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PRECLINICAL STUDY

4-Hydroxytamoxifen enhances sensitivity of estrogen receptor α-positive breast cancer to docetaxel in an estrogen and *ZNF423* SNP-dependent fashion

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Abstract

Purpose In early stage, ERα-positive breast cancer, concurrent use of endocrine therapy and chemotherapy has not been shown to be superior to sequential use. We hypothesized that genetic biomarkers can aid in selecting patients who would benefit from chemo-endocrine therapy. Our previous studies revealed that ZNF423 is a transcription factor for BRCA1 and an intronic single nucleotide polymorphism (SNP) in *ZNF423*, rs9940645, determines tamoxifen response. Here, we identified mitosis-related genes that are regulated by ZNF423 which led us to investigate taxane response in a rs9940645 SNP- and tamoxifen-dependent fashion.

Methods The Cancer Genome Atlas (TCGA) breast cancer dataset was used to identify genes correlated with ZNF423. Quantitative reverse transcription PCR, chromatin immunoprecipitation, and luciferase reporter assays were used to validate the gene regulation. We used CRISPR/Cas9 to engineer paired ZR-75-1 cells which differ only in *ZNF423* rs9940645 SNP genotype to test SNP-dependent phenotypes including cell cycle and cell viability. We validated our findings in an additional two breast cancer cell lines, Hs578T-ERα and HCC1500.

Results Mitosis-related genes *VRK1* and *PBK*, which encode histone H3 kinases, were experimentally validated to be regulated by ZNF423. ZNF423 knockdown decreased VRK1 and PBK expression and activity. Additionally, ZNF423 knockdown enhanced docetaxel-induced G2/M arrest and cytotoxicity through VRK1 or PBK regulation. Lastly, cells carrying the rs9940645 variant genotype had increased G2/M arrest and decreased cell viability when treated with docetaxel in combination with estradiol and 4-OH-TAM.

Conclusions We identified ZNF423 regulated genes involved in the G2/M phase of the cell cycle. 4-OH-TAM sensitized ERα-positive breast cancer cells to docetaxel in a *ZNF423* SNP-dependent manner. Our findings suggest that patients with rs9940645 variant genotype may benefit from concurrent tamoxifen and docetaxel. This would impact a substantial proportion of patients because this SNP has a minor allele frequency of 0.47.

Keywords Breast cancer · ZNF423 · rs9940645 · Chemo-endocrine therapy · Single nucleotide polymorphism · Precision medicine

Gen Wang, Sisi Qin and Jacqueline Zayas have equally contributed to this work.

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Abbreviations

4-OH-TAM	4-Hydroxytamoxifene
CALML3	Calmodulin-like protein 3
CRISPR	Clustered, regularly interspaced short palindromic repeats
DCT	Docetaxel
E2	17β-Estradiol
ER	Estrogen receptor
ERE	Estrogen response element
GO	Gene ontology
GWAS	Genome-wide association study
LCL	Lymphoblastoid cell line

NSABP	National surgical adjuvant breast and bowel project
OE	Overexpress
PARP	Poly(ADP-ribose) polymerase
PBK	PDZ binding kinas
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
SERM	Selective estrogen receptor modulator
SNP	Single nucleotide polymorphism
TCGA	The Cancer Genome Atlas
WT	Wild type
VRK1	Vaccinia-related kinase 1
ZNF423	Zinc finger protein 423

Background

Breast cancer is the most common form of cancer in women both in the USA [1] and worldwide [2]. Endocrine therapy is the most important treatment modality in the majority (about 70%) of women who have estrogen receptor α (ER α)-positive breast cancer. In many women with early stage breast cancer, chemotherapy is considered appropriate based on the risk of recurrence. However, the accepted approach is to utilize the chemotherapy and endocrine therapy sequentially. This approach is based on prospective clinical trials that failed to show an advantage for combining the two modalities over their sequential use [3, 4]. It is of note that both of these trials utilized tamoxifen as the endocrine agent but neither study utilized regimens including taxanes such as paclitaxel and docetaxel, as is commonly used currently. Related to this is the report that ERα mediates paclitaxel resistance in breast cancer cells through inhibition of apoptotic cell death [5]. These findings raise the possibility that concomitant use of endocrine therapy and chemotherapy may be of value in a subset of patients.

In our previous study utilizing patients from the National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1 and P-2 trials, we identified single nucleotide polymorphisms (SNPs) in *ZNF423*, including rs9940645, that were associated with decreased risk of breast cancer occurrence during selective estrogen receptor modulation (SERM) treatment. We also found that *ZNF423* gene expression, and downstream *BRCA1*, is modulated by 4-hydroxytamoxifen (4-OH-TAM) in an rs9940645 SNP-dependent manner. Specifically, after adding 4-OH-TAM in the presence of 17β-estradiol (E2), *ZNF423* and *BRCA1* expression was increased in *ZNF423* rs9940645 variant, but not wild-type, cells. Because of this striking effect on BRCA1, patients with *ZNF423* rs9940645 wild-type genotype could potentially be selected for combination treatment with tamoxifen and olaparib, a PARP inhibitor which has shown significant therapeutic benefit in BRCA1/2 deficient patients on [6, 7]. Importantly, the minor allele frequency for this *ZNF423* SNP (rs9940645) is reported to be 0.47 in Ensembl based on all individuals in Phase 3 of the 1000 Genomes Project [8].

In the present study, we used gene correlation analysis using The Cancer Genome Atlas (TCGA) breast cancer dataset to identify candidate genes correlated with ZNF423 and BRCA1 expression. Interestingly, most of the genes correlated with ZNF423 and BRCA1 expression in silico were related to mitosis such as VRK1 (vaccinia-related kinase 1) and PBK (PDZ binding kinase) which encode histone H3 kinases. Based on our previous studies, we hypothesized that 4-OH-TAM might regulate these mitosis-related genes in a *ZNF423* rs9940645 SNP genotype-dependent manner and affect drug response to mitosis-targeting agents such as docetaxel. We found that 4-OH-TAM sensitizes cells to docetaxel treatment in *ZNF423* rs9940645 variant, but not wild-type, cells. This cytotoxicity phenotype is dependent on VRK1 and PBK. Our data suggest that combined chemo-endocrine therapy may provide additional benefit to breast cancer patients with *ZNF423* rs9940645 variant genotype.

Materials and methods

Cell culture

The breast cancer cell lines ZR-75-1 and HCC1500 were purchased from American Type Culture Collection (ATCC). ZR-75-1 cells are homozygous variant for rs9940645, referred to as ZR-75-1 variant. ZR-75-1 cells that are homozygous wild-type for rs9940645, referred to as ZR-75-1 WT, were generated by CRISPR/Cas9 as previously described [7]. Hs578T breast cell line with ERα overexpression, referred to as Hs578T-ERα, was a generous gift from Thomas Spelsberg, Ph.D. (Mayo Clinic, Rochester, MN, USA). ZR-75-1 WT, ZR-75-1 variant, and HCC1500 were maintained in RPMI 1640 medium (Gibco) with 10% fetal bovine serum (FBS) (Atlanta Biologicals), while Hs578T-ERα was maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco) with 10% FBS. Lymphoblastoid cell lines (Coriell Cell Repository) with known genotypes for the *ZNF423* SNP were transfected with ERα and maintained as previously described [6, 7]. Only cell lines that are homozygous for the *ZNF423* SNP were chosen.

Drug treatment

17β estradiol (E2), 4-hydroxytamoxifen (4-OH-TAM), and docetaxel (DCT) were purchased from Sigma-Aldrich. Docetaxel was diluted in dimethyl sulfoxide (DMSO), and E2 and 4-OH-TAM were diluted in ethanol. Prior to all drug treatments, cells were cultured in phenol-free media supplemented with 5% charcoal-stripped serum (CSS) (Thermo

Fisher Scientific) for 24 h then in serum-free medium for an additional 24 h to remove all endogenous hormones. Then, cells were cultured in 1% CSS medium with 1 nM E2 for 24 h to stimulate ERα. Afterward, 1 μM 4-OH-TAM was added to the medium. Docetaxel or DMSO control was added 10 min after 4-OH-TAM.

Transient siRNA knockdown and gene overexpression

Control siRNA (D-001206-13), ZNF423 siRNA (D-012907-01, D-012907-02, D-012907-03, D-012907-04), VRK1 siRNA (M-004683-02), PBK siRNA (M-005390-00) were obtained from Dharmacon. Overexpression plasmids pCMV6-XL4 empty vector (EV) and pCMV6-XL4-ZNF423 were generated as previously as described [9]. Cells were reverse transfected in complete media using Lipofectamine RNAiMAX and Lipofectamine 2000 (Life Technologies) for knockdown and overexpression, respectively, as per manufacturer's recommendations. For the ZNF423 knockdown screen, Hs578T overexpressing ERα cells (Hs578T-ERα cells) were used [6].

Real-time quantitative reverse transcription-PCR

Total RNA was isolated using Qiagen RNeasy kit (QIAGEN). One-step qRT-PCR was performed using 50 ng of RNA template and Power SYBR® Green RNA-to-CT™ kit (Life Technologies) and Step One Plus Real-Time PCR (Applied Biosystems). Primers are provided in Online Resource 1. All assays were performed three times in triplicate.

Luciferase reporter assay

The VRK1 promoter (1018 bp) and PBK promoter (1301 bp) were amplified with Hs578T genomic DNA using primers listed in Online Resource 1. The PCR products were subsequently cloned into pGL4.10 [luc2] plasmid using KpnI and XhoI restriction enzyme sites for VRK1 and XhoI and HindIII restriction enzyme sites for PBK. Plasmid sequences were verified by Sanger sequencing.

24 h after knockdown or overexpression of ZNF423, Hs578T-ERα cells were co-transfected with Firefly and pGL4.74[hRluc/TK] Renilla plasmids at a 10:1 ratio using Lipofectamine 2000 in the presence of 0.1 nM E2 treatment. Luciferase activity was measured with a dual-luciferase reporter assay system (Promega) 48 h after transfection. Firefly luciferase activity was then normalized to the Renilla activity. Primers are provided in Online Resource 1. Each assay was performed in three independent experiments in triplicate.

Chromatin immunoprecipitation assays

ZNF423 protein and DNA complexes were immunoprecipitated and purified using the EpiTect ChIP OneDay kit (QIAGEN). Normal IgG served as negative control. PCR was performed as previously described [6]. PCR products were then loaded on 2% agarose gels for electrophoresed for 2 h in 1 × TAE buffer. PCR primers are provided in Online Resource 1.

Flow cytometry assays

For cell cycle experiments, cells were harvested, fixed in 70% ethanol at 4 °C and stained with propidium iodide (PI) for 30 min. All flow cytometry assays were measured using Attune NxT Flow Cytometer (Thermo Fisher Scientific), and raw data were analyzed with ModFit LT 5.0.

Cell viability assays

For docetaxel assays, ZNF423, VRK1, or PBK were individually knocked down and treated with indicated doses of docetaxel in RPMI 1640 medium with 10% FBS. For rescue experiments, cells were reverse transfected with ZNF423 siRNA or control siRNA and allowed to adhere to the plate. Twelve hours later, ZNF423 knockdown cells were transiently overexpressed with the VRK1 or PBK plasmids (Origene, Rockville, MD). All cells were treated with the indicated docetaxel dose after an additional 12 h. For combination treatment assays, after cells were charcoal stripped for 24 h and serum starved for another 24 h, cells were trypsinized and neutralized with 10% CSS medium. Then, cells were centrifuged and 10,000 cells were seeded into the 96-well plates using 1% CSS medium containing 1 nM E2 for 24 h. Cells were then treated with 1 μM 4-OH-TAM 10 min prior to the indicated docetaxel doses. Cell viability was assessed 72 h after docetaxel treatment using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assays (Promega) as previously described [10]. All assays were performed in three independent experiments in triplicate.

Western blot

Protein samples were harvested, electrophoresed, and transferred to PVDF membranes using standard wet transfer techniques. Membranes were incubated with either anti-ZNF423 (ABN410, Sigma; ab169096, Abcam), VRK1 (sc-271061, Santa Cruz Biotechnology), PBK (ab75987, Abcam), or β-actin (Sigma) overnight at 4 °C. After washing and incubation of secondary antibody at room temperature for 90 min, membranes were washed and incubated with ECL. Bands were visualized using ChemiDoc Imaging Systems (BioRad).

Statistical analysis

Experimental data were analyzed with GraphPad Prism Software, and statistical methods used are indicated in the figure legends.

Results

Candidate genes selection: identifying VRK1 and PBK

In order to identify ZNF423-regulated genes which might be modulated by 4-OH-TAM in an rs9940645 SNP-dependent manner, we employed the strategy depicted in Fig. 1. First, we performed a BLAST search for the putative ZNF423 binding motif 5′-CCGCCC-3′ [10] in the human genome. We found approximately 16,000 genes which contained one or more ZNF423 binding sequences within the promoter region. Then, we performed gene correlation analysis using The Cancer Genome Atlas (TCGA) ER+ breast cancer dataset ($n = 430$) to identify genes highly correlated with *ZNF423*. We also correlated genes with *BRCA1*, an estrogen-induced and direct target of ZNF423, to further narrow down the gene list. We found 368 genes were correlated with *ZNF423* as well as *BRCA1* expression with a p value $< 10^{-10}$. Then, to determine which of these 368 genes might be transcriptionally regulated by ZNF423, we performed a ZNF423 siRNA knockdown screen in Hs578T-ERα cells and measured change in gene expression relative to siRNA control by real-time quantitative reverse transcription-PCR (qRT-PCR). There were 109 genes with at least a two-fold change in gene expression upon ZNF423 knockdown; 99 downregulated and 10 upregulated genes (Online Resource 2). We were also interested in genes that were modulated by 4-OH-TAM in a *ZNF423* rs9940645 SNP-dependent manner, similar to our previous *BRCA1* discovery [6]. To evaluate this possibility, we used lymphoblastoid cell lines (LCLs) overexpressing ERα with known rs9940645 genotypes with treatments that included control, E2, or E2 plus 4-OH-TAM. We used at least seven different LCLs per genotype for each treatment. We then measured mRNA expression of the 368 genes by qRT-PCR. There were 119 genes that displayed SNP-dependent expression pattern changes in the E2 plus 4-OH-TAM treatment compared to E2 alone (Online Resource 3). Eighty-two of the 119 genes had decreased expression in wild-type cells treated with 4-OH-TAM and increased expression in variant cells treated with 4-OH-TAM, which is the same direction of change that we observed previously with ZNF423 and BRCA1 [6].

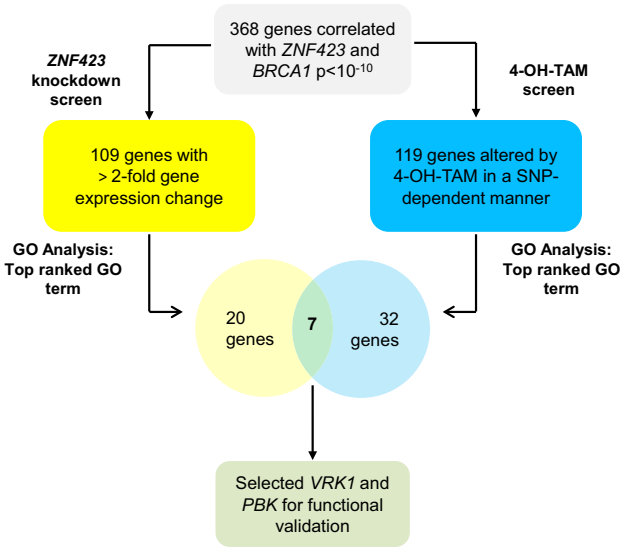

Fig. 1 Candidate gene selection strategy. The expression of 368 genes was found to be correlated with *ZNF423* and *BRCA1* expression with a p value $< 10^{-10}$ in The Cancer Genome Atlas ER+ breast cancer dataset ($n = 430$). In order to narrow down the candidate gene list, two screens were performed. (Left) ZNF423 was knocked down in Hs578T-ERα cells, and qRT-PCR was performed 48 h later. Expression of the 368 genes was determined relative to β-actin and was normalized to control siRNA. There were 99 genes downregulated and 10 genes upregulated by more than two-fold after ZNF423 knockdown. (Right) Lymphoblastoid cell lines with known *ZNF423* SNP genotypes were treated with control, E2, or E2 plus 4-OH-TAM, and gene expression was determined relative to β-actin and normalized to control treatment. Gene expression change was compared in E2 plus 4-OH-TAM treatment relative to E2 alone treatment. 119 genes had a SNP and drug-dependent changes in gene expression. Gene Ontology (GO) analyses were performed to narrow down these two gene lists. The top ranked GO term from each gene list was compared, and VRK1 and PBK were selected for functional validation

To narrow down the candidate gene list further, we performed Gene Ontology (GO) analyses. The gene lists from the two screens, including genes that were significantly changed upon ZNF423 knockdown and genes that showed an rs9940645 SNP and 4-OH-TAM-dependent expression profile, were separately inputted into the ClueGO plugin (version 2.3.5) of the Cytoscape software (3.2.0) using default parameters and a kappa score ≥ 0.4. GO terms were visualized as nodes and grouped based on similar function (Fig. 2a, Online Resource 4). Interestingly, many of the grouped GO terms were related to mitosis (Fig. 2b, c). GO terms were ranked by p value (Online Resource 5–6), and the genes within the most significant GO term from each screen list were compared. There were seven overlapping genes: *NCAPH, NEK2, NUSAP1, OIP5, PBK, STIL*, and *VRK1* (Online Resource 7). We hypothesized that these 7 genes were transcriptionally regulated by ZNF423 and were modulated by 4-OH-TAM in a *ZNF423* SNP-dependent manner.

VRK1 and PBK were chosen for futher functional validation based on literature review. VRK1 is ubiquitously expressed in human tissues and is increased in actively

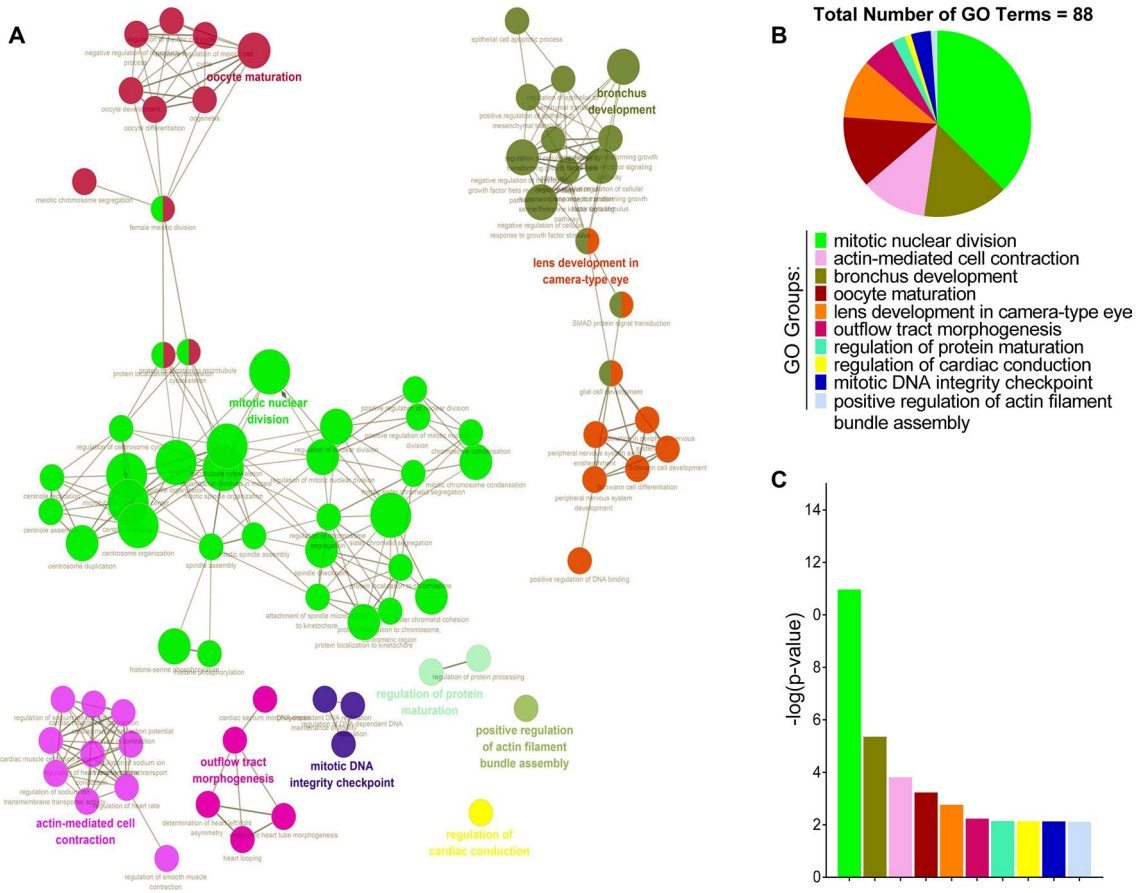

Fig. 2 Gene ontology analysis of top genes from the ZNF423 knockdown screen. GO Analysis was performed by inputting genes identified from the ZNF423 knockdown or 4-OH-TAM screen into the Cluego plugin of the Cytoscape software. Depicted is the GO analysis for the ZNF423 knockdown screen. **a** GO terms are visualized as nodes and grouped into a network based on function (kappa score level ≥ 0.4). The size of each node represents the enrichment significance. **b, c** Each functional group (GO group) is labeled with a unique color and named using the most significant GO term within the group. Some GO terms were shared by different GO groups. **b** The pie chart illustrates the proportion by which each GO group was represented among the GO terms. Mitotic nuclear division was the most abundant GO group. **c** The significance of GO groups is shown ranked by Bonferroni-corrected p value

dividing cells. It controls cell cycle progression [11] and is a predictive marker of drug response in multiple tumor types, such as rectal adenocarcinoma [12], hepatocellular carcinoma [13, 14], and ER positive breast cancer [15]. Moreover, VRK1 was identified as a marker for poor prognosis in human breast cancer, particularly in the ER positive subgroup [16, 17]. In addition, increased PBK has been implicated in tumorigenesis [18–20]. Its disruption may cause tumor-specific radio-sensitization in several cancer types [21]. Importantly, VRK1 and PBK have both been identified as histone H3 kinases [22, 23]. Because VRK1 and PBK are involved in the same gene ontology of mitosis regulation and because there are agents that target mitosis, we chose these two genes for subsequent functional validation.

Histone H3 Kinases VRK1 and PBK are downstream of ZNF423

First, we evaluated *VRK1* and *PBK* mRNA expression after ZNF423 knockdown or overexpression. We found that ZNF423 knockdown resulted in significantly decreased *VRK1* and *PBK* mRNA expression in cells that were homozygous wild type (CRISPR-Cas9 engineered ZR-75-1 and Hs578T-ERα) and homozygous variant (ZR-75-1 and HCC1500) for the *ZNF423* SNP (Fig. 3a, Online Resource 8). Similarly, ZNF423 overexpression significantly increased *VRK1* and *PBK* mRNA expression (Online Resource 8). Luciferase reporter assays were then conducted in ERα stably transfected Hs578T cells (Hs578T-ERα) to measure the transcriptional activity of *VRK1* and *PBK* upon ZNF423

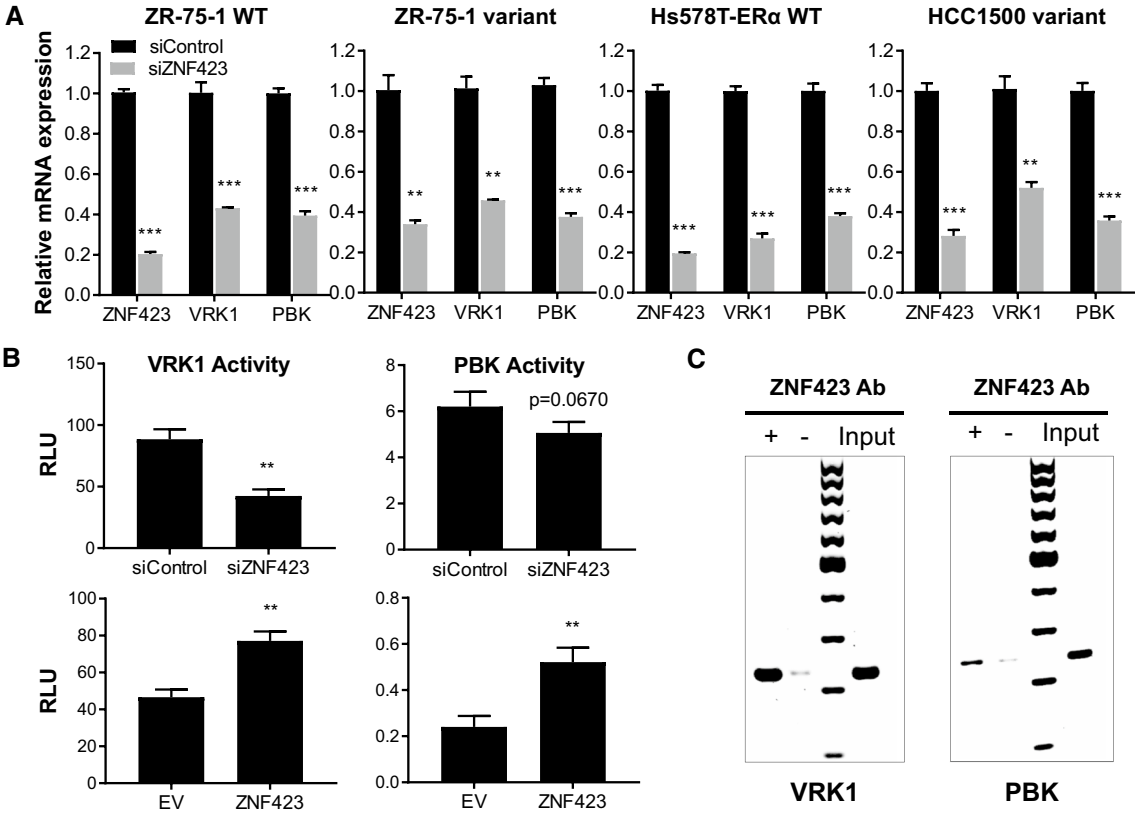

Fig. 3 *VRK1* and *PBK* are ZNF423-regulated genes. **a** ZNF423 was knocked down in the indicated cell lines, and *VRK1* and *PBK* mRNA expression was determined. Gene expression was normalized to actin and is shown relative to siControl transfected cells. **b** The VRK1 and PBK transcriptional activities were measured by Dual-Luciferase Reporter Assay under ZNF423 knockdown (top) or overexpression (bottom) conditions in ERα stably transfected Hs578T (Hs578T-ERα) cell line which is homozygous wild-type WT for the *ZNF423* SNP. The firefly luciferase activity was normalized to the corresponding Renilla activity and is shown as relative luciferase units (RLU). **c** Chromatin immunoprecipitation assay (ChIP) was performed in Hs578T-ERα and confirmed the binding of ZNF423 protein to the promoter regions of *VRK1* (left) and *PBK* (right). Experiments were repeated in at least three independent experiments. Error bars represent SEM. Student *t* test was performed relative to control: $*p<0.05$, $**p<0.01$, and $***p<0.001$

modulation. The luciferase activity of *VRK1* and *PBK* decreased when ZNF423 was knocked down and increased when ZNF423 was overexpressed (Fig. 3b). In order to assess whether ZNF423 can directly bind to *VRK1* and *PBK* promoters, chromatin immunoprecipitation (ChIP) assays were performed in Hs578T-ERα using an anti-ZNF423 antibody. Our results showed that ZNF423 protein binds to the promoter region of both genes (Fig. 3c). Together, these data suggest that ZNF423 acts as a transcription factor by binding to the promoter region and regulating the transcription of histone H3 kinases VRK1 and PBK.

VRK1, PBK, and ZNF423 regulate docetaxel response

Given the known function of VRK1 and PBK in mitosis regulation, we investigated whether ZNF423 could also affect the cell cycle. To test this possibility, we used siRNA to knockdown ZNF423, VRK1, and PBK in ZR-75-1 WT and variant cells and assessed cell cycle phases by propidium iodide staining. We did not observe any significant differences in DMSO-treated cells (data not shown). However, in docetaxel treated cells, knockdown of ZNF423, VRK1, and PBK significantly increased the proportion of cells arrested in the G2/M phase of cell cycle compared to control in both WT and variant ZNF423 genotypes (Fig. 4b). Overexpression of VRK1 and PBK in siZNF423 knockdown cells rescued this phenotype (Fig. 4b). Knockdown and overexpression efficiency was assessed by qRT-PCR (Fig. 4c).

Because there was an increase in docetaxel-induced G2/M arrest upon knockdown of ZNF423 and VRK1 and PBK, we questioned whether cytotoxic response to docetaxel was also affected. Knockdown of ZNF423, VRK1, and PBK all resulted in decreased cell viability upon docetaxel treatment in all breast cancer cell lines regardless of *ZNF423* SNP genotype (Fig. 5a). Sensitivity to docetaxel was decreased in ZNF423 knockdown cells when VRK1 and PBK were overexpressed (Fig. 5b). Taken together, these results provide evidence that VRK1 and PBK are downstream of ZNF423

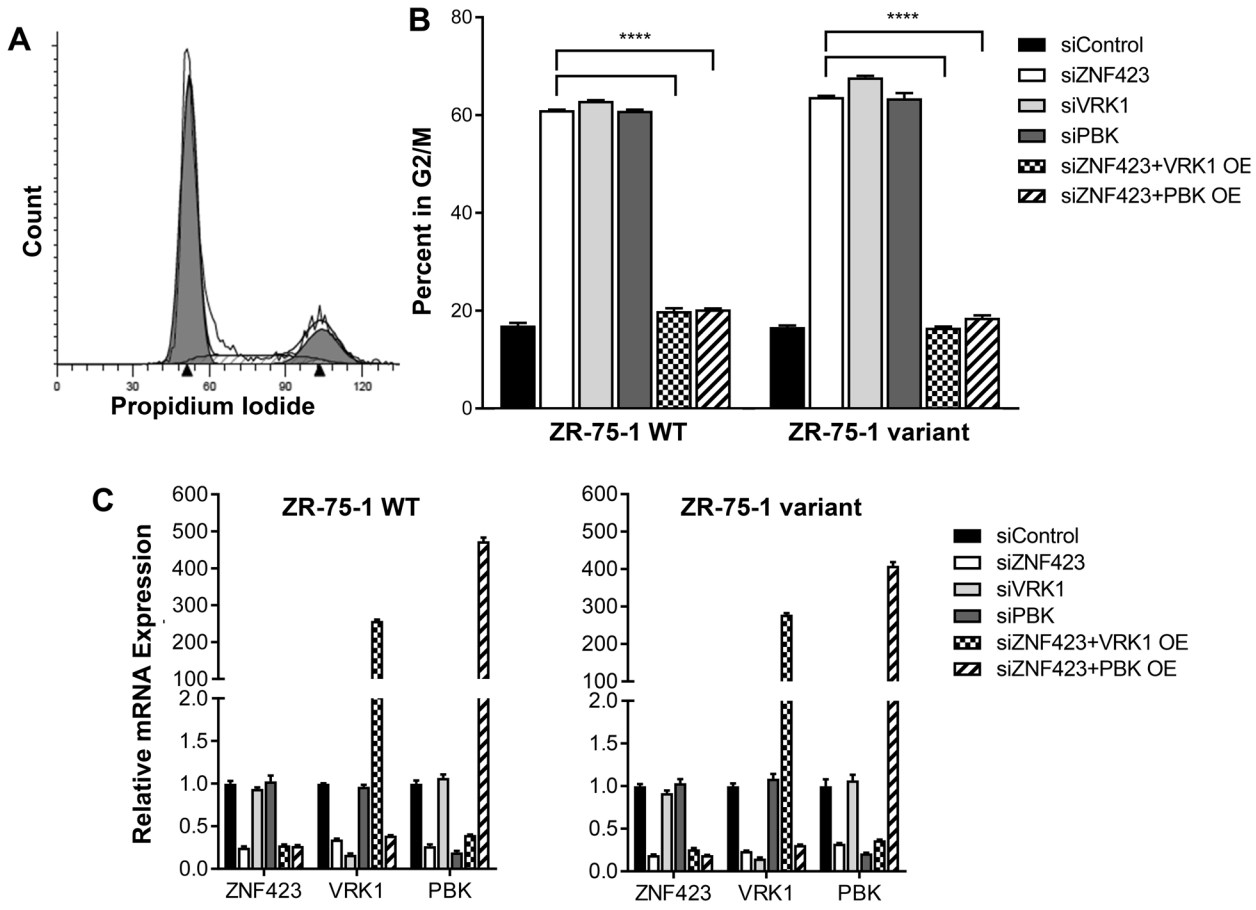

Fig. 4 Knockdown of ZNF423, VRK1, and PBK affect docetaxel-induced G2/M arrest. Cell cycle was assessed by propidium iodide staining followed by flow cytometry in CRISPR-Cas9 engineered ZR-75-1 WT and parental ZR-75-1 variant cell lines after the indicated knockdown and overexpression (OE) transfections under DMSO or docetaxel (DCT) treatment. **a** Representative peaks from an individual experiment are shown. **b** Quantification of cells in G2/M phase of the cell cycle using ModFit. **c** Knockdown and overexpression efficiency as measured by qRT-PCR normalized to actin and relative to siControl. Error bars represent SEM. Student *t* test was performed relative to siZNF423: ****$p < 0.0001$

and that these three genes regulate mitosis (Fig. 4) and subsequent chemotherapeutic response to docetaxel (Fig. 5).

4-OH-TAM sensitizes cells to docetaxel in *ZN423* rs9940645 variant genotype

Furthermore, we assessed cell cycle changes in a 4-OH-TAM and docetaxel combination treatment setting. In comparison with docetaxel and E2 alone, addition of 4-hydroxytamoxifen (4-OH-TAM) showed an increase in cells arrested in G2/M phase in cells harboring the variant *ZNF423* SNP (Fig. 6a, b). This effect was observed in paired ZR-75-1 cell lines which had been CRISPR-Cas9 engineered to differ only in the rs9940645 genotype.

We then tested whether there was a SNP-dependent cytotoxic effect after combination 4-OH-TAM and docetaxel treatment using a cell viability assay and western blot. ZR-75-1 variant cells were more resistant than ZR-75-1 WT cells when treated with docetaxel in the presence of E2 (Fig. 7a, open circles). However, this resistant phenotype in variant cells can be abolished with the addition of 4-OH-TAM. The same SNP-dependent phenotypes were observed in Hs578T-ERα WT and HCC1500 variant breast cancer cells. At the protein level, ZNF423 and its downstream targets VRK1 and PBK are decreased in the combination 4-OH-TAM and docetaxel setting only in variant cells (Fig. 7b). This is consistent with our previous findings that knockdown of ZNF423, VRK1, or PBK resulted in enhanced docetaxel sensitivity when treated in combination 4-OH-TAM (Fig. 5a).

Discussion

ERα-positive breast cancer is a heterogeneous disease. Using intrinsic subtype classification, ERα-positive tumors can be divided into Luminal A and Luminal B. Compared

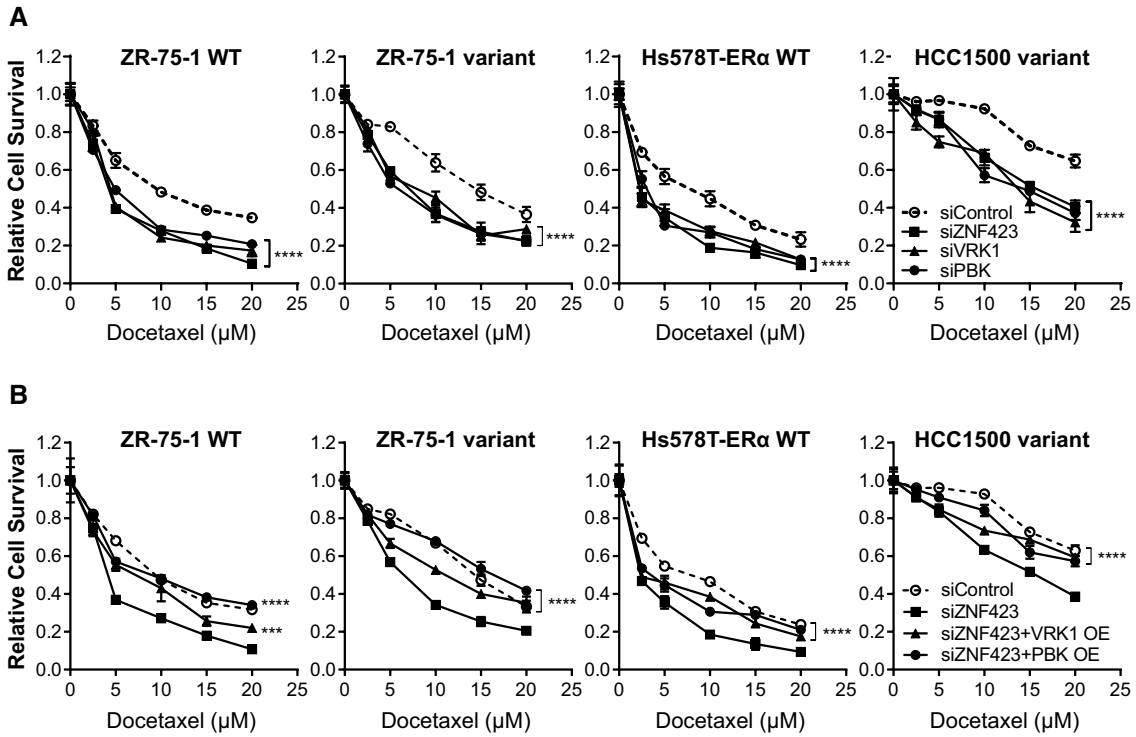

Fig. 5 Docetaxel sensitivity is dependent on VRK1 and PBK. **a** Knockdown of ZNF423, VRK1, and PBK was performed in the indicated cell lines which were treated with increasing doses of docetaxel (DCT) for 72 h prior to MTS cell viability assay. **b** ZNF423 knockdown cells were overexpressed (OE) with VRK1 or PBK plasmids and treated with the indicated docetaxel dose. Graphs were plotted, and area under the curve was calculated using GraphPad Prism 7. Data represented as mean ± SEM. For the knockdown experiments shown in (**a**), AUC in each experiment was compared to siControl (open circle) by one-way ANOVA. For the rescue experiments in (**b**), AUC for each overexpression condition (closed circle and closed triangle) was compared to siZNF423 (closed square) by one-way ANOVA. ***$p < 0.001$, ****$p < 0.0001$

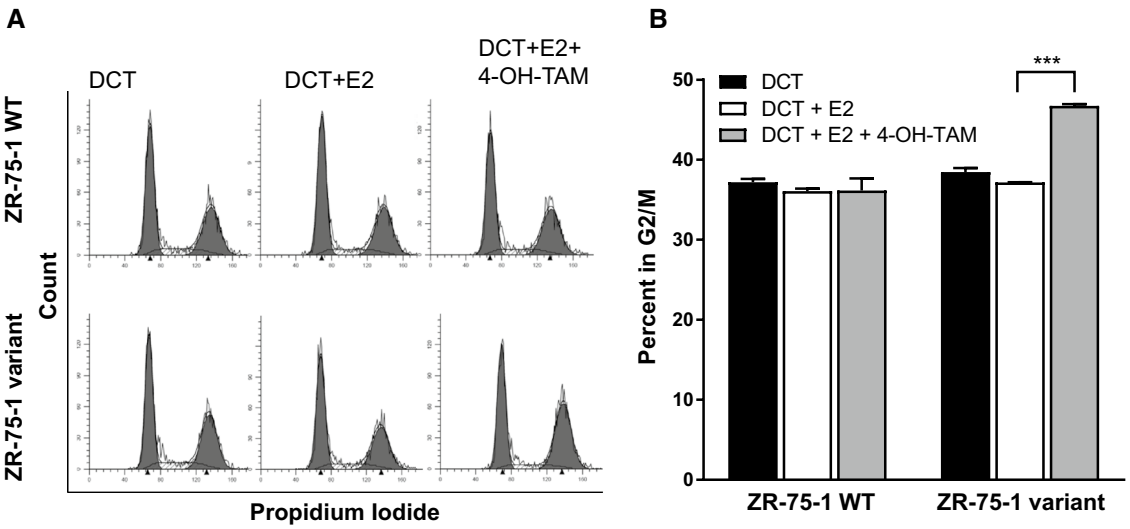

Fig. 6 Combination of 4-OH-TAM and docetaxel treatment increases G2/M arrest in *ZNF423* variant breast cancer cells. ZR-75-1 WT and ZR-75-1 variant cells were treated as indicated and subjected to cell cycle analysis by propidium iodide staining. **a** Representative peaks are shown, and **b** quantification of cells in G2/M is shown. Error bars represent SEM. Student *t* test was performed relative to E2 plus docetaxel (DCT) treatment: ***$p < 0.001$

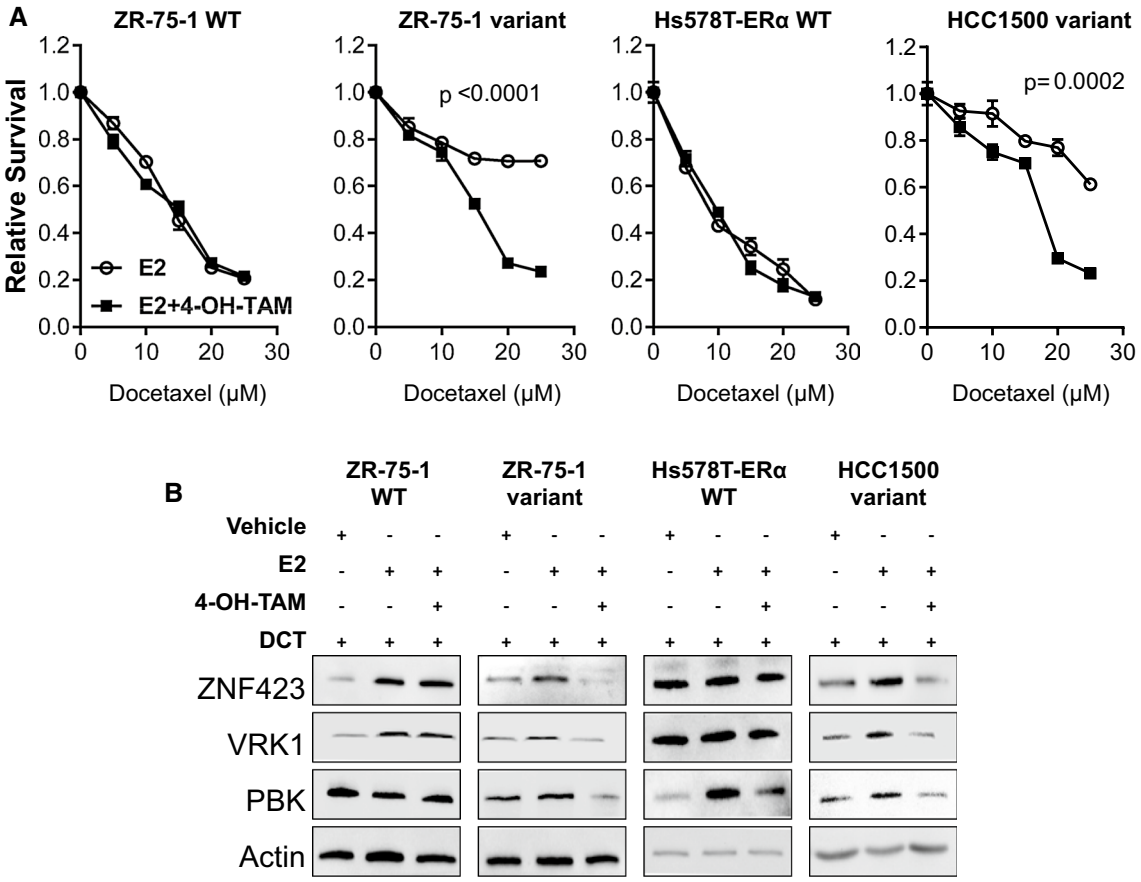

Fig. 7 Tamoxifen sensitizes ZNF423 variant breast cancer cells to docetaxel treatment. ZR-75-1 WT, ZR-75-1 variant, Hs578T-ERα WT and HCC1500 variant cells treated with docetaxel at the indicated concentrations alone or in combination with 4-OH-TAM. **a** Sensitivity to docetaxel was assessed by MTS cell viability assay 72 h after treatment. **b** Western blot was performed after 24 h of combination docetaxel (DCT) and 4-OH-TAM treatment. Experiments were performed in three independent experiments in triplicate. Graphs were plotted and areas under the curve were calculated using GraphPad Prism 7. Data represented as mean ± SEM. Area under the curve was compared to siControl by Student *t* test. ***$p < 0.001$, ****$p < 0.0001$

with luminal A breast cancer, luminal B breast cancer patients have a worse prognosis and typically have a lower response to endocrine therapy or chemotherapy [24] with a large number recurring even after sequential chemo-endocrine adjuvant therapy. Therefore, there is a growing need to develop biomarkers and additional therapeutic strategies to help improve treatment response for these patients [25].

One proposed mechanism of chemoresistance in ER+ breast tumors is activation of ERα-driven cancer survival pathways [26]. It has been theorized that treating with ER blocking agents, such as tamoxifen, concurrently with chemotherapy would help circumvent the ERα-induced chemoresistance mechanism. However, prospective clinical trials failed to show an advantage for combining the two modalities over their sequential use [3, 4]. The results from our current study suggest that a single nucleotide polymorphism, (rs9940645) which is located in *ZNF423* intronic region, may help predict which patients would benefit from combined endocrine and chemotherapy.

In our current study, the transcription factor ZNF423 was found to play an important role in regulating the G2/M phase of the cell cycle. This is in accordance with recent reports that ZNF423 regulates cell cycle progression and cell division in Purkinje neuron progenitors [27]. We found that ZNF423 exerts its cell cycle effects partially through the histone H3 kinases VRK1 and PBK. We also observed that VRK1 and PBK expression is directly regulated by ZNF423. Most intriguingly, ZNF423 ChIP-seq (GEO:GSE91794) from the ENCODE project showed binding peaks in the *PBK* promoter region in HEK293 cells, which is consistent with what we found in breast cancer cells. Although ZNF423 did not showing significant binding peaks in the *VRK1* gene in the same dataset, this may be due to cell line-specific differences.

Previous studies have revealed that suppressing the expression of histone H3 kinases, including VRK1 and PBK, causes perturbation of the G2/M phase leading to cell death [22, 23]. It has also been reported that combining histone

H3 kinase inhibitors and paclitaxel is synergistic [28, 29]. Similarly, in this study, when VRK1 and PBK were down-regulated, it resulted in increased sensitivity to docetaxel. However, this effect was only observed in cell lines harboring the variant *ZNF423* rs9940645 SNP genotype when treated in combination with 4-OH-TAM. The underlying mechanism for this SNP-dependent phenotype is mediated through a sensor protein called calmodulin-like protein 3 (CALML3). CALML3 binds to the rs9940645 SNP in the *ZNF423* promoter, forms a protein complex with ERα, and cooperates with ERα in estrogen response element (ERE) binding [7].

Whereas our study suggests that concomitant docetaxel and tamoxifen may provide an increase in efficacy in a genotype-determined subset of patients, we have no data on the impact of this combination on toxicity. Multiple attempts were made to integrate tamoxifen into chemotherapy regimens several decades ago [30], however, none of these regimens involved taxanes. Our own experience in adding tamoxifen to a non-taxane chemotherapy regimen in postmenopausal [31] and premenopausal women [32] did not identify any major toxicity signals. However, monitoring for toxicity in clinical trials of a combination of docetaxel plus tamoxifen would be necessary.

There are some limitations in our study. First, the genes selected for the two screens in this study were chosen using an arbitrarily drawn p value of less than 10^{-10} s, and these results were obtained using cell line models. It is important to validate these findings further using animal models before proceeding in a clinical setting.

Conclusions

In summary, our findings suggest that high risk ERα-positive breast cancer patients with the variant *ZNF423* rs9940645 SNP genotype may benefit from the concomitant administration of endocrine therapy (tamoxifen) and chemotherapy (docetaxel). Because the minor allele frequency of rs9940645 is 0.47 [8], there could be a large number of patients who might benefit from this combination treatment.

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Author contributions GW, SQ, and JZ participated in data acquisition, data analysis, and manuscript writing. GW designed the study and drafted the manuscript. SQ generated the ZR75-1 CRISPR Cas9 genome edited cell line. ML carried out the ZNF423 and 4-OH-TAM screens. JNI provided invaluable clinical expertise and assisted in manuscript preparation. RMW conceived the study, participated in the study design, and provided guidance in data interpretation. KS provided clinical advice and helped apply for the ChuYing Charity Foundation support. LW conceived the study, participated in the study design, coordinated the study, and is responsible for all data as described. All authors approved the final manuscript.

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Data availability All experimental datasets generated are available in the supplementary materials submitted with this manuscript. Any additional information is available upon reasonable request to the corresponding author.

Compliance with ethical standards

Conflict of interest ML is currently affiliated with AbbVie and, however, was not at the time of involvement in this study. LW and RMW are co-founders and stockholders in OneOme, LLC, a pharmacogenomic decision support company.

Ethical approval All experiments performed in this publication comply with US laws. There were no human participants or animals used in this study.

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