Clinical Cancer Research

EZH2 Is Overexpressed in *BRCA1*-like Breast Tumors and Predictive for Sensitivity to High-Dose Platinum-Based Chemotherapy

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Abstract

Purpose: BRCA1-deficient breast cancers carry a specific DNA copy-number signature ("*BRCA1-like*") and are hypersensitive to DNA double-strand break (DSB) inducing compounds. Here, we explored whether (i) EZH2 is overexpressed in human *BRCA1*-deficient breast tumors and might predict sensitivity to DSB-inducing drugs; (ii) EZH2 inhibition potentiates cisplatin efficacy in *Brca1*-deficient murine mammary tumors.

Experimental Design: EZH2 expression was analyzed in 497 breast cancers using IHC or RNA sequencing. We classified 370 tumors by copy-number profiles as *BRCA1*-like or non-*BRCA1*–like and examined its association with EZH2 expression. Additionally, we assessed *BRCA1* loss through mutation or promoter methylation status and investigated the predictive value of EZH2 expression in a study population of breast cancer patients treated with adjuvant high-dose platinum-based chemotherapy compared with standard anthracycline-based chemotherapy. To explore whether EZH2 inhibition by GSK126 enhances sensitivity to platinum drugs in EZH2-overexpressing breast cancers we used a *Brca1*-deficient mouse model.

Results: The highest EZH2 expression was found in BRCA1-associated tumors harboring a *BRCA1* mutation, *BRCA1*-promoter methylation or were classified as *BRCA1* like. We observed a greater benefit from high-dose platinum-based chemotherapy in *BRCA1*-like and non-*BRCA1*–like patients with high EZH2 expression. Combined treatment with the EZH2 inhibitor GSK126 and cisplatin decreased cell proliferation and improved survival in *Brca1*-deficient mice in comparison with single agents.

Conclusions: Our findings demonstrate that EZH2 is expressed at significantly higher levels in *BRCA1*-deficient breast cancers. EZH2 overexpression can identify patients with breast cancer who benefit significantly from intensified DSB-inducing platinum-based chemotherapy independent of *BRCA1*-like status. EZH2 inhibition improves the antitumor effect of platinum drugs in *Brca1*-deficient breast tumors *in vivo*.

Introduction

The histone methyltransferase EZH2 (enhancer of zeste homolog 2) is a member of the polycomb repressive complex 2 (PRC2) and catalyzes the trimethylation of lysine 27 on histone 3 (H3K27me^3; ref. 1). This chromatin mark is associated with gene silencing and involved in developmental regulation (2, 3). EZH2 is frequently overexpressed in a wide variety of cancers such as breast, melanoma, bladder, and prostate cancers (4, 5). EZH2 is implicated in cancer cell proliferation, invasion, as well as metastasis, and it is associated with a poor outcome in breast cancer (6–10).

BRCA1-mutant breast cancers are characterized by a triple-negative (hormone receptor–negative and HER2-negative) phenotype (TNBC), a basal-like molecular subtype and associated

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Translational Relevance

BRCA1-mutant breast cancers harbor a triple-negative phenotype and are associated with poor survival, highlighting the need for novel treatment options. In the present study, we show that Polycomb-group protein EZH2 is significantly higher expressed in breast tumors with a *BRCA1* mutation, *BRCA1*-promoter methylation, or *BRCA1*-like DNA copy-number profile, indicating that *BRCA1* loss is associated with high EZH2 expression levels. Moreover, EZH2 overexpression can identify *BRCA1*-like and non-*BRCA1*–like patients who benefit significantly from high-dose platinum-based chemotherapy. Potentially, EZH2 could be used as a predictive biomarker to categorize patients according to their likelihood to benefit from intensified DSB-inducing platinum-based chemotherapy independent of *BRCA1*-like status. The *in vivo* drug intervention study shows that EZH2 inhibition might be useful to enhance the antitumor effect of platinum drugs in BRCA1-associated breast tumors.

with poor survival (11–14). Although *BRCA1* germline mutations are rare in the general population, it is proposed that a subgroup of sporadic breast cancers have similar characteristics without harboring a *BRCA1* mutation, which is called BRCAness (15). BRCA1 is important for DNA double-strand break (DSB) repair through the homologous recombination (HR) pathway. In the absence of HR, DSB repair is mostly carried out by error-prone nonhomologous end joining (NHEJ), further promoting genomic instability (16–18). This genomic instability was shown to predispose to familial breast and ovarian cancer in *BRCA1* or *BRCA2* mutation carriers (19–21) and can be measured through DNA copy-number profiles by array comparative genomic hybridization (aCGH). It has been previously established that BRCA1-mutant breast cancers show a specific DNA copy-number profile, which are described as *BRCA1*-like signature (22, 23). Recent studies have shown that *BRCA1/2*-mutant breast cancers are hypersensitive to DSB-inducing agents, such as bifunctional alkylators or platinum drugs, and poly(ADP-ribose)polymerase (PARP) inhibitors (24–28). This sensitivity seems not to be restricted to *BRCA1*-mutant breast cancers, because the subgroup of sporadic breast cancers with the BRCAness phenotype also shows defects in HR (15). Interestingly, the *BRCA1*-like classifier can also identify tumors with a loss of BRCA1 function by other mechanisms such as *BRCA1*-promoter methylation (29). This indicates that the *BRCA1*-like classifier captures aspects of the BRCAness group and identifies patients who benefit from an intensified platinum-based chemotherapy (30, 31).

Novel studies describe a possible connection between BRCA1 and the PRC2 complex (32–35). In addition to the well-established role of BRCA1 in the HR process, a few studies describe a function of EZH2 in DNA repair by regulating the cancer cell fate in response to DNA damage and contribute to DSB repair (36, 37). Therefore, EZH2 emerged as a promising target for BRCA1-associated tumors. In this context, we have previously demonstrated that EZH2 overexpression is functionally relevant in *Brca1*-deficient tumor cells derived from a mouse model of hereditary breast cancer (38). Recently, GSK126, a highly selective inhibitor of EZH2 methyltransferase activity, was developed and has shown antitumor activity in several cancer cell lines, including breast cancer (39, 40).

In the current study, we set out to validate our findings by investigating global EZH2 expression in different cohorts of sporadic and BRCA1-associated human breast cancers, which were stratified by *BRCA1* mutation, *BRCA1*-promoter methylation, and *BRCA1*-like status. Additionally, we analyzed whether patients with EZH2 overexpression benefit from an intensified DSB-inducing platinum-based chemotherapy and tested *in vivo* if EZH2 inhibition alone or in combination with platinum drugs provides a novel treatment option for EZH2-overexpressing breast cancer.

Materials and Methods

Tumor sets

In vivo set. Twenty-one *Brca1*-deficient mammary tumors [arising in $K14^{cre};Brca1^{F/F};Tp53^{F/F}$ (KB1P) mice] and 33 Brca2-deficient mammary tumors [arising in $K14^{cre};Brca2^{F/F};Tp53^{F/F}$ (K21P) mice] were compared with 32 Brca1-proficient control tumors [arising in $K14^{cre};Tp53^{F/F}$ (KP) mice] and expression values ($\log_2$ ratio) of *Ezh2* were obtained from oligonucleotide microarrays representing 18,173 genes. Methods for RNA extraction, RNA amplification, microarray hybridization, and data processing were described in detail by Liu and colleagues (41).

For this study, we included four well-defined patient series. An overview of all analyzed cohorts is shown in Table 1. In the first retrospective collected patient cohort from the University Hospital of Cologne (set I), patients with breast cancer with deleterious mutations in the *BRCA1* ($n = 66$), *BRCA2* ($n = 27$), and sporadic breast cancers (WT; $n = 53$) were investigated for EZH2 expression by IHC. Thirty-nine samples were excluded (eight *BRCA1*- mutant tumors, 11 *BRCA2*-mutant tumors, 10 sporadic tumors) from analysis due to missing material or improper staining. The majority of WT tumors (86% = 37/43) and *BRCA2*-mutant tumors (69% = 11/16) were estrogen receptor positive (ER^+). Seventy-one percent (34/48) of the *BRCA1*-mutant tumors were triple negative. Patient clinical data were previously published (42).

The second tumor set from the NKI and Cambridge, a consecutive series of 112 triple-negative breast cancers (TNBC; set II, RATHER), was included. Microarray hybridization and analysis was performed as previously described (43), and microarray data were analyzed for *EZH2* expression levels. Class prediction (*BRCA1*-like/non-*BRCA1*–like) was carried out on the normalized data according to published instructions (22). Semiquantitative *BRCA1*-promoter methylation was determined using the methylation-specific-MLPA method according to the manufacturer's protocols using the SALSA MLPA ME001-tumor suppressor probemix 1 (MRC-Holland). Accordingly, this cohort was divided into four subgroups: *BRCA1*-like ($n = 62$), *BRCA1*-mutant ($n = 10$), *BRCA1*-promoter methylated ($n = 14$), and non-*BRCA1* (cleaned for *BRCA1*-like, *BRCA1* mutation and promoter methylation; $n = 44$).

In addition, the third patient cohort (set III, Neoadjuvant) consists of a gene-expression data set of 117 TNBC patients treated at the Antoni van Leeuwenhoek Hospital (NKI-AvL) and scheduled to receive neoadjuvant chemotherapy. All patients had a breast carcinoma with either a primary tumor size of at least 3 cm or the presence of axillary lymph node metastases. Sixty-three were labeled and hybridized to Illumina 6v3 arrays (Illumina),

Table 1. Clinical characteristics and *BRCA* mutation status: overview of all patient cohorts

Variable	Set I (Cologne) *N* = 107	Set II (RATHER) *N* = 112	Set III (Neoadjuvant) *N* = 117	Set IV (N4+) *N* = 161
ER				
Negative	49	112	117	58
Positive	58	0	0	103
PR				
Negative	52	112	117	78
Positive	55	0	0	83
HER2				
Negative	97	112	117	161
Positive	10	0	0	0
TNBC				
No	63	0	0	106
Yes	44	112	117	55
Grade				
1	5	0	0	35
2	44	11	27	52
3	38	86	68	68
Missing	20	15	22	6
Mutation analysis				
BRCA1	48	10	19	9
BRCA2	16	0	2	0
Wild-type	43	94	41	54
ND	0	8	55	98
aCGH				
BRCA1-like	0	62	59	37
Non-*BRCA1*–like	0	50	38	124
ND	107	0	19	0
BRCA1 promoter methylation				
Methylated	0	14	11	16
Unmethylated	0	80	45	44
ND	107	18	61	101
EZH2 expression analysis	Protein	RNA	RNA	Protein

Abbreviations: aCGH, array comparative genomic hybridization; ER, estrogen receptor; ND, not determined; PR, progesterone receptor; TNBC, triple-negative breast cancer.

Table 2. SET IV (N4+, HER2-negative) cohort: Clinical characteristics

	Non-BRCA1–like (%)			
Variable	EZH2 low (≤50%) *(N = 53)*	EZH2 high (>50%) *(N = 71)*	BRCA1-like (%) *(N = 37)*	*P*
ER				
Negative (<10%)	8 (15%)	18 (25%)	32 (86%)	<0.001
Positive (≥10%)	45 (85%)	53 (75%)	5 (14%)	
PR				
Negative (<10%)	20 (38%)	23 (32%)	35 (95%)	<0.001
Positive (≥10%)	33 (62%)	48 (68%)	2 (5%)	
HER2				
Negative	53	71	37	
Positive	0	0	0	
TNBC				
No	46 (87%)	55 (77%)	5 (14%)	<0.001
Yes	7 (13%)	16 (23%)	32 (86%)	
pT stage				
1	6 (11%)	14 (20%)	12 (32%)	0.036
2	32 (61%)	48 (67%)	20 (54%)	
3	15 (28%)	9 (13%)	5 (14%)	
Grade				
1	21 (39%)	14 (20%)	0 (0%)	<0.001
2	19 (36%)	28 (39%)	5 (14%)	
3	11 (21%)	28 (39%)	29 (78%)	
Missing	2 (4%)	1 (1%)	3 (8%)	
LN				
4–9 LN+	37 (70%)	42 (59%)	23 (62%)	0.469
≥10 LN+	16 (30%)	29 (41%)	14 (38%)	
Surgery				
Breast-conserving	8 (15%)	18 (25%)	12 (32%)	0.146
mastectomy	45 (85%)	53 (75%)	25 (68%)	
BRCA1 mutation status				
Germline	1	2	6	0.012
Promoter methylation	1	1	14	
Normal	7	14	12	
ND	44	54	5	

NOTE: *P* values were calculated with χ^2 test or Fisher exact test. The *P* values are for the comparisons of the three groups within one variable.
Abbreviations: ER, estrogen receptor; LN, lymph node; ND, not determined; PR, progesterone receptor; TNBC, triple-negative breast cancer.

and 50 samples were profiled using RNA-seq (44). *BRCA1* mutation status ($n = 19$) was obtained from patient records through our family cancer clinic. aCGH *BRCA1*-like scores were obtained by three different assays as described by Lips and colleagues (45) and classified as *BRCA1*-like ($n = 59$) or non-*BRCA1* (cleaned for *BRCA1*-like, *BRCA1* mutation and promoter methylation; $n = 33$). Hypermethylation of the *BRCA1* promoter ($n = 6$) was determined using methylation-specific MLPA analysis, according to manufacturers' protocol (45).

The final set IV (N4+) originates from a randomized trial of patients with breast cancer with tumor-positive lymph nodes, which were treated with either adjuvant high-dose therapy or conventional chemotherapy (46). The conventional regimen consisted of intravenous injection of 5-fluorouracil (500 mg/m^2), epirubicin (90 mg/m^2), and cyclophosphamide (500 mg/m^2; FEC). In the high-dose group (HD-CTC, intensified DSB-inducing platinum-based chemotherapy) patients were administered FEC followed by stem cell harvesting and carboplatin (1,600 mg/m^2), thiotepa (480 mg/m^2), and cyclophosphamide (6,000 mg/m^2). For this study, we included 523 patient samples available on the TMA for EZH2 staining. Of these groups from 161 patients aCGH profiles for *BRCA1*-like status and data on *BRCA* mutation and *BRCA1*-promoter methylation were available and were used for this study (set IV, N4+; Table 2; refs. 23, 30). This study was approved by the local ethics committee of the NKI.

IHC staining and evaluation

Set I. The samples were incubated for 30 minutes with a mouse monoclonal antibody against EZH2/Ezh2 (1:100, BD Biosciences) with an automated staining system (Medac Autostainer). Antibody specificity was previously tested on formalin-fixed tissue (47, 48). Once immunostained, the amount of EZH2-expressing epithelial cell nuclei was counted in a blinded manner by three persons under guidance of an experienced pathologist. The tumors were assed for the proportion of positive nuclei (0% and 100%).

Set IV (N4+). Tumor tissue of 523 patients was available on a tissue microarray (TMA). Tumors were arrayed in triplicates of 0.6 mm cores. Automated IHC was performed using an automated staining system (Ventana Benchmark ultra). The slides were incubated for 1 hour with a mouse monoclonal antibody against EZH2/Ezh2 (1:800, Leica, NCL-L-EZH2) at room temperature. The NCL-L-EZH2 antibody was previously validated in EZH2 expressing formalin-fixed tissue (49, 50). The TMA slides were scanned with a slide scanner (Aperio ScanScope XT system) and evaluated by one person in a blinded fashion under guidance of an experienced pathologist. Staining was seen in nuclei and cases with <20 cancer cells or lost TMA cores were excluded. The tumors

were assed for the prevalence of positive nuclei. Values ranged between 0% and 100%. The average was calculated among the three cores. From 523 patients presented in the TMA EZH2 staining could be evaluated in 400 cases. Array CGH-based BRCA1-like status was available for 161 patients of the randomized trial. Therefore, we focused on this subgroup for further analyses. Expression was assessed using the proportion of cells stained for EZH2, and expression categories were based on distribution plots and number of cases when determining the final cutoff between low and high expression (>50% = high EZH2, ≤50% = low EZH2). The protocols of further histopathologic stainings (ER, PR, and HER2) were described previously (46). Representative samples of IHC stainings are shown in Supplementary Fig. S1.

***In vivo* target inhibition of GSK126.** KB1P tumors were harvested after 10 days of treatment with GSK126 150 mg/kg daily or vehicle control (Captisol), and IHC stainings for H3K27me^3 were performed using formalin-fixed paraffin-embedded tumor tissue. Automated IHC was performed using an automated staining system (Ventana Benchmark ultra). The slides were incubated with a mouse monoclonal antibody against H3K27me^3 (1:100, Abcam, ab6002) overnight at 4°C. This antibody was extensively tested for target specificity (51).

Statistical methods

The Mann–Whitney *U* test was used to compare EZH2 protein expression among non-*BRCA1*–associated breast cancers with BRCA1-associated tumors. For the data set IV, we defined three groups of interest: all *BRCA1*-like patients, non-*BRCA1*–like patients with EZH2 ≤ 50%, non-*BRCA1*–like patients with EZH2 > 50%. Associations between these groups and clinicopathologic features were assessed using χ^2 test or Fisher exact test. The *P* values are for the comparisons of the three groups within one variable. Disease-free survival (DFS) and overall survival (OS) were defined as in ref. 43. Survival curves were generated with the Kaplan–Meier approach and compared with the log-rank test between EZH2 low and high expression levels. The Cox proportional hazards model was used to evaluate the group and treatment regimen effects on survival. A stepwise backward-selection method with $P > 0.1$ as a removal criterion was used to find possible confounders and other prognostic factors. Candidate factors were patients' age, pT stage, number of positive lymph nodes (4–9 or ≥10), type of surgery (breast conserving or mastectomy), triple-negative status (yes or no), ER status (positive or negative), PR status (positive or negative), grade (1, 2, 3, or unknown). To assess whether the effect of adjuvant high-dose therapy versus conventional chemotherapy on survival differs in the three groups of interest, an interaction term between the treatment regimen and the three groups was included in the regression models. Proportionality of the hazards was evaluated using Schoenfeld residuals and in case of nonproportionality for a particular covariate, an interaction between that covariate and follow-up time was included in the models. All *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

Derivation and maintenance of mouse tumor cell lines

Tumor cell lines were generated from individual tumors arising in female mice with a KB1P or KP genotype as described previously (52). Established cell lines were cultured at 37°C with 5% carbon dioxide under low oxygen conditions (3%) in DMEM-F12 medium (Gibco) supplemented with 10% FCS, 50 U/mL penicillin, 50 μg/mL streptomycin (Gibco), 5 μg/mL insulin (Sigma), 5 ng/mL epidermal growth factor (Invitrogen), and 5 ng/mL cholera toxin (Gentaur). The KB1P G3 BRCA1 reconstituted cell line was generated by transfecting KB1P G3 cells (53) with a human *BRCA1* cDNA expression construct (54) using Lipofectamine 2000 (Thermo Fisher Scientific). One day after transfection, cells were passaged and cultured with 300 μg/mL G418 (Thermo Fisher Scientific) to select for human *BRCA1*–complemented colonies. G418-resistant colonies were picked and checked for human *BRCA1* integration by PCR with *BRCA1* exon 11–specific primers.

Real-time cell proliferation assay

One hundred fifty (KP 3.33) to 300 (KB1P G3 and SC8) cells per well were plated in 384-well plates. Twenty-four hours later, cells were treated with GSK126 (8 μmol/L; Syncom), cisplatin (0.5 μmol/L; Mayne Pharma), and the combination or DMSO (vehicle control) as indicated and allowed to grow for 144 hours. Phase-contrast images were automatically acquired by IncuCyte FLR (Essen Bioscience) from the incubator at 2-hour intervals. Proliferation was monitored by analyzing the occupied area (% confluence) of cell images over time by IncuCyte software (Essen Bioscience).

Clonogenic survival assay

Cells were seeded at 1,000 per well into 12-well plates and continuously treated with GSK126 (8 μmol/L) or with cisplatin (0.125 μmol/L) alone or in combination for 8 days. DMSO was used as vehicle control. After the treatment period, colonies were stained using 0.5% crystal violet in methanol.

In vivo drug intervention studies

This study is compliant with all relevant ethical regulations regarding animal research; all animal experiments were approved by the Animal Ethics Committee of The Netherlands Cancer Institute (Amsterdam, the Netherlands) and performed in accordance with the Dutch Act on Animal Experimentation (November 2014). Orthotopic transplantations, tumor monitoring, and sampling were performed as described before (55). Briefly, KB1P tumor pieces were transplanted into FvB/Ola females and treatments were initiated following tumor outgrowth to approximately 200 mm^3 (100%). GSK126 (Syncom) was reconstituted in 20% Captisol (CyDex Pharmaceuticals), and brought to a pH of 4.5 with 10 M potassium hydroxide, to create a working stock of 15 mg/mL. Cisplatin was obtained from Mayne Pharma. Tumor-bearing mice were randomized into treatment groups blindly and treated with vehicle (Captisol, intraperitoneally), cisplatin (3 mg/kg, on days 1 and 14 once, intraperitoneally), and GSK126 inhibitor (150 mg/kg, daily, intraperitoneally) alone for 28 consecutive days or in combination with cisplatin (3 mg/kg, on days 1 and 14 once, intraperitoneally). Animals were euthanized with CO_2 when the tumor reached a volume of 1,500 mm^3. All of the procedures were carried out by animal technicians who were blinded regarding the hypothesis of the treatment outcome.

Results

EZH2 expression among *BRCA1*- and *BRCA2*-mutated breast tumors

We previously found high protein and gene expression of EZH2 in tumors derived from a *Brca1*-deficient mouse model (38). To

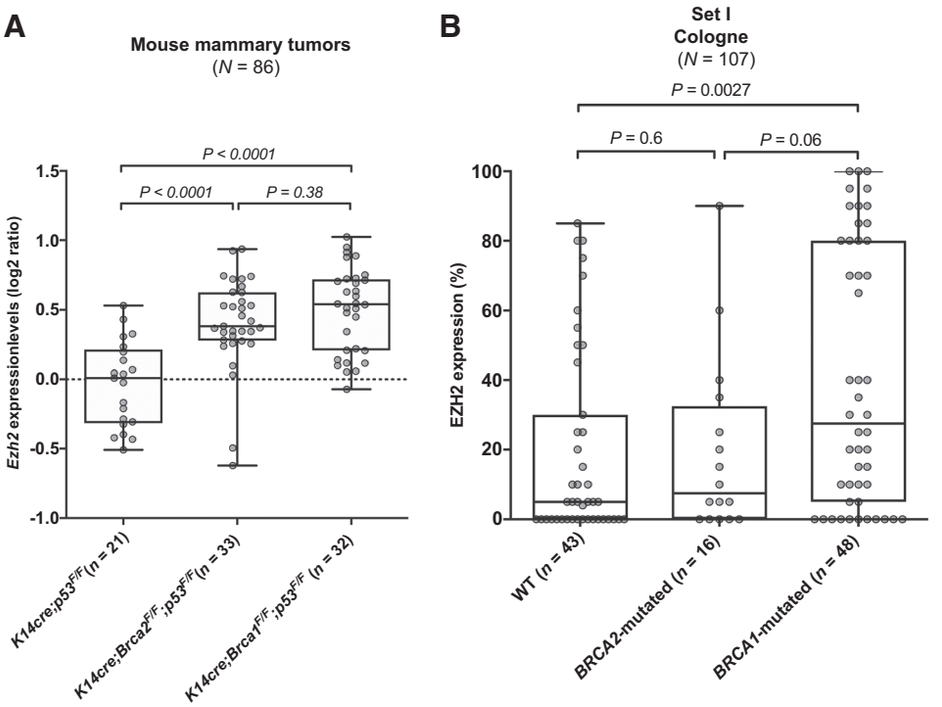

Figure 1.
EZH2 expression among *BRCA1* and *BRCA2*-mutant breast cancers. **A,** *In vivo* set: *Ezh2* RNA expression among tumors derived from a *Brca1/2*-deficient compared with a *Brca1/2*-proficient mouse model. **B,** Set I: EZH2 protein expression among sporadic breast tumors (WT, wild-type) compared with *BRCA1/2*-mutant human breast tumors. Groups were compared using an unpaired Mann–Whitney *U* test with $\alpha = 0.05$.

further validate this finding in human samples, we initially investigated the EZH2 expression in a cohort tested for BRCA1/2 status (set I; ref. 42). Because the BRCA2 protein has a similar role in DNA repair, we included *BRCA2*-mutant tumors in our analysis. We found an expression pattern identical in this cohort to our previously published mouse data with the highest EZH2 expression in *BRCA1*-mutant breast tumors. Interestingly, EZH2 levels were lower in *BRCA2*-mutant tumors. To confirm this result, we assessed our microarray data of tumors derived from *Brca1*- and *Brca2*-deficient mice in comparison with a *Brca1/2*-proficient mouse model and found that Ezh2 expression was still the highest in *Brca1*-deficient tumors, followed by an intermediate expression in *Brca2*-deficient tumors and lowest in *Brca1*- or *Brca2*-proficient tumors (Fig. 1).

EZH2 expression among breast cancers with *BRCA1* germline mutation, promoter methylation, and *BRCA1*-like DNA copy-number profile

Human *BRCA1*-mutant breast tumors carry a specific DNA copy-number signature ("*BRCA1*-like"), which is also found in tumors with a loss of BRCA1 function by other mechanism such as *BRCA1*-promoter methylation. To evaluate if EZH2 overexpression is consistent with a loss of BRCA1 function in breast tumors, we analyzed three large patient cohorts and stratified EZH2 expression by *BRCA1*-germline mutation status, *BRCA1*-promoter methylation and *BRCA1*-like-status (Table 1). Gene-expression data were available for sets II (RATHER) and III (neoadjuvant), which consist of TNBC samples. In both sets, the *EZH2*-RNA expression was significantly higher in *BRCA1*-mutant tumors and tumors with *BRCA1*-promoter methylation or tumors with a *BRCA1*-like DNA copy-number profile compared with non-BRCA1–associated tumors (Fig. 2). Next, we investigated EZH2 protein expression in a prospectively collected clinical study population, consisting of patients with node-positive and HER2-negative breast cancer (set IV: N4+). In accordance with the gene-expression data of sets II and III, EZH2 was overexpressed in BRCA1-associated tumors compared with non-BRCA1–associated tumors (Fig. 3). Of note, the vast majority of *BRCA1*-like patients showed a high EZH2 expression (92% = 34/37) and only three had a low EZH2 expression (one patient had EZH2 = 33% and two patients had EZH2 = 40%).

Association between EZH2 abundance and clinicopathologic characteristics

We used the prospective evaluated data set IV to investigate associations between EZH2 and (non)-*BRCA1*–like groups with different clinical characteristics. The results are presented in Table 2. Most of non-*BRCA1*–like tumors were ER^+ and PR^+, while most of the *BRCA1*-like tumors were ER^- and PR^-. Taking into account that 92% of all *BRCA1*-like tumors were EZH2 high, we found the highest EZH2 expression within the subgroup of triple-negative breast tumors. Non-*BRCA1*–like tumor with low EZH2 levels were more often > 5 cm diameter, less likely to be ER^- and less often grade 3 compared with the non-*BRCA1*–like tumors with high EZH2 levels and the *BRCA1*-like tumors (Table 2).

Predictive value of EZH2 expression in patients with breast cancer treated with conventional (CONV) or high-dose (HD) platinum-based chemotherapy

Kaplan–Meier curves were generated for non-*BRCA1*–like and *BRCA1*-like patients to compare the outcome of low and high EZH2 expression in relations to conventional and HD platinum-based chemotherapy (Fig. 4). The cohort of *BRCA1*-like patients benefited from HD platinum-based chemotherapy, and all of these tumors had high levels of EZH2 (Fig. 4C). No Kaplan–Meier curves could be drawn for the three *BRCA1*-like patients with low EZH2 levels. Inclusion of these three cases to the *BRCA1*-like with high EZH2 levels would result in Kaplan–Meier

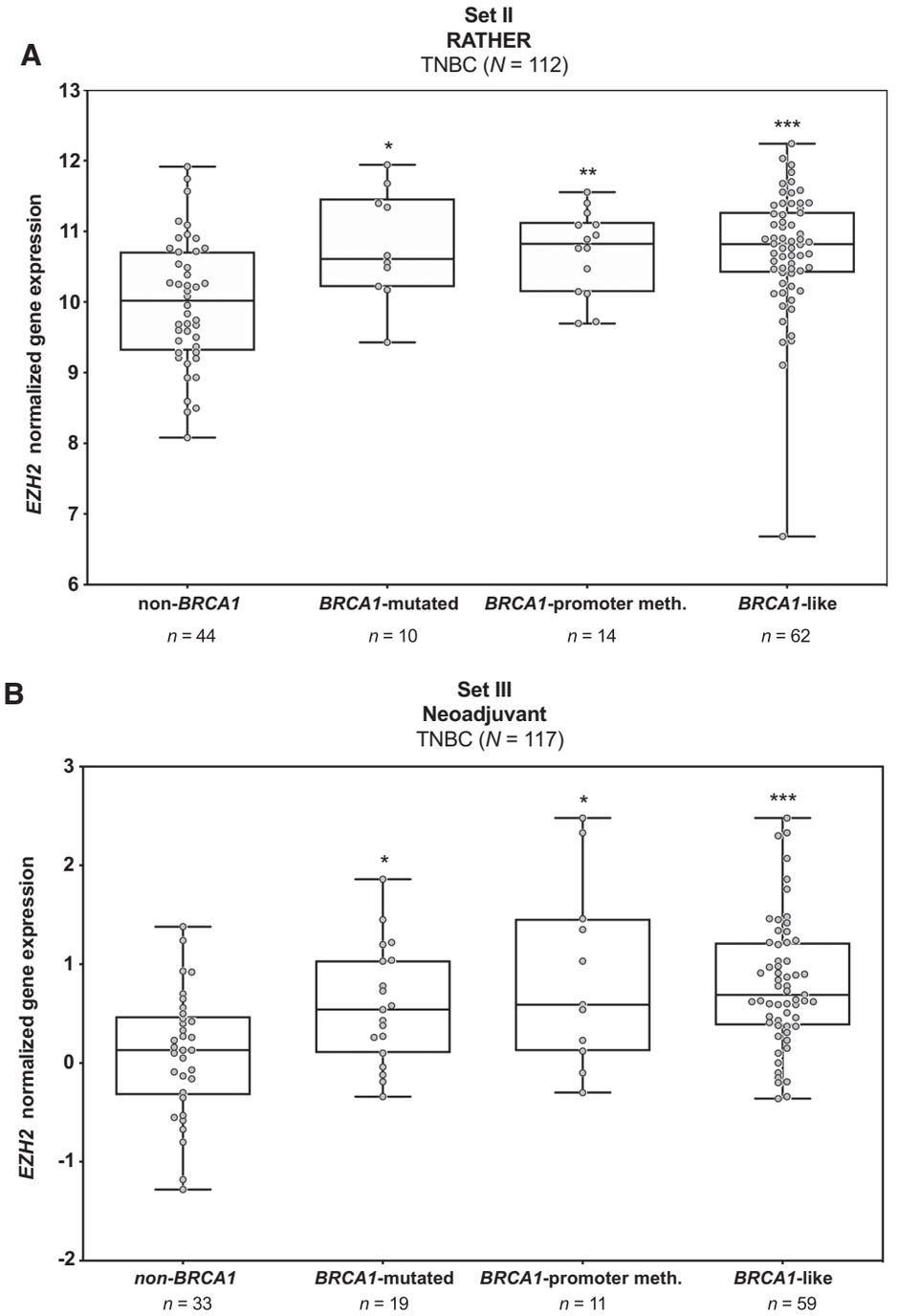

Figure 2.
EZH2-RNA expression among TNBC in association with *BRCA1* germline mutation, promoter methylation, and *BRCA1*-like DNA copy-number profile. **A,** Set II: sporadic tumors (non-*BRCA1*) vs. tumors with a *BRCA1* mutation, *BRCA1*-promoter methylation expression, or *BRCA1*-like DNA copy-number profile within TNBC. **B,** Set III: sporadic tumors (non-*BRCA1*) versus tumors with a *BRCA1* mutation, *BRCA1*-promoter methylation, or *BRCA1*-like DNA copy-number profile within TNBC. For each comparison, an unpaired Mann–Whitney *U* test was used (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

curves as shown by Vollebergh and colleagues (23, 30), which looks very similar to Fig. 4C. In analogy to this, we further assessed whether EZH2 can be used to identify a population among non-*BRCA1*–like tumors that benefit from intensified DSB-inducing platinum-based chemotherapy. We observed that the effect of adjuvant HD therapy versus conventional chemotherapy on DFS survival differs in the three groups of interest (DFS $P_{\text{interaction}} = 0.015$, OS $P_{\text{interaction}} = 0.115$). Both non-*BRCA1*–like patients with high levels of EZH2 and *BRCA1*-like patients benefit from HD chemotherapy. Such tumors have 70% to 80% reduced chance of experiencing DFS and OS events after HD chemotherapy in comparison with conventional chemotherapy (DFS: HR = 0.35, $P = 0.004$ and HR = 0.18, $P = 0.001$, respectively; OS: HR = 0.31, $P = 0.007$ and HR = 0.21, $P = 0.007$, respectively). For non-*BRCA1*–like tumors with low levels of EZH2, we found no significant difference in survival between the two treatment regimen (DFS: HR = 0.94, $P = 0.89$; OS: HR = 0.71, $P = 0.48$; Table 3). Non-*BRCA1*–like tumors were more often ER^+ (Table 2). Thus, we further investigated whether the benefit of HD platinum-based chemotherapy is related to ER status and calculated Kaplan–Meier

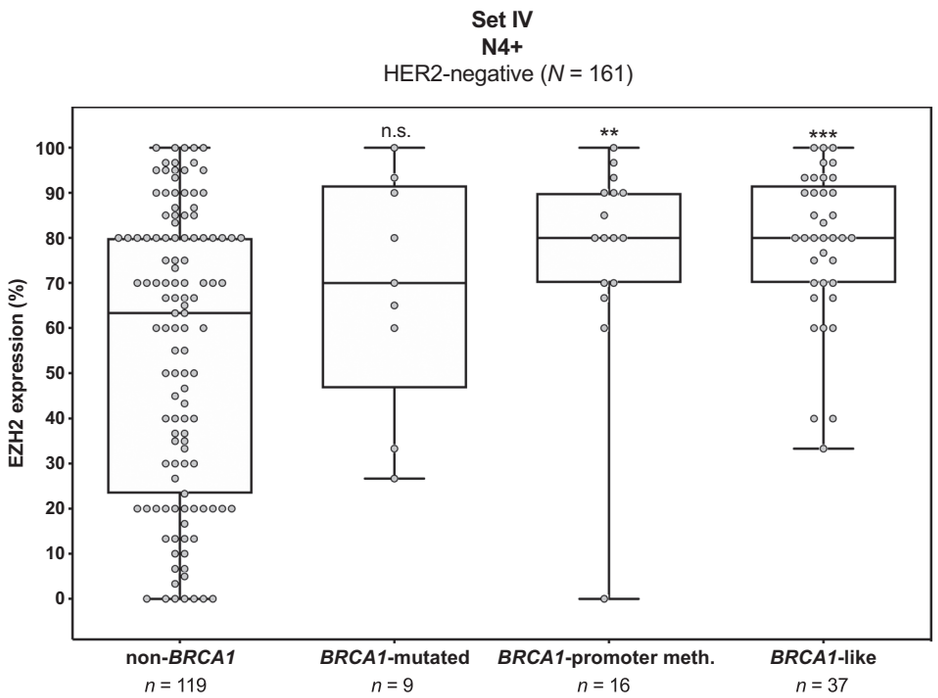

Figure 3.
Set IV (N4+, HER2-negative): EZH2 protein expression among breast cancers with *BRCA1* germline mutation, *BRCA1*-promoter methylation, and *BRCA1*-like DNA copy-number profile. EZH2 expression in non-*BRCA1*–associated breast tumors vs. *BRCA1* mutation, *BRCA1*-promoter methylation and *BRCA1*-like DNA copy-number profile. The non-*BRCA1*–like control group was cleaned for *BRCA1*-like, *BRCA1* mutation and promoter methylation cases ($n = 119$). For each comparison, an unpaired Mann–Whitney *U* test was used (**, $P < 0.01$; ***, $P < 0.001$).

curves for the non-*BRCA1*–like ER^+ and ER^- cases (Supplementary Fig. S2). The Cox proportional hazards model showed significant differences between treatments for non-*BRCA1*–like and EZH2-high and ER^+ cases favoring HD platinum-based chemotherapy (DFS: HR = 0.28, $P = 0.004$; OS: HR = 0.18, $P = 0.004$). We checked for potential confounding variables

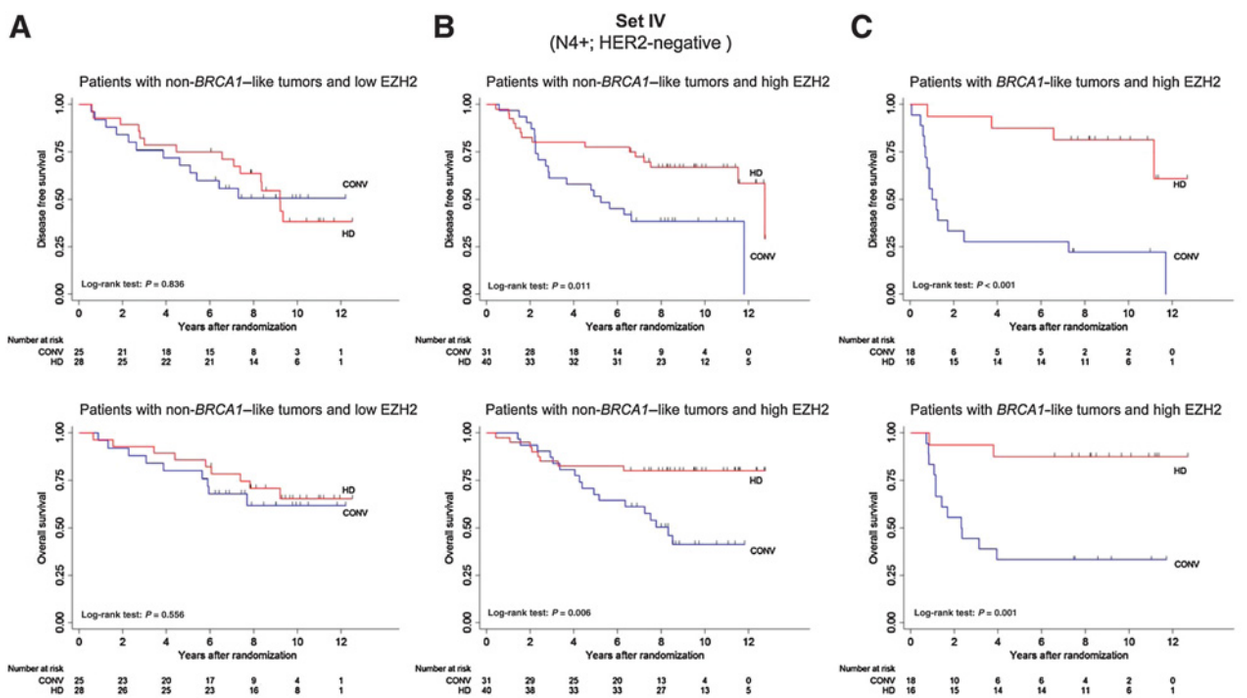

Figure 4.
Predictive value of EZH2 expression in patients with breast cancer treated with conventional (CONV) or high-dose (HD) platinum-based chemotherapy (set IV N4+, HER2-negative). **A,** Kaplan–Meier curves of DFS and OS of non-*BRCA1*–like patients with low EZH2 expression ($n = 53$). **B,** Kaplan–Meier curves of DFS and OS of non-*BRCA1*–like patients with high EZH2 ($n = 71$). **C,** Kaplan–Meier curves of DFS and OS of *BRCA1*-like patients with high EZH2 expression ($n = 34$). No Kaplan–Meier curves could be drawn for the three *BRCA1*-like patients with low EZH2 levels. Log-rank test was applied, and $P < 0.05$ was considered statistically significant.

Table 3. SET IV (N4+, HER2-negative) cohort: Cox proportional hazards models for DFS and OS

	DFS			OS		
Variable	**Events/patients (*n*)**	**HR (95% CI)**	***P***	**Event/patients (*n*)**	**HR (95% CI)**	***P***
BRCA1-like status						
Non-*BRCA1*–like EZH2 low.	27/53	0.27 (0.05–1.37)	0.116	18/53	0.27 (0.04–1.78)	0.174
Non-*BRCA1*–like EZH2 high	35/71	1.00		25/71	1.00	
BRCA1-like	22/37	8.76 (1.62–47.17)	0.012	17/37	2.93 (0.44–19.61)	0.267
BRCA1-like*time		0.98 (0.97–0.99)	0.029			
Non-BRCA1–like EZH2 low						
Conventional	12/25	1.00		9/25	1.00	
High dose	15/28	0.94 (0.44–2.03)	0.892	9/28	0.71 (0.28–1.80)	0.479
Non-*BRCA1*–like EZH2 high						
Conventional	20/31	1.00		17/31	1.00	
High dose	15/40	0.35 (0.17–0.71)	0.004	8/40	0.31 (0.13–0.72)	0.007
BRCA1-like						
Conventional	16/19	1.00		13/19		
High dose	6/18	0.18 (0.07–0.48)	0.001	4/18	0.21 (0.06–0.65)	0.007
pT stage						
1	18/32	1.00		14/32	1.001.1.00	
2	47/100	0.66 (0.37–1.17)	0.158	28/100	0.59 (0.30–1.16)	0.128
3	19/29	3.90 (1.54–9.88)	0.004	18/29	1.85 (0.85–4.00)	0.116
3*time		0.97 (0.95–0.99)	0.006			
LN						
4–9 LN+	48/102	1.00		31/102	1.00	
≥10 LN+	36/59	1.76 (1.11–2.80)	0.016	29/59	1.66 (0.98–2.83)	0.059

NOTE: Cox proportional hazards model of DFS and OS adjusted for significant clinical factors.
Interaction tests between the treatment regimen and the three groups: DFS, $P = 0.015$; OS, $P = 0.115$.
Interaction terms with time and *BRCA1*-like group and pT stage 3 are included in the DFS model due to nonproportionality of hazards, and time unit is 30 days. The estimated hazard ratio for *BRCA1*-like comparison with non-*BRCA1*–like EZH2 high is a function of follow-up time: HR(time) = exp{ln(8.76) + time*ln(0.98)}.
Abbreviations: LN, lymph node; T, tumor.

and prognostic factors in multivariate analysis and ER status and tumor grade were not identified as significant confounders (data not shown).

Antitumor activity of combination treatment with GSK126 and cisplatin in *Brca1*-deficient breast tumors

EZH2 has been found to be a potential target in cancer therapy. To determine whether EZH2 inhibition by GSK126 enhances sensitivity to platinum drugs in EZH2-overexpressing breast tumors, we made use of cell lines that were derived from *Brca1*-deficient (KB1P) and *Brca1*-proficient (KP) murine mammary tumors. As depicted in Fig. 1, tumors arising in *Brca1*-deficient mice show high levels of Ezh2. Next, we reintroduced the complete human *BRCA1* gene into the *Brca1*-deficient cell line (KB1P G3) to test if the therapy effect of GSK126 alone or in combination with cisplatin is a direct consequence of *Brca1* loss. Cell proliferation analysis, using live cell imaging, showed increased growth inhibition of the GSK126 and cisplatin combination, compared with single agents only in *Brca1*-deficient tumor cells (Fig. 5A). We conducted clonogenic assays to evaluate the long-term effect of GSK126 alone or in combination with cisplatin (Fig. 5B). Among all the tested cell lines, the *Brca1*-deficient breast tumor cells were the most sensitive to the combination treatment with GSK126 and cisplatin. For this reason, we performed an *in vivo* drug intervention study in our *Brca1*-deficient tumor model. GSK126 alone induced a minor survival benefit that was not significant, compared with vehicle control ($P = 0.076$; Fig. 5C). As expected, cisplatin treatment led to an increased survival, compared with GSK126 or vehicle exposure (Fig. 5C). However, a combination therapy consisting of GSK126 and cisplatin increased the OS significantly compared with cisplatin ($P = 0.0042$) or GSK126 ($P < 0.0001$) only. GSK126 alone and in combination with cisplatin was well tolerated with no relevant weight loss. As depicted in Fig. 5D, GSK126 significantly reduced $H3K27me^3$ levels, indicating a strong target inhibition of EZH2-associated enzymatic activity.

Discussion

In the present study, we demonstrated in various patient cohorts that EZH2 is overexpressed in BRCA1-associated breast tumors (Figs. 1–3). Moreover, EZH2 could be used to identify non-*BRCA1*–like patients who will benefit from intensified DSB-inducing platinum-based chemotherapy. We and others found high EZH2 expression in triple-negative and basal-like breast tumors (7, 9, 56). Here, we could show that high EZH2 expression could identify a subpopulation associated with a loss of *BRCA1* function. This subgroup is classified by *BRCA1* mutation status, *BRCA1*-promoter hypermethylation or a *BRCA1*-like DNA copy-number profile. EZH2 overexpression may also be an indirect surrogate marker for a high proliferation rate given its known role in regulation of the cell cycle and differentiation (4). In this study, we could demonstrate, especially in the large data sets of pure TNBC (sets II and III), that EZH2 is significantly higher expressed in BRCA1-associated tumors indicating that EZH2 is more than just a proliferation marker. Accordingly, EZH2 might be useful as a stratifying biomarker to identify breast tumors harboring a defect in HR-mediated DSB repair. Because BRCA2 is also involved in HR, we wanted to know if EZH2 has a similar role in *BRCA2*-deficient tumors. Interestingly, *BRCA1*-mutant tumors still show the highest EZH2 expression of all subgroups. This could be explained by novel studies that established a connection between BRCA1 and EZH2, demonstrating that BRCA1 is involved in DNA DSB repair and a negative modulator of EZH2 expression (34–36). Given the lack of high EZH2 expression levels in *BRCA2*-deficient tumors, on the other hand, HR defects may

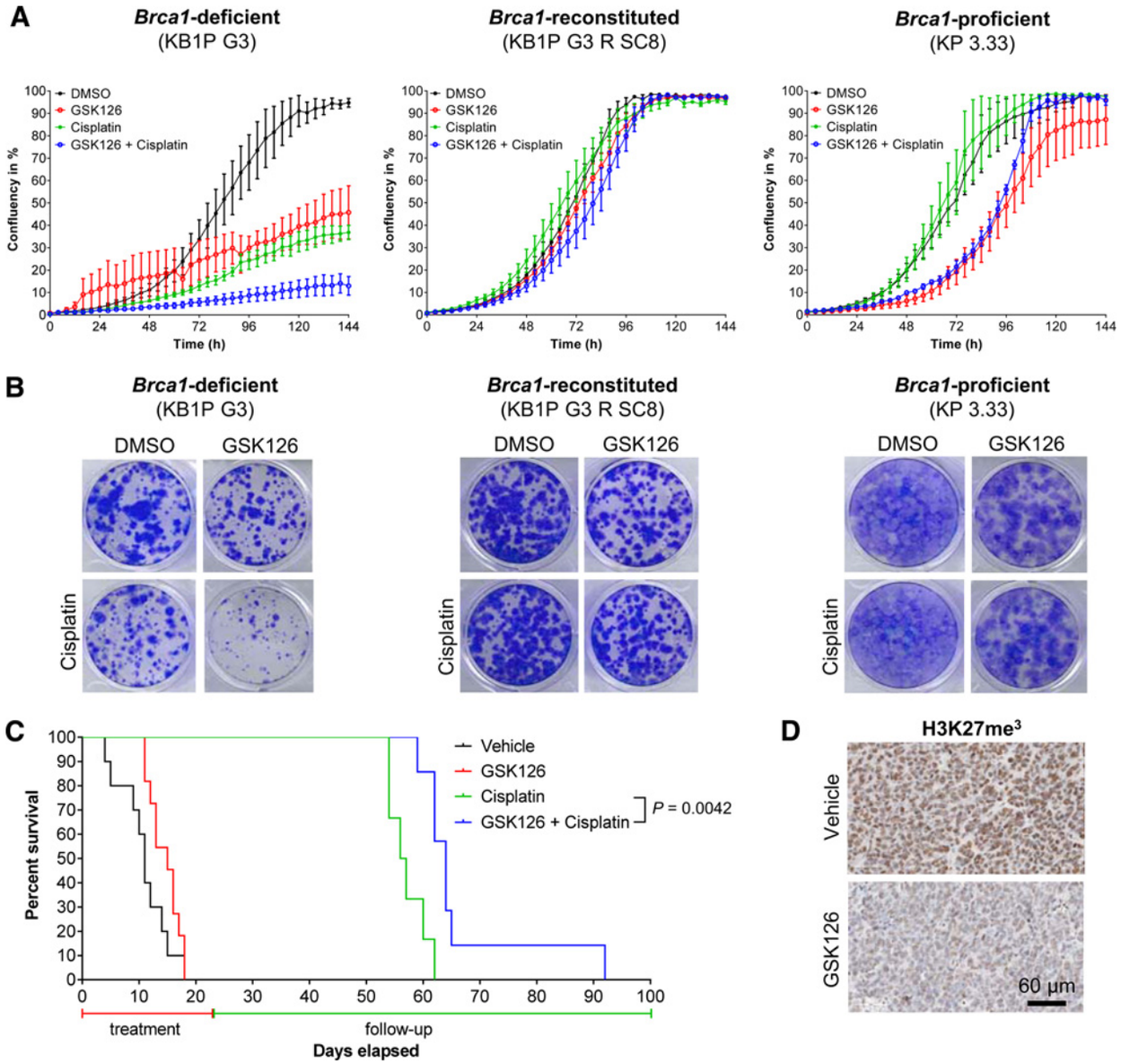

Figure 5.

Antitumor activity of combination treatment with GSK126 and cisplatin in *Brca1*-deficient breast tumors. **A,** Cell proliferation analysis by live cell imaging shows potent growth inhibition of GSK126 (8 µmol/L), cisplatin (0.5 µmol/L), and the combination in *Brca1*-deficient tumor cells (KB1P G3) compared with *Brca1*-reconstituted tumor cells (KB1P G3 SC8) and *Brca1*-proficient tumor cells (KP 3.33). The experiment shows three technical replicates. **B,** Effects of GSK126 (8 µmol/L), cisplatin (0.125 µmol/L), or the combination treatment on cell colony formation of *Brca1*-deficient tumor cells (KB1P G3), *Brca1*-reconstituted tumor cells (KB1P G3 SC8) and *Brca1*-proficient tumor cells (KP 3.33) after incubation for 8 days. DMSO was used as untreated control. **C,** *In vivo* activity of GSK126 and cisplatin: following tumor outgrowth to approximately 200 mm^3, mice were treated with either vehicle (Captisol; $n = 10$), GSK126 150 mg/kg daily ($n = 11$), cisplatin 3 mg/kg on days 1 and 14 ($n = 6$), or the combination of both ($n = 7$) for 28 days. OS was defined as the time needed for the tumors to reach a volume of 1,500 mm^3. Survival curves were generated with the Kaplan–Meier approach and compared with the log-rank test as indicated. **D,** Target inhibition of GSK126: representative staining of $H3K27me^3$ expression by IHC in vehicle versus GSK126 150 mg/kg treated KB1P-derived tumors. Scale bar, 60 µm.

not underlie changes in EZH2 expression. The mechanism that links *BRCA1* deficiency, but not *BRCA2* deficiency, to high EZH2 levels should be explored in future studies. Therefore, our data suggest that loss of *BRCA1* function could be sufficient but not necessary for EZH2 overexpression.

EZH2 has previously been identified as a prognostic factor in several types of cancers. In breast cancer, EZH2 overexpression was associated with early recurrence and shorter survival time (4, 9, 10, 57). High EZH2 expression in hormone receptor–positive, early breast cancer has been associated with unfavorable outcome after first-line tamoxifen therapy for metastatic disease (58). In the current study, we observed a greater benefit from HD platinum-based chemotherapy in *BRCA1*-like and non-*BRCA1*–like patients with high levels of EZH2

(Fig. 4; Table 3). Here, we present evidence that EZH2 could be used as a predictive marker for patients with breast cancer treated with intensified DSB-inducing platinum-based chemotherapy. An explanation could be that EZH2 might identify tumors with BRCAness features that are generally more sensitive to DNA crosslinking agents, such as cisplatin (15). This hypothesis is supported by a recent study showing that EZH2 expression predicts outcome in patients with *BRCA2*-mutant ovarian tumors by regulating genomic stability at stalled replication forks (59). However, EZH2-overexpressing non-*BRCA1*–like tumors also benefit from HD platinum-based chemotherapy. This suggests that EZH2 is more likely a predictive marker for intensified DSB-inducing platinum-based chemotherapy benefit independent of *BRCA1*-like status.

Despite the fact that HD platinum-based chemotherapy is not widely used anymore, these HD alkylating regimens undergo a revival after several studies have shown benefit for *BRCA1*-like breast cancer (30, 31) and new clinical trials are under way (http://clinicaltrials.gov: NCT01898117, NCT01057069, and NCT01646034).

Blocking EZH2 enzymatic activity might be a valid strategy for the treatment of EZH2-overexpressing tumors. For this reason, EZH2 is emerging as a promising target for BRCA1-associated tumors. We could show previously that *Brca1*-deficient tumor cells are sensitive to the EZH2 inhibitor DZNep and dependent on EZH2 function (38). Recently, novel selective Inhibitors of EZH2-associated H3K27me^3 methyltransferase, including GSK126 and ZLD1039, using concentrations that can be achieved in humans, have shown potent antitumor effects in preclinical breast cancer models (40, 60). In addition, phase I studies with EZH2 inhibitors have shown a favorable safety profile and antitumor activity in patients with relapsed B-cell non-Hodgkin lymphoma and solid tumors (61). However, GSK126 alone did not have a substantial antitumor effect in our *Brca1*-deficient mouse model. Because EZH2 acts as a transcriptional repressor, inhibition of its trimethylase activity could restore gene expression of cell-cycle inhibitors, tumor suppressors, and DNA-damage response mechanisms, making these tumors more vulnerable to additional DNA damage rather than inducing direct cell death. In the present study, we demonstrated that pharmacologic inhibition of EZH2 by using GSK126 potentiates the effect of cisplatin in *Brca1*-deficient breast tumors. In line with our finding, Riquelme and colleagues found evidence that EZH2 depletion sensitizes lung adenocarcinoma cells to platinum-based therapy (62). Interestingly, we did not observe this effect in *Brca1*-reconstituted or *Brca1*-proficient tumor cells. This indicates that sensitivity of *Brca1*-deficient cells to the combination therapy is mainly due to a loss of BRCA1 function.

This study has some limitations because the drug combination of the clinical trial (set IV) made it impossible to dissect whether the survival benefit stems from a particular drug or dose. Further research should investigate whether patients with high EZH2 benefit also from less toxic treatments with DNA-DSB–inducing agents, such as bifunctional alkylators or platinum drugs. Because there is no gold standard for scoring EZH2, we counted the proportion of cells stained for EZH2 and based expression categories on distribution plots and number of cases when determining the final cutoff to quantify tumors as EZH2 high (>50%). Other studies on EZH2 used different cutoffs (6, 57) or used a score of quantity and intensity (4). However, analysis by Reijm and colleagues concluded for EZH2 IHC that quantity and not intensity is associated with survival (58). We observed a stronger correlation of EZH2 overexpression and *BRCA1*-associated tumors in our RNA expression data sets compared with IHC stainings. This could be due to a bigger sample size especially for the *BRCA1*-mutant tumors or higher accuracy of RNA analysis. *EZH2* gene-expression profiling can probably better identify the subpopulation associated with a BRCA1ness phenotype. However, no RNA expression data for set I and IV was available for additional correlation studies. Another restriction of this study was that *BRCA1*-like status in set IV was not analyzed for all patients. Therefore, only 161 patients could be included in this group. Additionally, in some tumors, information on *BRCA1* mutation and *BRCA1*-promoter methylation status was missing. In mice, loss of Ezh2 enzymatic activity in *Brca1*-deficient breast tumors did not result in a significantly improved survival, pointing out differences between *in vitro* and *in vivo* experimental settings. Presumably, targeting the EZH2 protein directly might be more effective in EZH2-overexpessing tumors, than reducing its enzymatic activity. Further studies are needed to evaluate the underlying mechanism of combination treatment strategies with platinum drugs and EZH2 inhibitors in *BRCA1*-deficient breast cancers. Nevertheless, the combination therapy showed antitumor activity in all experiments, suggesting that EZH2 inhibitors may be combined with DSB-inducing compounds in EZH2-overexpressing tumors.

Conclusion

This study confirmed that EZH2 is significantly higher expressed in breast tumors with a *BRCA1* mutation, *BRCA1*-promoter methylation or *BRCA1*-like DNA copy-number profile indicating that EZH2 overexpression is associated with loss of *BRCA1* function. Furthermore, *BRCA1*-like and non-*BRCA1*–like patients with high EZH2 expression recur less often after treatment with HD platinum-based chemotherapy, compared with standard chemotherapy. We report that EZH2 inhibition might be useful to enhance the antitumor effect of platinum drugs in BRCA1-associated breast tumors. Potentially, EZH2 could be used as a predictive biomarker to categorize patients according to their likelihood to benefit from intensified DSB-inducing platinum-based chemotherapy independent of *BRCA1*-like status.

Disclosure of Potential Conflicts of Interest

P.C. Schouten is an employee of AstraZeneca. C. Caldas reports receiving commercial research grants from AstraZeneca, Servier, Genentech, and Roche, and is a consultant/advisory board member for AstraZeneca. K. Rhiem is a consultant/advisory board member for Tesaro and AstraZeneca. E. Hahnen is a consultant/advisory board member for AstraZeneca. H.C. Reinhardt reports receiving commercial research grants from Gilead Pharmaceuticals, speakers bureau honoraria from AbbVie and AstraZeneca, and is a consultant/advisory board member for AbbVie. R. Büttner is an employee of and holds ownership interest (including patents) in Targos Molecular Pathology Inc, reports receiving speakers bureau honoraria from AbbVie, MSD, Bristol-Myers Squibb, Novartis, and AstraZeneca, and is a consultant/advisory board member for AbbVie, MSD, Bristol-Myers Squibb, and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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