of pluripotent and differentiated cells, Hawkins et al. compared the abundance of H3K27me3 in human embryonic stem (ES) cells with several types of lineage-committed cells (Hawkins et al., 2010). They found an expansion of the H3K27me3 mark in all lineage-committed cells, with the number of base pairs spanned by H3K27me3 domains in lineage-committed cells typically more than three times as large as in ES cells. Such expansion could possibly explain the high abundance of H3K27me3 that we observe in normal-like and ER-positive tumors with low proliferation (Table 5). It is clear that experiments identifying polycomb targets in each of the breast cancer subtypes have to be performed to further elucidate these patterns.

Figure 5 — Survival analysis stratified by abundance of EZH2 and H3K27me3. Survival analysis stratified by high or low expression of EZH2 in (a) set I and (b) set II. Abundance of H3K27me3 was divided into low, intermediate and high in (c) set I and (d) set II. Shortest survival was found for patients with high EZH2 and low H3K27me3. Log-rank tests and Kaplan–Meier curves were censored at five years of distant disease free survival (DDFS).

Table 4 — Correlations between EZH2 and H3K27me3.

	Spearman correlation	P-value	No of samples
All tumors	0.03	0.5	404
Set I			
Triple negative	0.09	0.6	34
ER+/HER2-/Ki67low	0.2	0.05	91
ER+/HER2-/Ki67high	0.05	0.9	16
HER2+	0.6	0.007	20
Non-classified	−0.02	1	39
Set II			
Triple negative	0.1	0.5	46
ER+/HER2-/Ki67low	0.2	0.1	58
ER+/HER2-/Ki67high	0.1	0.6	13
HER2+	0.3	0.05	33
Non-classified	0.3	0.04	54
GEX set			
Basal-like	0.2	0.3	29
Luminal A	0.3	0.1	36
Luminal B	0.4	0.08	16
HER2-enriched	0.3	0.3	16
Normal-like	−0.1	0.5	19
Non-classified	−0.3	0.2	25

Significant P-values in bold.

Interestingly, high expression of EZH2 is not correlated with high abundance of H3K27me3 in our material. The correlation across all samples is low but increases when stratified for subtypes (Table 4). These results enhance the notion that the different breast tumor subtypes evolve through different progression pathways and may originate from different cell types. We found high expression of EZH2 in basal-like, triple negative and HER2-positive tumors, and low abundance of H3K27me3 in basal-like, triple negative, luminal B and ER-positive tumors with high proliferation (Table 5). In luminal A and ER-positive tumors with low proliferation the expression of EZH2 was low while abundance of H3K27me3 was relatively high. The high expression of EZH2 in proliferating tumors may have a canonical explanation as it could be required to maintain H3K27me3 marks during cell divisions to preserve transcriptional programs and cellular identity (Hansen et al., 2008). Overexpression of EZH2 might also lead to formation of another variant of the polycomb complex called PRC4, which contains a shorter isoform of EED (a member of the PRC2 complex) and has been shown to be enriched in cancer cells and to methylate H1K26 rather than H3K27 (Kuzmichev et al., 2005). Additionally, high expression of EZH2 could lead to non-canonical functions besides its histone methyltransferase activity and be directly related to breast cancer development and progression independently

Table 5 – Epigenetic patterns in breast cancer subtypes.

	Basal-like	Luminal A	Luminal B	Her2-enriched
EZH2 abundance	High	Low	Intermediate	High
H3K27me3 abundance	Low	High	Low	High
PRC2 targets ^a				
Undifferentiated cells				
Expression	Low	High	Low	Intermediate
DNA methylation	Low	Low	High	Intermediate
Differentiated cells				
Expression	High	Low	Low	High
DNA methylation	Low	Low	High	Intermediate

a A summary of results published in Holm et al. (2010), in which published PRC2 target genes obtained from undifferentiated and differentiated cell types were investigated for expression and DNA methylation status in breast tumor subtypes.

of histone modifications. For example, overexpression of EZH2 has been found to induce genomic instability (Chang et al., 2011; Gonzalez et al., 2011, 2009), to lead to increased proliferation through nuclear export of BRCA1 (Gonzalez et al., 2011, 2009), as well as to amplification of RAF1 signaling resulting in expansion of an aggressive population of tumor initiating cells (Chang et al., 2011). It has also been shown that EZH2 can regulate NF- κ B targets in a context-specific manner leading to activation of NF- κ B targets in ER-negative basal-like breast cancer cells and repression in ER-positive breast cancer cells (Lee et al., 2011). EZH2 can also exist in a cytosolic complex and control cell signaling and actin polymerization, possibly by methylating proteins (Su et al., 2005). The underlying cause for the overexpression of EZH2 has not been investigated in the current study, however, some possible explanations in breast cancer such as gain of its genomic region (Holm et al., 2010), or loss of its negative regulators microRNA-101 (Varambally et al., 2008) or microRNA-214 (Derfoul et al., 2011) have been suggested.

Survival analyses for both markers displayed significant differences depending on expression levels. High levels of EZH2 and low levels of H3K27me3 were associated with poor survival. This is not surprising since basal-like, triple negative and HER2-positive tumors generally have high levels of EZH2, while basal-like, triple negative, ER-positive tumors with high proliferation and HER2-positive tumors generally have low or intermediate levels of H3K27me3, and patients with tumors characterized by these subtypes commonly have a shorter survival. BMI1 is part of polycomb repressive complex 1 (PRC1), which is involved in maintenance of gene silencing and is suggested to be recruited to the histone after PRC2 has methylated H3K27 (Simon and Kingston, 2009). In line with our results, an inverse expression pattern of EZH2 and BMI1 has been found in breast tumors, with high expression of EZH2 associated with shorter patient survival, and high expression of BMI1 associated with longer survival (Pietersen et al., 2008).

In conclusion, the current work adds further layers of information to the epigenetic characteristics of breast tumor subtypes as differential pattern of histone modification can be added to our previous findings of differential methylation between subtypes. In particular, we corroborate earlier findings of high expression of EZH2 in breast tumors with poor outcome. Moreover, we validate our earlier findings of

differential transcript levels of EZH2 across breast cancer subtypes and we present new data demonstrating identical patterns of protein expression across the subtypes. Finally, we show that the abundance of H3K27me3 across the subtypes varies, intriguingly, not necessarily following the pattern of EZH2 expression. Relatively few studies on PRC2 target genes in differentiated cells have been published. Therefore, an important next step would be to identify PRC2 targets in specific breast tumors and cell types.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.molonc.2012.06.002>.

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