RESEARCH ARTICLE

Transcriptional Regulation of CYP2D6 by Nrf2 and Its Implications in Breast Cancer Therapy: Bioinformatics and Experimental Evidence

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Abstract

BACKGROUND: Tamoxifen (TAM) resistance in patient with breast cancer is the leading cause of mortality among women globally. Cytochrome P450 2D6 (CYP2D6) is involved in the metabolism of TAM, and recently NF-E2-related factor 2 (Nrf2) has recently been found as its regulator. However, the impact of Nrf2-mediated *CYP2D6* regulation in the context of breast cancer and TAM resistance are currently unknown. Therefore, this study was conducted to examine the role of CYP2D6 and Nrf2 in breast cancer prognosis.

METHODS: The roles of CYP2D6 and Nrf2 were investigated in the T47D breast cancer cell line and T47D-derived TAM-resistant cells by examining the gene expression, cell viability, and transcriptional regulation by quantitative reverse transcription polymerase chain reaction (qRT-PCR), MTT, and reporter gene assay, respectively. Additionally, comprehensive *in silico* analysis of the transcriptomic and clinical data from The Cancer Genome Atlas database were performed to uncover the prognostic role of CYP2D6 and its regulator in breast cancer patients.

RESULTS: *CYP2D6* mRNA was low and Nrf2 protein was high in TAM-resistant T47D cells compared to parental cells. Nrf2 knockdown upregulated *CYP2D6* mRNA levels and enhanced the cytotoxicity of TAM. Similarly, *in silico* analysis revealed that low *CYP2D6* mRNA and high Nrf2 protein were related to a lower probability of survival. The rs1238662089 within the identified Nrf2-binding site was found to greatly affect *CYP2D6* expression levels, indicating its role as predictor for better prognosis.

CONCLUSION: This study revealed for the first time that Nrf2 regulates *CYP2D6* expression in breast cancer and is involved in TAM sensitivity; thus, plays a role in breast cancer patient prognosis.

KEYWORDS: breast cancer, CYP2D6, Nrf2, pharmacoepigenetics, SNPs

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Introduction

Cytochromes P450 enzymes (CYPs) are a membrane-bound hemoprotein superfamily of enzymes that play an important role in the metabolism of various medicines and endogenous compounds. The diverse ways in which patients react to medications, particularly anticancer drugs, are of salient interest to CYPs. It has been noted that most of anticancer medicines are metabolized by the CYP family. Alterations in the activity and expression of CYPs have been associated with various cancers.(1) One of the CYP

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members, CYP2D6, is one of the most investigated CYPs that participates in about 25% marketed drugs metabolism, including the anti-cancer drug tamoxifen (TAM).(2) The *CYP2D6* gene has twelve highly polymorphic exons due to significant structural variations such as tandem arrangements, hybridizations, deletions, and multiplication, as well as smaller changes such as single-nucleotide polymorphisms, insertions, and deletions. Over 100 allelic variations of *CYP2D6* have been reported and are classified phenotypically as poor (PM), intermediate (IM), extensive (EM), and ultra-rapid (UM) metabolizers with activity scores of 0, 0.25–1, 1.25–2.25, and > 2.25, respectively.(3)

TAM has been the gold standard adjuvant therapy for estrogen receptor-positive (ER+) breast cancer for many decades, as it reduces breast cancer mortality by 31%. Its pharmacological action is reliant on the CYP2D6 activity that transformed it into 4-hydroxytamoxifen and endoxifen, both of which have stronger antiestrogenic potential than the parent compound.(4) The clinical outcome of TAM treatment has been proposed to be influenced by interindividual variance in the CYP2D6 genotype. Patients with PM and IM phenotypes are expected to produce lower plasma concentrations of active metabolites, which decreases the effectivity of TAM.(5) Based on this concept, the Clinical Pharmacogenetic Implementation Consortium (CPIC) supports the significance of the CYP2D6 genotype in TAM therapy by publishing genotype-based TAM dose guidelines in 2018.(6) Numerous investigations have looked into the relationship between the CYP2D6 genotype and plasma metabolite levels and clinical outcome. Majority of research has shown a connection between CYP2D6 genotype and clinical outcomes, but many other findings yielded contradictory results.(4) In fact, European Society for Medical Oncology (ESMO) indicated that CYP2D6 genotyping should not be used in clinical settings.(7)

The pharmacogenetic features of *CYP2D6* are of great interest to researchers and clinicians, and a number of studies have been published in this field. On the other hand, little is known about the pharmacoepigenetic aspects of CYP2D6, which include modifications in the mRNA expression by transcription factor(s). According to recent data, epigenetic control plays a crucial role in the expression and overall activity of CYPs. For instance, hypermethylation of the *CYP1B1* promoter in colon cancer leads to reduced expression and worse outcome.(8) Histone modification at the *CYP24A1* promoter contributes to its suppression and worse outcome in prostate cancer.(9) Additionally, a study showed a strong correlation between CYP2D6 enzymatic activity and mRNA levels.(10) Several transcription factors have been demonstrated to control CYP2D6 transcriptional levels, including CCAAT/enhancer-binding protein alpha (CEBPα), hepatocyte nuclear factor-4 alpha (HNF4α), krüppel-like factor 9 (KLF9), small heterodimer partner (SHP), and NF-E2-related factor 2 (Nrf2).(2,11)

Nrf2 is originally found as the transcriptional regulator of genes that produce enzymes and transporters that are involved in protecting cells from oxidative stress and damage caused by electrophiles, thereby maintaining redox balance.(12) In various types of cancer, aberrantly activated Nrf2 pathway enhances the expression of drug-metabolizing enzymes and numbers of cytoprotective factors.(13) This overexpression contributes to the reduced effectiveness of anticancer drugs by enhancing their biotransformation or removal during chemotherapy.(14) Although various studies have implicated Nrf2 and chemotherapeutic resistance in many types of cancer, the mechanism and impact of Nrf2-mediated *CYP2D6* regulation in the context of breast cancer and TAM resistance are currently unknown. Therefore, this study was conducted to explore *CYP2D6* regulation by Nrf2 and its implications on TAM sensitivity in breast cancer.

To explore the role of epigenetic regulation of *CYP2D6* in breast cancer, particularly the regulation by Nrf2, *in vitro* experiments were conducted in TAM-resistant breast cancer cell lines T47D. Next, datasets from the Cancer Genome Atlas (TCGA), the Clinical Proteomic Tumor Analysis Consortium (CPTAC), the Single Nucleotide Polymorphism Database (dbSNP), and the UALCAN databases were combined to examine differential expression and assess the prognostic value of *CYP2D6.* The potential transcriptional regulator(s) of *CYP2D6* expression that may be involved in the clinical outcome of breast cancer patients was explored in the present study.

Methods

Cell Culture

Human ER+ breast cancer cell line, T47D, was obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). Cells were grown in RPMI-1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Sigma-Aldrich, Darmstadt, Germany) at 37°C in a humidified incubator with 5% CO_2. An active metabolite of TAM, 4-OH-TAM (Sigma-Aldrich), was used to develop TAM-resistant T47D subline by continuous exposure to gradual increases in the concentrations of TAM (three serial passages at each concentration) up to 1 μM, which correspond to the

dosages utilized in the clinical context. When the growth of the cells cannot be inhibited with 1 μM TAM, the TAM-resistant T47D was established. The methods for CYP2D6 overexpression and the plasmid construct have been reported previously.(15) Knockdown experiments were performed with siCon (Cat. No. SI03650318) and siNrf2 (Cat. No. SI03246950) from Qiagen (Hilden, Germany). The siRNA was transfected with ScreenFect™ A (Wako, Osaka, Japan) following the manufacturer's instructions.

Cytotoxicity Assay

The cytotoxicity assay was performed using the MTT assay. Briefly, TAM-resistant T47D cells (5×10^4 cells/well) were seeded onto a 24-well plate. After 24 h, cells were exposed to a graded concentration of TAM. At 48 h post-incubation, the medium was removed and replaced with fresh medium containing 100 μL of MTT solution (5 mg/mL in PBS), then the cells were incubated for 2 h. Next, the culture medium was removed, and 500 μL of isopropanol containing 0.04 N HCl and 0.1% NP40 was added to each well to dissolve formazan. The absorbance of each well was read at 590 nm using a microplate reader (PerkinElmer, Waltham, MA, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

In the present study, the SV Total RNA System (Promega, Madison, WI, USA) was used to isolate total RNA, and the AccessQuick™ RT-PCR System (Promega) was used to convert it to cDNA. Quantitative PCR was carried out using the GoTaq™ Probe qPCR Master Mix (Promega) and a Thermal Cycler Dice Real Time System (Takara Bio, Shiga, Japan). The *GAPDH* was used for the normalization. The raw data were analyzed using the $2^{-\Delta\Delta Ct}$ technique. Primers for amplification of *CYP2D6* have been described previously.(2)

Reporter Gene Assay

Reporter gene assay was performed to measure the effect of Nrf2 on the transcriptional activity of *CYP2D6* gene according to previous method.(2) In brief, TAM-resistant T47D cells were transfected with reporter gene of interest (pGL3-containing wild-type or ARE mutants *CYP2D6* promoter), pRL-null, and expression plasmid (or empty vector) using GenePORTER TM2 (Gene Therapy Systems). The detailed procedure on the plasmid construction for reporter gene assay, including wild-type, ARE-mut1, and ARE-mut2 *CYP2D6* promoter and the method for insertion to pGL3 plasmids, have been described previously.(2) Two days after transfection, cells were harvested for luciferase activity examination using a Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Lumat LB9507; Berthold). Firefly luciferase activity was normalized to Renilla luminescence.

***In silico* Analysis**

Expression of *CYP2D6* and relevant clinical information were retrieved from the Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA, https://portal.gdc.cancer.gov/) database. Comparison of *CYP2D6* mRNA and the promoter methylation level between the tumor and matched normal samples were retrieved from the UALCAN database (https://ualcan.path.uab.edu/).(16) The enrichment levels of H3K4me3 were analyzed using ChIP-Seq datasets of the human genome (GRCh38/hg38) (http://chip-atlas.org). The Clinical Proteomic Tumor Analysis Consortium (CPTAC) was used to examine the proteomics data. The correlation between *CYP2D6* mRNA levels and the expression of its regulators were analyzed with the cBioPortal platform (http://www.cbioportal.org/).(17) The top 100 genes related to *CYP2D6* were obtained from GEPIA2 (http://gepia2.cancer-pku.cn/#index) (18), and were analyzed for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment using enrichR (https://maayanlab.cloud/Enrichr/) (19). Variations in the *CYP2D6* promoter were mapped using the single nucleotide polymorphisms (SNPs) location information from the NCBI dbSNP database (https://www.ncbi.nlm.nih.gov/snp/). To predict the effects of the variants on transcription factor binding sites (TFBS), the FABIAN-variant (https://www.genecascade.org/fabian/) tool with transcription factor flexible models was employed.(20) In brief, the variants were selected and the corresponding chromosomal annotation was entered into the SNV box. The reference genome used in the present study was GRCh38/hg38. The "Select individually" box was checked in the Transcription Factor section and NFE2L2 box was selected. To select models, the transcription factor flexible models (TFFM) detailed was chosen. Finally, the analysis was run and the results were downloaded in .tsv format.

Statistical Analysis

Data from three biological replicates with at least three technical replicates were displayed as mean±SD. Student's t-test or one-way ANOVA was used to determine statistical significance. Pearson's analysis was used to assess the association. The survival rate was evaluated using the Log-rank test. A p-value<0.05 was considered significant.

Results

CYP2D6 and *Nrf2* mRNA Levels are Altered in TAM-resistant T47D Cells

The IC_{50} of TAM for the parental and TAM-res T47D cells was 10.45±1.11 µM and 22.94±1.41 µM, respectively. Thus, TAM-res cells were successfully constructed with 2.2-fold higher TAM resistance than the parental cells. The mRNA levels of *CYP2D6* and *Nrf2* in TAM-res cells were significantly downregulated and upregulated, respectively (Figures 1A and 1B). Since Nrf2 is a negative regulator of *CYP2D6* in hepatoma, the impact of siRNA-mediated Nrf2 knockdown was then assessed. The knockdown of Nrf2 in TAM-res T47D cells resulted in enhanced *CYP2D6* expression (Figures 1C and 1D).

The function of CYP2D6 and Nrf2 in TAM-treated T47D cells was further explored by MTT assays. The results showed that the IC_{50} of TAM was 11.72 µM in mock T47D cells, while it was 5.50 µM in CYP2D6-overexpressed

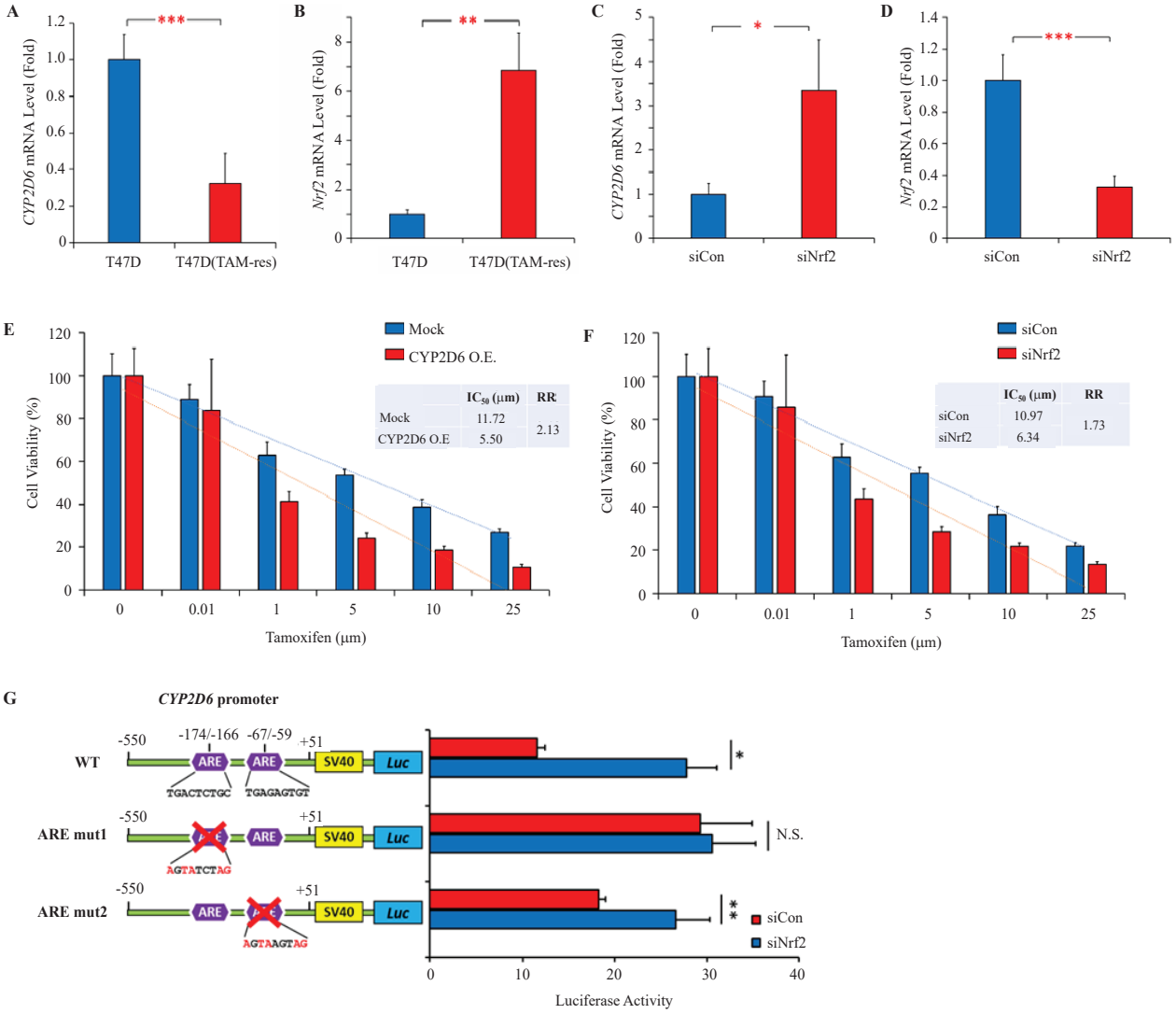

Figure 1. The role of CYP2D6 and Nrf2 on the TAM sensitivity in the breast cancer cell line T47D. A-B: The mRNA levels of *CYP2D6* (A) and *Nrf2* (B) were assessed using qRT-PCR, showing that *CYP2D6* and *Nrf2* were lower and higher, respectively, in T47D(TAM-res) than the parental T47D cells. C-D: TAM-res T47D cells transfected with siNrf2 showed an increased and decreased mRNA levels of *CYP2D6* (C) and *Nrf2* (D), respectively, as examined by qRT-PCR. E: T47D cells expressing empty vector (Mock) or CYP2D6 were treated with TAM for 48 h, and cell viability was measured by the MTT assay. CYP2D6-overexpressed cells were more sensitive to TAM. F: T47D cells were transfected with siCon or siNrf2, treated with TAM for 48 h, and examined for viability with MTT assay. Nrf2-knockdown cells were more sensitive to TAM. G: WT or ARE mutants reporter constructs (−550/+51 bp from transcription start site) and siCon or siNrf2 were transfected into T47D cells, and reporter activity was quantified 48 h after transfection using a Dual-Luciferase Reporter assay. ARE: Antioxidant Response Element; IC_{50}: Inhibitory Concentration 50%; Luc: Luciferase; N.S.: Not Significant; O.E.: Overexpression; RR: Reversal Fold; siCon: siRNA Control; siNrf2: siRNA Nrf2; SV40: Simian Vacuolating Virus 40; TAM-Res: Tamoxifen Resistant Cells; WT: wild-type.

cells (Figure 1E). Knockdown of Nrf2 reduced the IC_{50} of TAM from 10.97 μM to 6.34 μM (Figure 1F). These results demonstrated that overexpression of CYP2D6 and knockdown of Nrf2 strongly promoted the sensitivity of breast cancer cells to TAM.

Next, the mechanism of *CYP2D6* regulation by Nrf2 in breast cancer cells was explored. The −500/+51 region of the *CYP2D6* promoter was isolated, the Nrf2 binding sites were identified, site-directed mutagenesis was performed, and the reporter activity of these promoters was examined. The mutant constructs first Nrf2-binding site (ARE mut1) exhibited significantly higher basal promoter activities to the wild-type (WT) construct, whereas Nrf2-binding site (ARE mut2) basal promoter activity was slightly higher than that of the WT. Knockdown of Nrf2 significantly enhanced the reporter activity of WT and ARE mut2 construct, but not ARE mut1 construct (Figure 1G). These results suggest that Nrf2 binding to the first putative site plays an important role in the suppression of *CYP2D6* expression.

The Clinical Importance of *CYP2D6* Gene Expression in Breast Cancer

CYP2D6 and Nrf2 were unusually expressed at low and high levels, respectively, in TAM-resistant T47D cells, which may be connected to the cancer progression and prognosis. In order to ascertain whether CYP2D6 and Nrf2 are connected to patient prognosis, the public databases were explored. The 1085 breast cancer patients had a considerably lower *CYP2D6* expression than normal patients in the TCGA-BRCA database (Figure 2A). Additionally, the CPTAC database verified the elevated CYP2D6 protein levels (Figure 2B).

In the pooled TCGA-BRCA dataset, there was a significant correlation between low *CYP2D6* expressions and shorter overall survival (Figure 2C). Although not statistically significant, patients with low *CYP2D6* expression also had a lower disease-free survival rate (Figure 2D). In the TAM-treated patients, there was a significant correlation between low *CYP2D6* expressions and shorter overall survival (Figure 2E), as well as a lower disease-free survival (Figure 2F). Subsequently, the methylation and H3K4me3 enrichment in the promoter of *CYP2D6* was investigated, as these are the most often occurring molecular lesions in cancer cells that result in gene suppression. But methylation level and H3K4me3 enrichment were not altered in breast cancer patients (Figure 2G), suggesting that Nrf2 plays a major role in the suppression of *CYP2D6* gene expression.

The biological processes and pathways that were altered by CYP2D6 were then assessed. First, the top 100 genes related to *CYP2D6* from the GEPIA2 platform was retrieved. Next, the GO and KEGG pathway enrichment using enrichR were analyzed. Complement and coagulation cascade, fat metabolism, and xenobiotic metabolism were among the top pathways that were considerably enriched by the alteration in CYP2D6 (Figure 2H).

The Involvement of *CYP2D6* Regulators in the Prognosis of Breast Cancer Patients

To examine the clinical significance of *CYP2D6* regulators and to validate that Nrf2 plays a major role in breast cancer, as well as to predict their prognostic values, the correlation between *CYP2D6* and its regulators and their impact on patients' survival in the TCGA-BRCA dataset. This study focused on previously reported regulators of *CYP2D6* expression, including CEBPα, HNF4α, KLF9, SHP, and Nrf2. The expression of *CEBPα* and *KLF9* was significantly lower in breast cancer patients compared to normal patients, while *Nrf2* and *SHP* were not significantly different (Figure 3A-3D). HNF4α was not expressed in the breast tissue. Since Nrf2 is mainly regulated at post-transcriptional levels, this study focused on determining the levels of Nrf2 protein. Indeed, breast cancer tissues expressed a considerably higher level of Nrf2 protein than normal breast tissues, based on the Human Protein Atlas (HPA) database (Figure 3E).

Next, correlation analysis revealed that *CYP2D6* expression levels were positively correlated with *CEBPα*, negatively correlated with *KLF9* and *Nrf2* (Figure 4A-4C). Unfortunately, the expression levels of *SHP* were too low for the correlation analysis. In the pooled TCGA-BRCA dataset, the expression profiles of both *CEBPα* (Figure 4D) and *KLF9* (Figure 4E) did not determine the overall survival of breast cancer patients. In contrast, a significant correlation between high Nrf2 protein expression and shorter overall survival was observed in breast cancer patients (Figure 4F). In the TAM-treated patients, there was a correlation between high Nrf2 protein expressions and shorter overall survival (Figure 4G), as well as a lower disease-free survival (Figure 4H).

The Role of SNP in the Nrf2-binding Site within the CYP2D6 Promoter

Transcription factors regulate the expression of genes by interacting with the short sequence patterns in DNA. Many variants may alter gene expression because the great majority of genetic variants (mostly SNPs) are localized to a noncoding region of the genome and are enriched in the regulatory compartment. The primary mode of action is the modification of the binding site that causes the

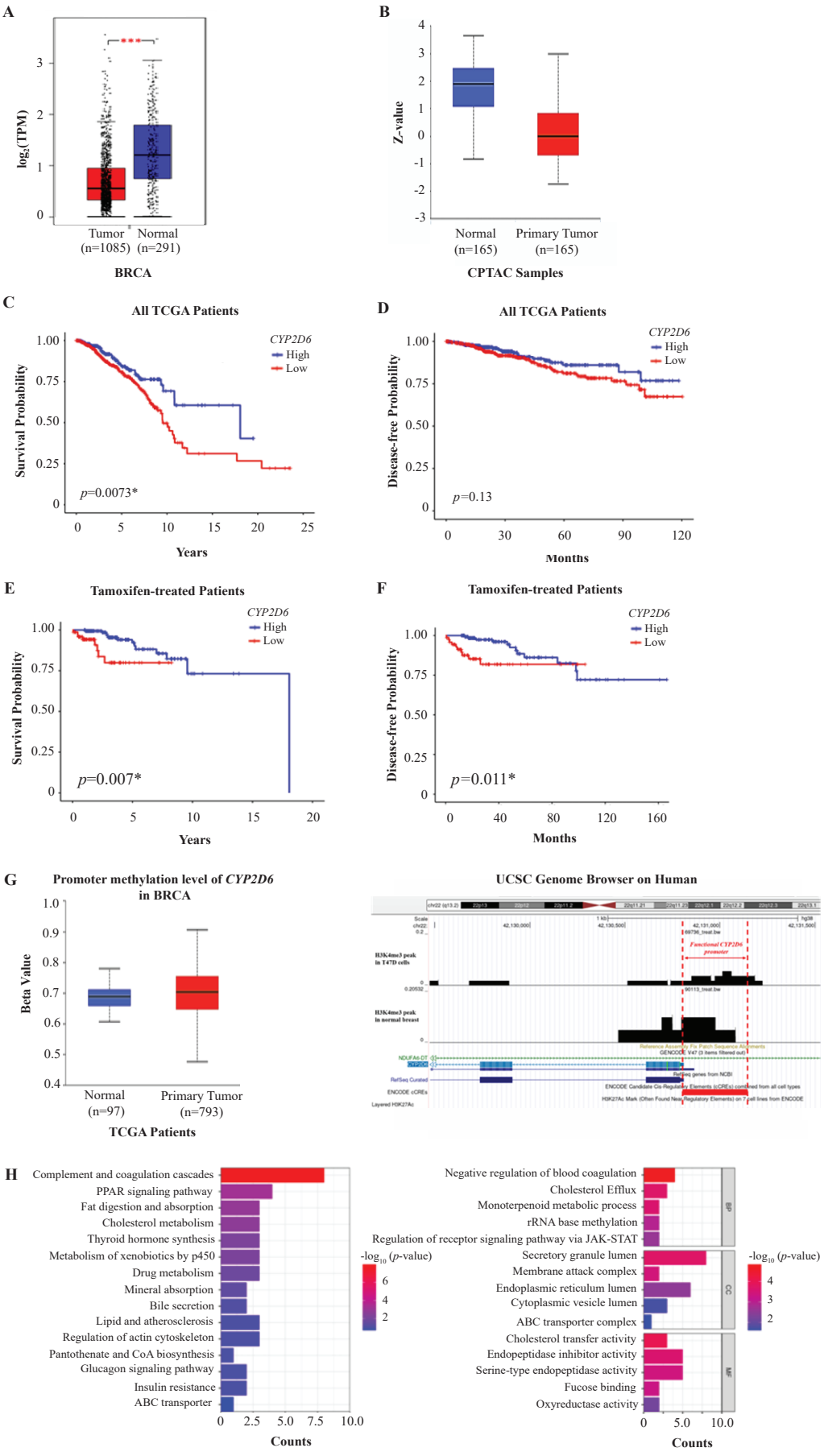

Figure 2. The role of CYP2D6 on the prognosis of breast cancer patients. A: The GEPIA analysis of *CYP2D6* mRNA levels in breast cancer and normal tissues based on the TCGA-BRCA dataset showed low *CYP2D6* expression in breast cancer tissue. B: The UALCAN analysis of CYP2D6 protein abundance based on the CPTAC dataset indicated that breast cancer tissues had lower CYP2D6 protein levels than the normal breast tissue. C-F: Kaplan-Meier analysis of the overall (C) and disease-free (D) survival of patients based on *CYP2D6* expression in the pooled TCGA-BRCA cohort revealed that low CYP2D6 expression is favorable for survival. Kaplan-Meier analysis of the overall (E) and disease-free (F) survival of patients based on *CYP2D6* expression in the TAM-treated patients of the TCGA-BRCA cohort. G: The UALCAN analysis of *CYP2D6* promoter methylation indicated that similar methylation levels between tumor and normal breast tissues (left panel). The enrichment levels of H3K4me3 analyzed using ChIP-Seq datasets and visualized with UCSC browser indicated that H3K4me3 peaks are similar between tumor and normal breast tissues (right panel). H: KEGG signaling pathway and GO analysis of the top 100 genes related to *CYP2D6* in breast cancer. BRCA: Breast Invasive Carcinoma Dataset; CPTAC: Clinical Proteomic Tumor Analysis Consortium; TCGA, The Cancer Genome Atlas; TPM, Transcripts per Million.

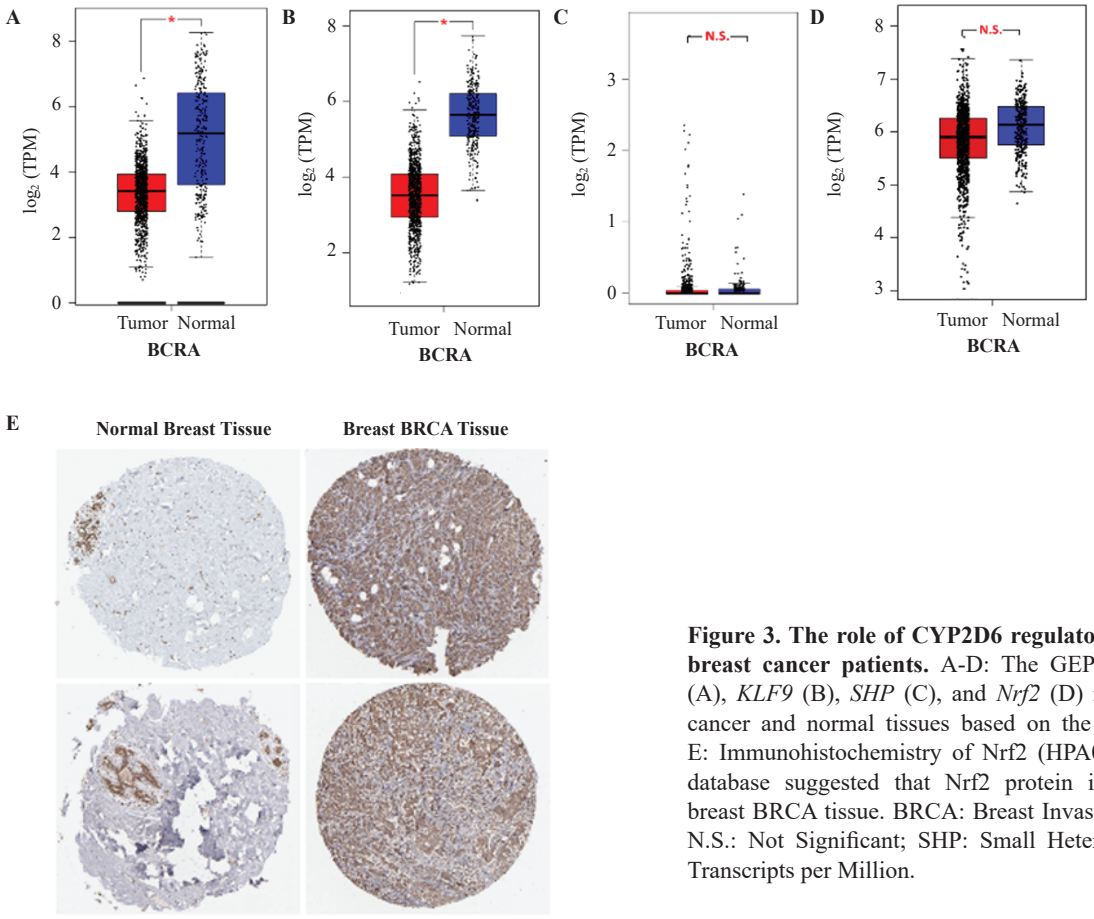

Figure 3. The role of CYP2D6 regulators on the prognosis of breast cancer patients. A-D: The GEPIA analysis of *CEBPA* (A), *KLF9* (B), *SHP* (C), and *Nrf2* (D) mRNA levels in breast cancer and normal tissues based on the TCGA-BRCA dataset. E: Immunohistochemistry of Nrf2 (HPA002990) from the HPA database suggested that Nrf2 protein is highly expressed in breast BRCA tissue. BRCA: Breast Invasive Carcinoma Dataset; N.S.: Not Significant; SHP: Small Heterodimer Partner; TPM: Transcripts per Million.

alteration in the activity of transcription factor.(22) To study the role of SNPs in *CYP2D6* expression, the list of SNPs located in the previously reported Nrf2-binding site within the *CYP2D6* promoter was explored. Data retrieved from dbSNP suggested several SNPs in this region, including rs1264108606, rs1932103654, rs1326180688, rs1205159672, rs1602592933, rs1932101460, and rs1238662089 (Figure 5). The effects of these SNPs on the binding activity of Nrf2 were then predicted using the Markov model-based TFFMs of the FABIAN-variant tool. TFFM scores correlate with ChIP-seq peak signals that reflect TF-DNA interactions. It represents the probability of transcription factor binding to a certain region of the DNA, where the negative value means a higher probability of transcription factor-DNA interactions in the wild-type allele than the alternating allele. Almost all SNPs resulted in the loss of Nrf2 activity, except for rs1932103654. Rs1238662089 showed the most detrimental effects on the activity of Nrf2 (Table 1). Since rs1238662089 would results in loss Nrf2 activity and thus high *CYP2D6* expression, patients with this variation would have a better prognosis than that of wild-type allele.

Discussion

Breast cancer is currently one of the most common cause of cancer-related fatalities globally, with an annual increase in incidence.(21-23) It also one type of cancer that has been the most common cause of death in woman (23), therefore finding the suitable adjuvant treatments is necessary. One of the most popular adjuvant treatments for ER+ breast cancer is TAM. Although it lowers the death rate, long-term TAM treatment can cause acquired resistance in certain patients. TAM-treated breast cancer patients develop resistance to the treatment in about 40% of cases, which increases the risk of metastasis and recurrence, the two main causes of death. (24) Therefore, it is now critical to identify the mechanisms that lead to TAM resistance development and to use novel strategies to enhance breast cancer treatment. The processes underlying drug resistance are intricate and typically arise from the synergistic interaction of many pathways. The typical changes underlying drug resistance encompass heightened xenobiotic metabolism, improved drug efflux, growth hormones, amplified DNA repair capability, and

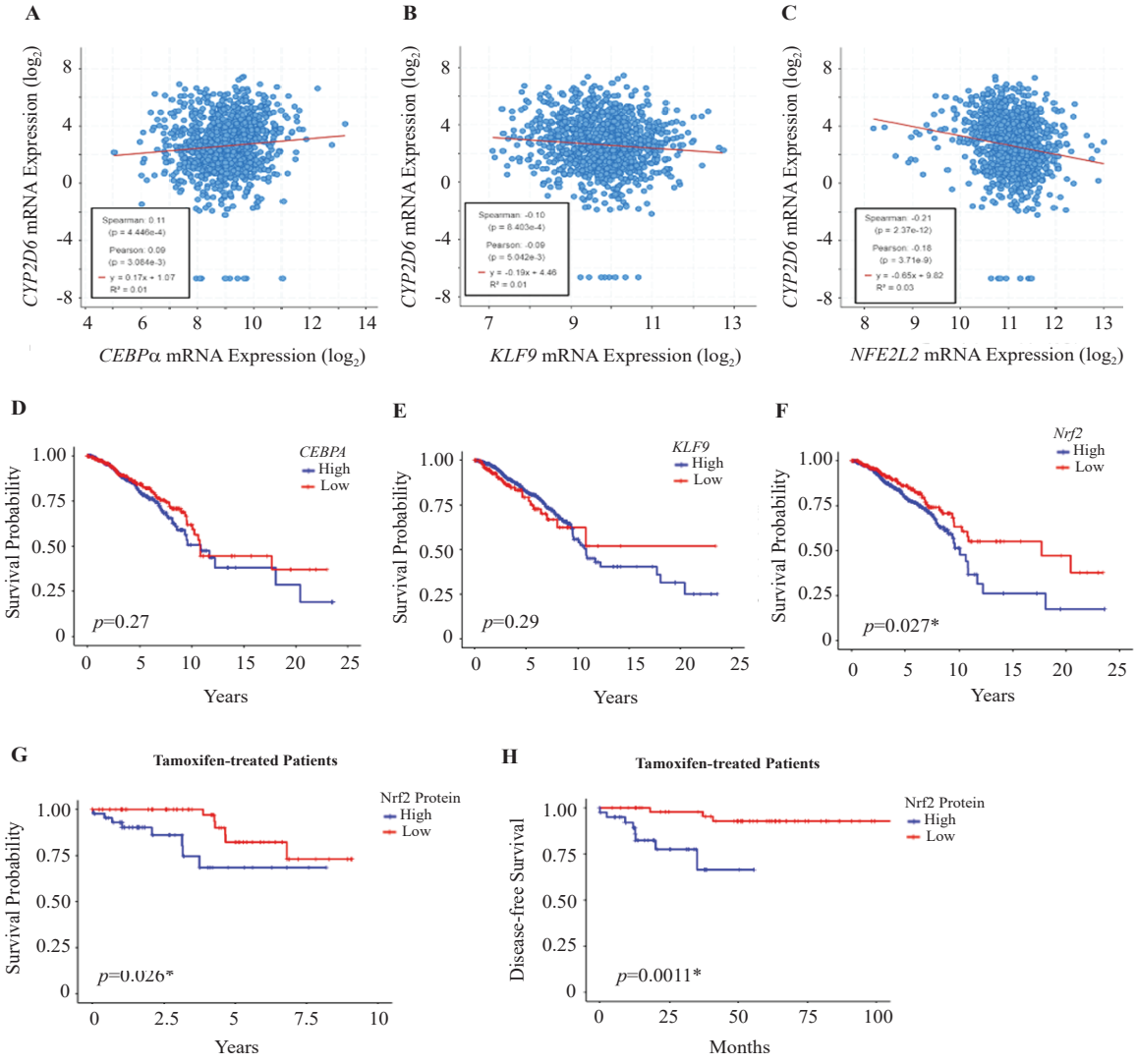

Figure 4. The role of CYP2D6 regulators on the prognosis of breast cancer patients. A-C: Correlation analysis between *CYP2D6* and *CEBPα* (A), *KLF9* (B), and *Nrf2* (C) in the cBioPortal platform. D-H: Kaplan-Meier analysis of the overall survival of patients based on *CEBP* (D), *KLF9* (E), and *Nrf2* (F) expression in the pooled TCGA-BRCA cohort. Kaplan-Meier analysis of the overall (G) and disease-free (H) survival of patients based on *Nrf2* expression in the TAM-treated patients of the TCGA-BRCA cohort revealed that high Nrf2 expression is favorable for survival. CEBPα: CCAAT/Enhancer-Binding Protein Alpha; KLF9: Krüppel-Like Factor 9; Nrf2: NF-E2-related factor 2.

genetic alteration (mutations, amplifications, and epigenetic modifications).(25) The altered activity and expression of phase I drug metabolizing enzymes have been proposed as a hallmark of chemotherapy resistance in various study, as metabolic activation is necessary for many anticancer medicines. Since the pharmacological action of TAM is reliant on the CYP2D6 activity that transformed it into its active forms (4-OH-TAM and endoxifen) (4), the activity and expression of CYP2D6 could predict resistance to TAM treatment. In this study, low CYP2D6 expression and high Nrf2 levels were found to confer resistance to TAM and are prospective targets for therapeutic intervention in breast cancer.

The genetic variation in *CYP2D6* has been used as guidance for personalized TAM treatment in ER+ breast cancer. There are about 100 known genetic variations in CYP2D6, and these variants are categorized into four different phenotypes in the population: PM, IM, EM, and UM, which correspond to no activity, reduced activity, normal activity, and high activity, respectively. The CPIC guidelines for CYP2D6 and TAM therapy published in 2018 recommends individuals with UM phenotype to take tamoxifen at the recommended dosage (strong recommendation), while PM and IM phenotypes are suggested to take alternative hormonal therapy, such as an aromatase inhibitor (strong recommendation).(6) These

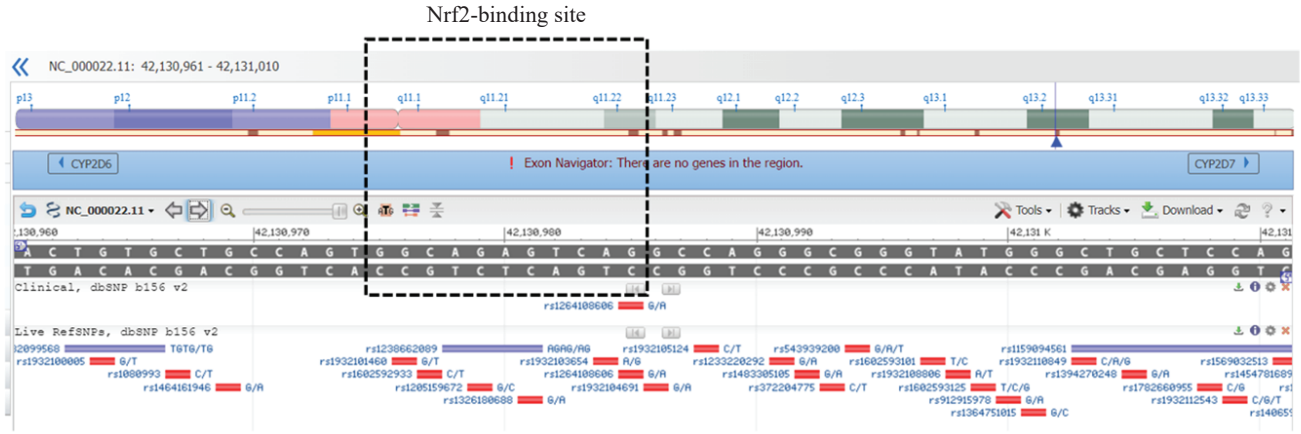

Figure 5. The list of SNPs in the Nrf2-binding site within *CYP2D6* promoter downloaded from dbSNP.

recommendations are based on the theory that genetic variation in *CYP2D6* gene affects its enzymatic activity, *e.g.*, low activity of CYP2D6 is expected to produce lower plasma concentrations of active metabolites, which decreases the effectivity of TAM.(5) However, conflicting results have been reported, raising questions about the relationship between the CYP2D6 genotype and the clinical outcome of TAM treatment.(4) Apart from the CYP2D6 genotype, other variables that may impact endoxifen levels include treatment compliance, drug-drug interaction (26), and epigenetic regulation (27).

The present study was the first to show that *CYP2D6* is extremely downregulated in TAM-resistant breast cancer cells. The reduced *CYP2D6* causes insensitivity to TAM and is correlated with a poor prognosis in breast cancer patients. Exogenous overexpression of *CYP2D6* was further validated to sensitize breast cancer cells to TAM *in vitro*. This study is the first to report that the mRNA expression levels of *CYP2D6* play a significant role in TAM treatment efficacy and the prognosis of breast cancer patients. Previous studies using human liver tissue have reported that CYP2D6 enzymatic activity is strongly correlated with its mRNA levels.(10) Another study has also shown a moderate correlation between *CYP2D6* mRNA expression and activity levels.(28) In contrast to the widely reported genetic aspect of *CYP2D6*, the role of epigenetic regulation that modifies *CYP2D6* expression is rarely explored. Epigenetic control (methylation, histone modification, and regulation by transcription factors) plays a crucial role in the expression and overall activity of CYPs.(29) In the present study, methylation and histone modification do not contribute to the high *CYP2D6* expression in breast cancer, suggesting that regulation by transcription factor(s) might play a major role. In view of the fact that reduced *CYP2D6* expression promotes TAM-resistance, the identification of factor(s) that regulate *CYP2D6* gene transcription in breast cancer cells is of great clinical importance, as it may be exploited to enhance CYP2D6-dependent TAM sensitivity.

To this end, the most important regulator of *CYP2D6* that determines the prognosis of breast cancer patients was searched. The *Nrf2* mRNA is highly upregulated in TAM-resistant breast cancer cells and causes insensitivity to TAM. High levels of *Nrf2* expression are correlated with a poor prognosis in breast cancer patients. Manipulating Nrf2 by knockdown strategy could sensitize breast cancer cells to TAM. The roles of Nrf2 as a key player in chemotherapeutic and radiotherapeutic resistance have been

Table 1. The list of variations in the promoter of CYP2D6 and prediction of their effects on Nrf2 binding activity.

SNP ID	Variation	Score	Effects
rs1264108606	chr22:42130985G>A.1	-0.10058	Loss
rs1932103654	chr1:160001799G>C.1	-0.00032	NA
rs1326180688	chr22:42130981G>A.1	-0.09242	Loss
rs1205159672	chr22:42130979G>C.1	-0.10106	Loss
rs1602592933	chr22:42130977C>T.1	-0.20544	Loss
rs1932101460	chr22:42130976G>T.1	-0.20082	Loss
rs1238662089	CTGGCCTGACTCTGCCATCGG>CTGGCCTGACTGCCATCGG.1	-0.42550	Loss

extensively reported in various cancer, including breast cancer.(30) A higher risk of death following TAM therapy has been associated with elevated Nrf2 expression at the time of diagnosis in breast cancer patients.(31) The highly activated Nrf2 in breast cancer could be due to elevated Nrf2 transcription by oncogenic proteins (Myc, K-Ras, *etc.*), somatic mutations, reduced Keap1, elevated Keap1-competing proteins, or epigenetic changes amplifying Nrf2 level.(32) However, based on the results of present study, the highly activated Nrf2 is not likely due to elevated Nrf2 transcription by oncogenic proteins since the *Nrf2* mRNA was not elevated in BRCA, but the protein of Nrf2 is highly expressed in BRCA based on HPA database. These results suggest that activation of Nrf2 in breast cancer patient is due to post-transcriptional mechanism, such as inactivation of Keap1 or gain-of-function mutation in *Nrf2* genes.

In TAM-treated patients, Nrf2 might be elevated due to TAM-induced overproduction of reactive oxygen species that alters Keap1 configuration, thus, liberating Nrf2 from proteasomal degradation.(33) Nrf2 is a transcription factor responsible for the expression of genes involved in redox regulation, glycolysis, fatty acid metabolism, inflammation, proteostasis, and most importantly, drug metabolism.(13) In the present study, whether Nrf2 inhibits the expression of CYP2D6 was investigated. *Nrf2* is negatively correlated with CYP2D6 in breast cancer patients. Nrf2 is known for its role as a master regulator of phase II drug metabolizing enzymes; however, many studies currently suggest that Nrf2 also regulates phase I enzymes.(34) The present study and previous report clearly show that Nrf2 inhibits CYP2D6 expression, likely by competing with KLF9 for DNA binding.(2) Several SNPs within the Nrf2 binding site that may reduce Nrf2 binding capacity were identified in the present study. The rs1238662089 showed the lowest TFFM score, suggesting that this variation causes the biggest loss of Nrf2 binding activity. Currently, there is no evidence on the role of these SNPs in breast cancer, particularly regarding TAM resistance, thus, further study is warranted. At least, the results of this study inferred that rs1238662089 would results in loss Nrf2 activity and thus high *CYP2D6* expression; hence, patients with this variation would have a better prognosis than that of wild-type allele. In brief, reduced Nrf2 levels or activity may enhance *CYP2D6* levels and lead to improved sensitivity to TAM. So, the exploitation of Nrf2 inhibitors in patients treated with TAM will be of great significance in clinical settings.

Thus far, several naturally occurring substances have been recognized as Nrf2 inhibitors, including luteolin, apigenin, brusatol, and brucein D. It has been discovered that these inhibitors can be effectively used as tumor sensitizers for chemotherapy and conventional radiation, as well as chemo-preventive and chemo-therapeutic drugs. (35) Indeed, studies have shown that luteolin, berberine, and brusatol are effective Nrf2 inhibitors in breast cancer. Berberine and luteolin have both moved into clinical trials (http://clinicaltrials.gov/). These inhibitors may enhance TAM sensitivity, although further study is required.

There are a number of limitations of this study that used cell lines instead of primary cells or *in vivo* models, including changes in the transcriptome and proteome over time (especially after prolonged passage), lack of representation of *in vivo* (cell lines may not accurately represent what happens *in vivo* particularly related to inter-organ communication), the use of cell culture media that differs from the biological fluids encountered *in vivo* that may be critical for the hypothesis being tested. Additionally, the use of *in silico* approach in this study also possesses several limitations such as the reliance on existing datasets that may lack comprehensive clinical variables, be outdated, or be inaccurate, model accuracy that may not accurately represent the real biological systems or processes, oversimplification of complex biological systems, and predictive limitations that may not capture unforeseen interactions or rare events that could significantly impact the results.(36) To this end, further research is warranted to determine the effects of Nrf2 and *CYP2D6* on breast cancer resistance and prognosis using an *in vivo* approach.

Conclusion

In conclusion, this study revealed for the first time that Nrf2 regulates the *CYP2D6* expression in breast cancer and is involved in the TAM sensitivity, contributing to the pharmacoepigenetic aspect of CYP2D6. Nrf2-mediated regulation of CYP2D6 can therefore potentially be modified to enhance TAM treatment efficiency.

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Authors Contribution

FMS, MDNH, LL, RD, ZA, and LH were involved in concepting and planning the research. FMS and PSA performed the data acquisition/collection. FMS calculated the experimental data and performed the analysis. FMS and PSA drafted the manuscript and designed the figures. FMS, MDHN, LL, and ZA aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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