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Network Pharmacological Analysis Identifies the Curcumin Analog CCA-1.1 Targeting Mitosis Regulatory Process in HER2-Positive Breast Cancer

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ABSTRACT

Recent studies have demonstrated that a curcumin derivative, namely, chemoprevention-curcumin analog 1.1 (CCA-1.1), impedes the proliferation of breast cancer (BC) cells, including luminal, human epidermal growth factor 2 (HER2)-overexpressed, and triple-negative BC cells. We analyzed the possible target of action of CCA-1.1, particularly in BC cells with HER2 amplification, using bioinformatics analysis. The differentially expressed genes (DEGs) of HER2-positive BC were retrieved from The Cancer Genome Atlas–Breast Invasive Carcinoma data (via UALCAN). Using the SMILE similarity feature, we used three web-based tools (Swiss Target Prediction, BindingDB, and TargetNet) to predict the potential target of CCA-1.1. The functional annotation and network enrichment were processed in WebGestalt. The alteration of selected genes was observed in CBioPortal. The protein–protein interaction network was constructed in STRING and then ranked based on the degree score using the Cytohubba feature in Cytoscape. The survival analysis of hub genes was determined in Gene Expression Profiling Interactive Analysis 2 (GEPIA2) with selection for HER2-positive BC cases only. The correlation between hub genes and tumor-infiltrating immune markers was determined using TIMER web tools. The pathway network analysis highlighted the cell cycle regulation in mitosis affected by signaling amid putative CCA-1.1 targets. We identified eight potential genes, including aurora A kinase (AURKA), aurora B kinase (AURKB), polo-like kinase 1, TPX2 microtubule nucleation factor, kinesin-like protein KIF11, maternal embryonic leucine zipper kinase, cyclin-dependent kinase 1 (CDK1), and serine/threonine-protein kinase Chk1 (CHEK1), that may inhibit mitosis regulation in response to CCA-1.1 treatment. Several potential markers (AURKB, AURKA, CDK1, and CHEK1) were correlated with immune cell infiltration markers. CCA-1.1 may regulate mitosis to induce cell cycle arrest and lead to cell death. The predicted targets of CCA-1.11 gave insights into the potency of CCA-1.1 to be applied with immunotherapy. Further validation of the data presented in the study is essential to develop CCA-1.1 for BC treatment.

Keywords: CCA-1.1; Bioinformatics; Mitosis; HER2-amplified breast cancer.

INTRODUCTION

Numerous studies have shown the anticancer properties of curcumin and its derivatives for breast cancer (BC) therapy with multitarget (Liu and Ho, 2018). In addition to the lack of stability and bioavailability, distinct approaches are needed to overcome the challenge of developing curcumin-based compounds as anticancer. We focused on chemoprevention curcumin analog 1.1 (CCA-1.1) (Figure 1A), a

synthesized compound based on the reduction reaction of the carbonyl group of pentagamavunone-1 (PGV-1). CCA-1.1 has displayed antitumor activities through *in vitro* screening (Novitasari *et al*., 2021b; Utomo *et al*., 2022). Studies have also evaluated the anticancer properties of CCA-1.1 in BC, notably in a highly aggressive metastatic subtype. The CCA-1.1 treatment halted the mitosis phase and induced reactive oxygen species generation and cellular senescence (Novitasari *et al*., 2021c) in 4T1 cells classified as triple-negative BC (TNBC) cells. Moreover, the treatment with CCA-1.1 enhanced the cytotoxicity of doxorubicin to inhibit cell proliferation in 4T1 and human epidermal growth factor 2 (*HER2*) gene-transfected MCF7 (MCF7/HER2) cells (Novitasari *et al*., 2021b). These capabilities of CCA-1.1 deserve further development to evaluate its molecular activities. A recent bioinformatic study suggested that CCA-1.1 possibly targets several mitotic markers in the TNBC subtype, namely, aurora A kinase (AURKA), aurora B kinase (AURKB), polo-like kinase 1 (PLK1), and other mitotic regulators in TNBC (Novitasari *et al*., 2021a). We focused on determining the putative therapeutic targets from CCA-1.1 in HER2-positive breast tumors.

As a part of the BC subtype with high malignancy features, HER2 amplification results in a poor prognosis in 20% of the presented cases. Despite the current HER2-targeted drugs and antibodies, cancer cells continue to adapt and induce resistance, limiting therapy effectiveness and causing difficulty in the development of therapies to combat resistance (Pernas and Tolaney, 2019). Given the advances in sequencing and high-throughput microarray assays, several differentially expressed genes (DEGs) have been linked to tumor progression, and they are available in many resources (Kolodziejczyk *et al*., 2015; Shapiro *et al*., 2013).

This study presented a series of bioinformatic analyses to evaluate the potential therapeutic target of CCA-1.1 in HER2-positive BC. Using the chemogenomic approach with the SMILES code based on chemical structure, we predicted the target interaction of CCA-1.1. Simultaneously, the DEGs of HER2-amplified BC were collected from UALCAN, a web resource for analysis of cancer omics data generated from The Cancer Genome Atlas (TCGA) (Chandrashekar *et al*., 2017). By visualization of overlapping genes in the Venn diagram, we further analyzed the possible target pathway of CCA-1.1, and the predictive value of each target gene was plotted on a Kaplan–Meier survival graph. The genetic alterations in selected genes were analyzed using cBioPortal. We also searched for the association of selected genes with immune marker sets via TIMER to gain insights into tumor–immune interactions in HER2-positive breast tumors. The present results can be expected to reliable data for the development of CCA-1.1 as a potential treatment option for HER2-positive BC.

MATERIAL AND METHODS

Analysis of predicted CCA-1.1 therapeutic targets

We utilized three different tools to determine the target gene for CCA-1.1 (Swiss Target Prediction (Daina *et al*., 2019), BindingDB (Gilson *et al*., 2016), and TargetNet (Yao *et al*., 2016)). The structure of CCA-1.1 was drawn in MarvinJS, and the SMILES code was retrieved and inputted to the databases. We selected the default settings while generating data in the databases. Then, we used Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) to determine the potential targets of CCA-1.1 in HER2-positive BC.

Acquisition of regulated genes in HER2-positive BC

We used the UALCAN webtool (Chandrashekar *et al*., 2017) (http://ualcan.path.uab.edu/) to determine the list of DEGs from HER2-positive BC patients, according to the TCGA BC database. A total of 500 genes (250 upregulated and 250 downregulated genes) were selected for further analysis.

Genetic Alteration Analysis

Genetic alterations of the target genes were processed through cBioPortal (Gao *et al*., 2013). We also checked for connectivity analysis using a METABRIC BC study with a threshold value of $p < 0.05$

Construction of the protein–protein interaction (PPI) network

The PPI network of the CCA-1.1 potential target was elucidated via STRING-DB 11.0 [11], with default settings selected during analysis; then, the graph was visualized through Cytoscape (Shannon *et al*., 2003). The degree score was used to rank the hub-genes using CytoHubba (Chin *et al*., 2014).

Functional annotation and pathway enrichment analysis

We used WebGestalt (Liao *et al.*, 2019) (http://www.webgestalt.org/) and selected overrepresentation enrichment analysis for the enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and functional annotation terms using Gene Ontology (GO) databases (false discovery rate less than 0.05 was selected as the threshold).

Classification of overlapping genes

Each gene was investigated based on its classification provided by MsigDB datasets (Subramanian *et al.*, 2005) (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp) using hallmark gene sets (Liberzon *et al.*, 2015).

Survival analysis of hub genes in HER2-positive BC patients

We processed the overall survival (OS) and disease-free survival (DFS) as a component of survival analysis through Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (http://gepia2.cancer-pku.cn/) (Tang *et al.*, 2019). We selected the Breast Invasive Carcinoma (BRCA) database and filtered information based on HER2-positive non-luminal data (n = 66). The data were plotted in a Kaplan–Meier graph with a median cut-off of 50% and a hazard ratio of 95% confidence interval. The p-value was also calculated and visualized on the graph.

Correlation analysis between predicted target genes and immune infiltration levels

We processed the relationship analysis between the expression of potential biomarkers of CCA-1.1 toward several immune cell infiltration markers (CD8, CD4, and B cells, neutrophils, macrophages, and cancer-related fibroblasts) using the TCGA database (BRCA-HER2; n = 82) via TIMER 2.0 (http://timer.comp-genomics.org/) (Li *et al.*, 2017). The data were presented as transcription level (Log2 TPM) against infiltration level. The association analysis results were automatically generated through the system using Spearman's correlation (positive correlation if $p < 0.05$, $\rho > 0$; negative correlation if $p < 0.05$, $\rho < 0$).

RESULT AND DISCUSSION

Data acquisition regarding significant genes in HER2-positive BC and CCA-1.1 putative target genes

In this study, we explored the putative marker genes correlated with CCA-1.1 by gathering TCGA data and selected HER2-positive breast tumor samples to determine genes that are critical in breast tumors. We explored the potential target genes of CCA-1.1 through online web tools using chemogenomic approaches as the computational drug-target analysis is notably beneficial in predicting the target and molecular mechanisms of candidate drugs (Kaushik *et al.*, 2020). We sorted the 500 genes that were identified in HER2-positive BC patients and mentioned them as DEGs of HER2-positive BC (Supplementary Data 1 and 2). We noticed that several genes are involved in the BC progression regulated by HER2. Then, we determine the possible target genes of CCA-1.1 from BindingDB, SwissTargetPrediction, and TargetNet. A total of 733 genes were obtained from the databases, and the overlapping genes were generated using a Venn diagram. A total of 12 overexpressed and 20 downregulated genes were selected as CCA-1.1-mediated genes (CMGs), which were processed for subsequent bioinformatics analysis (Figure 1B).

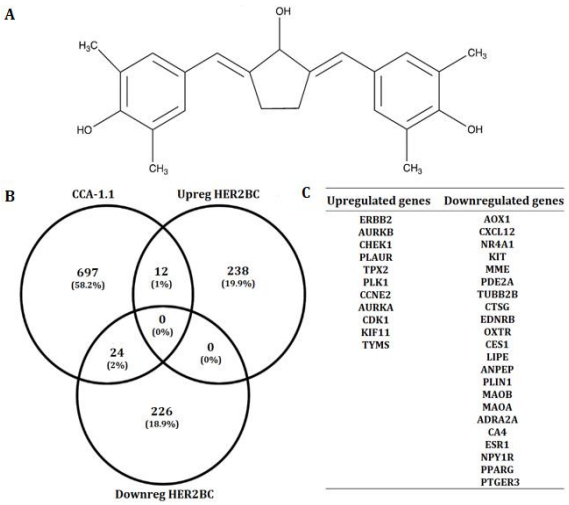


Figure 1. (A) The chemical structure of CCA-1.1. Several CCA-1.1 target genes crossed with HER2-positive BC biomarkers from TCGA–BRCA database, displayed in Venn diagram (B), resulting in 36 prospective therapeutic targets of CCA-1.1 listed in the table (C).

We noticed that CCA-1.1 targets *ERBB2*, which encodes the HER2 protein (Figure 1C). However, curcumin and PGV-1 did not alter the expression or localization of HER2 (Lai *et al.*, 2011; Meiyanto *et al.*, 2021); thus, the antiproliferative effect of curcumin and its analogs was mediated through another pathway.

Table I. Mutual exclusivity analysis of CCA-1.1 prospective target genes through cBioPortal

No.	A	B	Log2 Odds Ratio	*p*-Value	Tendency
1	CCNE2	AURKA	2.087	<0.001	Co-occurrence
2	ERBB2	CCNE2	1.218	<0.001	Co-occurrence
3	ERBB2	AURKA	1.532	<0.001	Co-occurrence
4	ERBB2	AURKB	2.656	<0.001	Co-occurrence
5	PLAUR	CCNE2	2.602	<0.001	Co-occurrence
6	PLK1	CCNE2	1.846	<0.001	Co-occurrence
7	ERBB2	CDK1	1.505	0.001	Co-occurrence
8	TPX2	CCNE2	1.916	0.002	Co-occurrence
9	AURKB	CCNE2	2.222	0.003	Co-occurrence
10	TPX2	CDK1	2.595	0.003	Co-occurrence
11	ERBB2	TYMS	2.248	0.003	Co-occurrence
12	AURKB	CDK1	2.892	0.004	Co-occurrence
13	CHEK1	TPX2	>3	0.005	Co-occurrence
14	ERBB2	KIF11	2.310	0.006	Co-occurrence
15	CDK1	KIF11	>3	0.010	Co-occurrence

Curcumin disrupts cell cycle progression during mitosis, and this event is characterized by the formation of monopolar spindles and aberrant chromatin structure (Holy, 2002); all these structures possibly cause the reduction in AURKA level and mislocalization of AURKB due to the downregulation of survivin. In addition, curcumin inhibits the progression from metaphase to anaphase by binding to APC/C member CDC27, which leads to mitotic arrest during metaphase. Notably, another distinct mechanism has been demonstrated by PGV-1. The compound revealed a remarkable anticancer effect against several cancer cell lines, and its anticancer activity is partly mediated through mitotic arrest in prometaphase (Lestari *et al*., 2019).

As cancer biomarkers are often used in clinical settings (i.e., for targeted cancer therapy), we focused on the upregulated genes interacting with CCA-1.1 for further bioinformatics analysis.

Analysis of genetic alterations of the predicted target genes

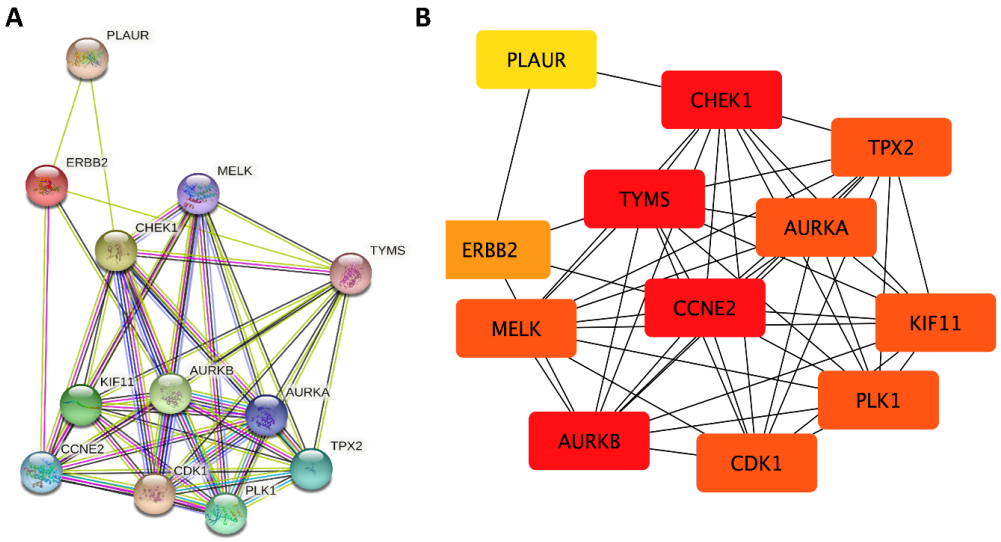
Twelve associated genes were processed via cBioPortal to analyze their genomic variations across selected cancer studies—most gene alterations were associated with amplification, deletion, and truncating mutation (Supplementary Figure 1). A study from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) was picked for analysis. The genetic alterations, such as *ERBB2* (which encodes HER2) (18%), maternal embryonic leucine zipper kinase (*MELK*) (1%), TPX2 microtubule nucleation factor (*TPX2*) (2%), cyclin E2 (*CCNE2*) (22%), and *AURKA* (6%) (Supplementary Figure 2), ranged from 0.3% to 22%. Mutual exclusivity analysis highlighted that 15 gene pairs presented significant co-occurrence (p<0.05) in the BC study project (Table I), suggesting their important role in CCA-1.1 treatment.

Analysis of PPI network of CCA-1.1 mediated hub-genes

We further evaluated the connection of CCA-1.1 target genes to explore their interactions (directly or with other interactor genes) and constructed the STRING pathway. A schematic network of 50 edges and a node degree average of 8.33 were displayed, with the enrichment of p < 1.0e−16 (Figure 2A). We subsequently ranked the nodes based on their degree and arranged them from the highest to lowest scores (Figure 2B). Thymidylate synthase (TYMS), CCNE2, serine/ threonine-protein kinase Chk1 (CHEK1), and AURKB had the highest degree score of 10 (Figure 2C).

GO and the KEGG pathway enrichment of CCA-1.1-mediated target

We investigated the functional annotations of potential genes using Webgestalt and selected the KEGG enrichment tools and GO for analysis. We categorized the GO available and displayed that CMGs are related to biological processes, metabolic processes, and cellular component organization (Figure 3A, red bar).



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Rank	Gene	Degree Score	Protein Name
1	*TYMS*	10	Thymidylate synthase
2	*CCNE2*	10	G1/S-specific cyclin-E2
3	*CHEK1*	10	Serine/threonine-protein kinase Chk1
4	*AURKB*	10	Aurora kinase B
5	*KIF11*	9	Kinesin-like protein KIF11
6	*MELK*	9	Maternal embryonic leucine zipper kinase
7	*PLK1*	9	Polo-like kinase 1
8	*AURKA*	9	Aurora kinase A
9	*TPX2*	9	Targeting protein for Xklp2
10	*CDK1*	9	Cyclin dependent kinase 1
11	*ERBB2*	4	Receptor tyrosine-protein kinase erbB-2
12	*PLAUR*	2	Urokinase plasminogen activator surface receptor

Figure 2. (A) PPI of CMGs established in STRING. (B) CMGs were ranked based on the degree scores (highest to lowest) and visualized in Cytohubba powered by Cytoscape. (C) Hub genes rank based on the degree scores from Cytohubba.

In addition, CMGs are connected with protein, nucleotide, and ion binding (Figure 3A, green bar) and located in the cytosol and nucleus (Figure 3A, blue bar). Furthermore, according to the KEGG pathway analysis, CMGs are enriched in cell cycle regulation, notably in the G2/M transition and mitotic process (Figure 3B). We then classified each CMG using cancer hallmark datasets provided by MSigDB. The majority of CMGs are responsible for cell cycle regulation: G2/M checkpoint (AURKA, AURKB, CDK1, CHEK1, KIF11, TPX2, and PLK1), cell cycle-related E2F (CDK1, AURKA, AURKB, CHEK1, PLK1, and MELK1), and mitotic spindle arrangement (AURKA, KIF11, PLK1, CDK1, and TPX2) (Figure 3C).

Mitotic cascade comprises a complicated process with many unique proteins controlling the progression during cell division. AURKA, for example, is responsible for the mitotic entry by activating CDC25B; it then phosphorylates CDK1, which forms a complex with cyclin B (named as the maturation promoting factor) to enable cells to enter mitosis (Kishimoto, 2015). In addition to AURKA, MELK interacts with CDC25B to induce progression from G2 to mitosis (Davezac *et al.*, 2002). Interestingly, MELK overexpression in BC occurs explicitly in basal-like and hormone receptor-negative BC (Wang *et al.*, 2014). AURKA reaches peak expression during mitosis because it controls numerous mitotic proteins. AURKA also interacts with TPX2 as its cofactor to protect the former from dephosphorylation starting from prometaphase. TPX2 binding regulates KIF11 to form a bipolar spindle (Waitzman and Rice, 2014).

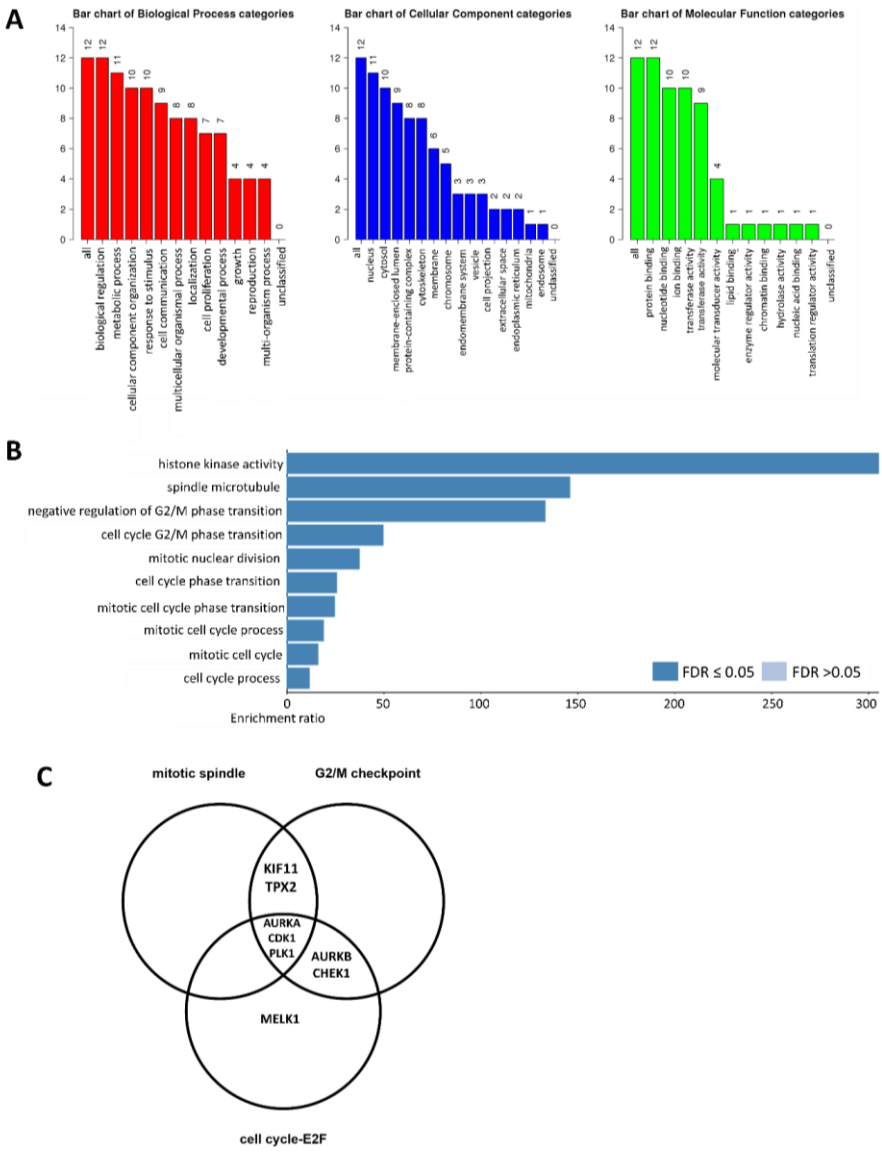

Figure 3. (A) Association of CMGs in biological process (red bar), molecular function (blue bar), and cellular component (green bar) using GO database. (B) KEGG pathway enrichment analysis from KEGG CMGs processed through Webgestalt. (C) CMGs

AURKA is directly upstream of PLK1 and phosphorylated at Thr-210 of the PLK1 loop during pre-entry mitosis; the activated PLK1 cooperates with CDK1, causing cells to enter mitosis (Lindqvist *et al*., 2009). The AURKA-PLK1 cascade, which operates in convergence with the cyclin B–CDK1 complex, represents the essential signaling pathway in mitotic control (Joukov and Nicolo, 2018). Upon mitosis, PLK1 activates pericentrin, which enables centrosome maturation and spindle assembly (Lee and Rhee, 2011).

Along with AURKA, AURKB serves as a PLK1-activating kinase and is involved in kinetochore formation (Carmena *et al*., 2012; Krenn and Musacchio, 2015). AURKB can be promoted through CHEK1 and activate the histone H3 variant CENP-A and inhibit mitotic centromere-associated kinesin, which are both responsible for correcting errors in kinetochore-microtubule attachment and spind leassembly checkpoint (Krenn and Musacchio, 2015). Moreover, the inactivation of PLK1 through CHEK1 is mediated by cyclosome (Lee *et al*., 2010).

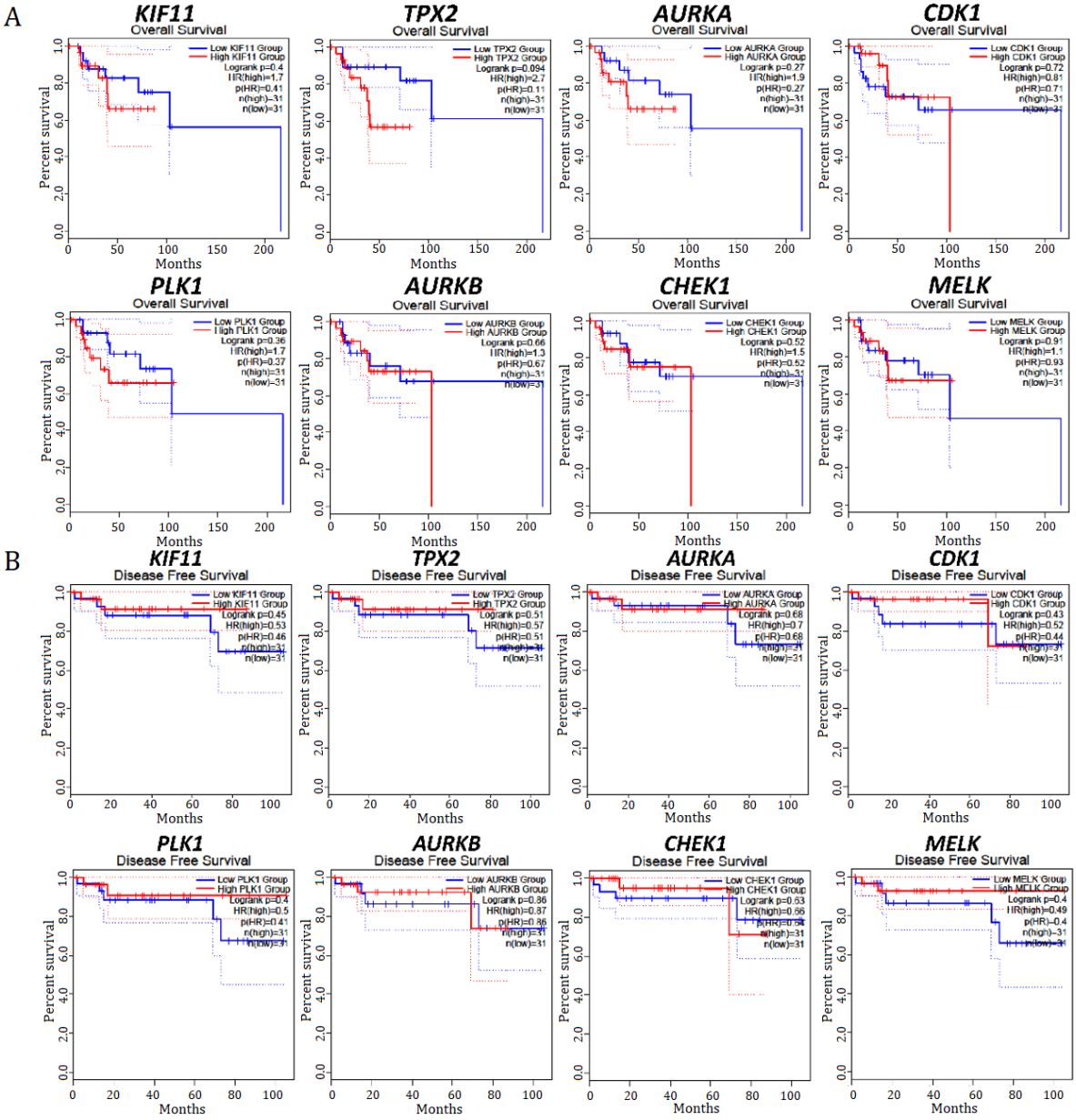

Figure 4. (A) OS analysis and (B) DFS analysis of KIF11, TPX2, AURKA, CDK1, PLK1, AURKB, CHEK1, and MELK genes in HER2-positive BC patients.

Given the results, we focused on genes involved in the cell cycle to evaluate them based on their survival analysis.

Survival analysis of associated CCA-1.1 target genes in HER2-positive BC patients

Using the dataset in GEPIA2, we used two parameters, namely, OS and DFS, for the survival analysis of HER2-positive BC patients. We mapped the results into a Kaplan–Meier plot for the CMGs involved in cell cycle regulation (*KIF11, TPX2, AURKA, CDK1, PLK1, AURKB, CHEK1,* and *MELK*). In 66 HER2-positive BC patients, the high level of these genes reduced the OS by 220 months. However, the log-rank tests did not show a significant prognostic score (Figure 4A). The patients with increased CDK1, AURKB, and CHEK1 levels showed a poorer chance of survival than those with lower expressions of genes. Concurrently, high CDK1, AURKB, and CHEK1 expressions indicated poor DFS in HER2-positive BC cases (Figure 4B).

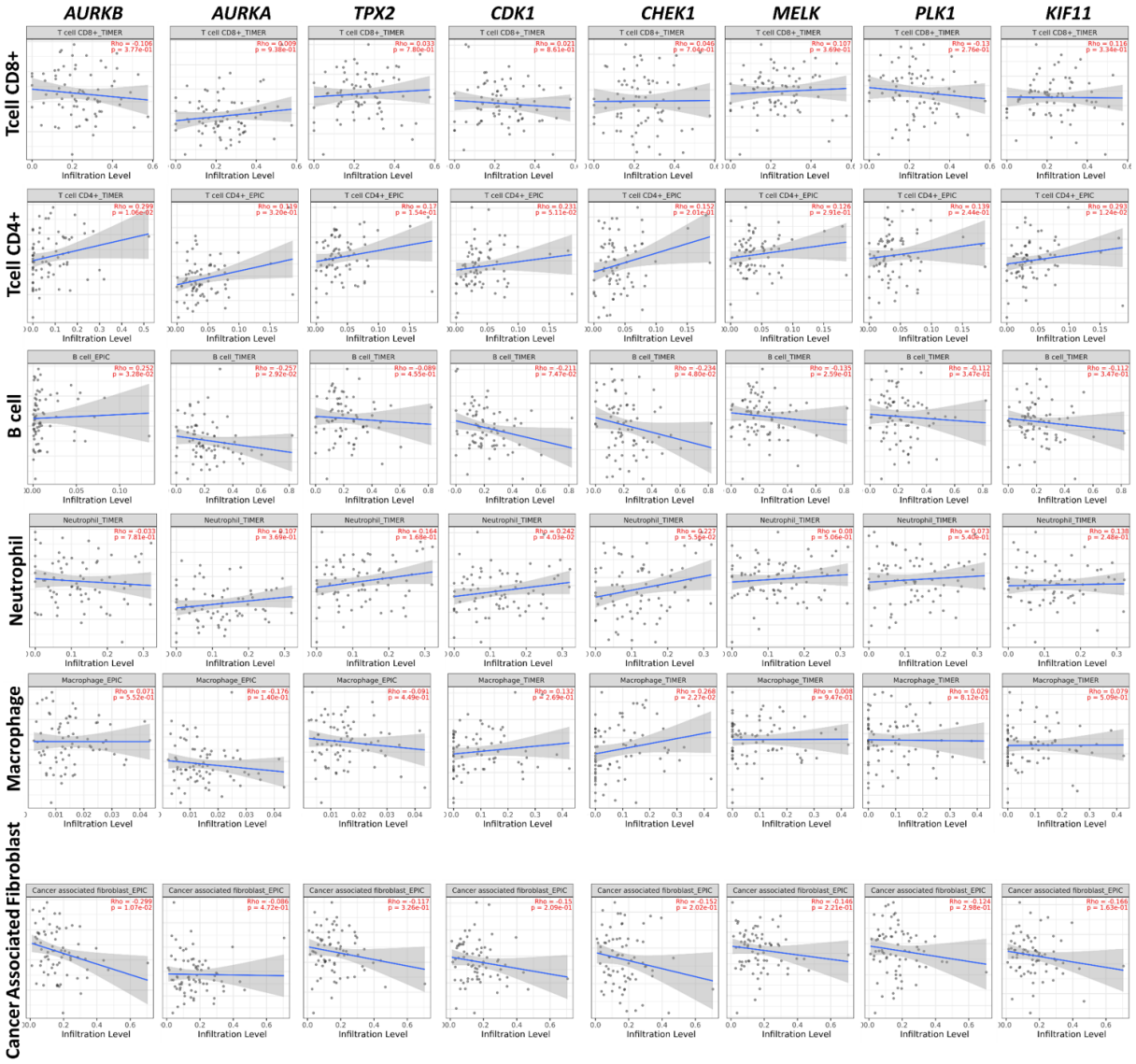

Figure 5. Associated CCA-1.1 target gene expressions correlated with immune infiltrating cells in BRCA-HER2 (n = 82). Several biomarkers used in this study included CD8+ T cells, CD4+ T cells, B cells, neutrophils, macrophages, and cancer-associated fibroblasts. The data presented were adjusted by their purity.

Correlations of the expressions of associated CCA-1.1 target genes with immune marker sets

Immune infiltrating cells in tumor tissues are a significant part of the tumor microenvironment. Therefore, the association of CCA-1.1 target genes and immune infiltrating cells was elucidated through TIMER (Figure 5). The correlation of target genes with diverse immune markers was adjusted by purity. Positive correlations were observed in CD4+ cells with AURKB (ρ = 0.299; p = 0.0106) and CDK1 (ρ = 0.231; p = 0.0511), in macrophage cells with CHEK1 (ρ = 0.268; p = 0.0227), and in neutrophil cells with CDK1 (ρ = 0.231; p = 0.0403). B-cell infiltration was negatively associated with AURKA (ρ = −0.257; p = 0.0292) and CHEK1 (ρ = −0.234; p = 0.048), whereas AURKB was revealed to have a positive correlation (ρ = 0.252; p = 0.0328). These data indicated that the potential of these target genes may be attributed to their effect, especially on the infiltration levels of CD4 and CD8 T cells, B cells, neutrophils, and macrophages in HER2-positive BC.

The most aggressive subtypes of BC (HER2-enriched type and basal-like) are correlated to plenty of tumor-infiltrating immune cells (TIICs) (Denkert *et al*., 2018). In a prior report, TIICs were associated with chemotherapy responses in HER2-positive and TNBC (Denkert *et al*., 2018). The low cytotoxic T cell expression in HER2-positive BC against TNBC possibly occurred because *ERBB2* oncogene lost its immunoproteasome subunits, resulting in the loss of significant histocompatibility class I epitopes, which supposedly can be recognized by $CD8^+$ T cells (Mimura *et al*., 2011). Among the eight genes presented in Figure 8, the experimental studies reported the downregulation of AURKA-induced immune response via the activation of $CD8^+$ T cell activity. AURKA also increased programmed death-ligand 1 expression via the MYC-dependent pathway, and this finding was attributed to immune avoidance (Sun *et al*., 2021). Consequently, BC was proven to be immunogenic, and targeting the tumor immune microenvironment will hopefully benefit cancer patients. These data indicated that the potential of these target genes may be attributed to their effects, especially on the infiltration levels of CD4 and CD8 T cells, B cells, neutrophils, and macrophages in HER2-positive BC.

This study also encountered shortcomings, such as the needed experimental studies to support all the findings in bioinformatics studies to determine the biomarkers for CCA-1.1 in BC. Moreover, the algorithm built for analysis is based on the database we selected for this study. Therefore, further bioinformatics analysis through different approaches is encouraged to gather data using more controlled parameters. Nonetheless, the data presented in this study will help narrow down the mechanism of action of CCA-1.1 in HER2-positive BC.

CONCLUSIONS

To sum up, we collectively noted potential therapeutic targets of CCA-1.1 (CMGs) that presumably contribute to mitosis: AURKA, AURKB, CDK1, CHEK1, KIF11, TPX2 PLK1, and MELK. This study also unfolded the association of CMGs in tumor immune infiltration with HER2-BC. However, further validation of the results of this study is required to refine the scientific evidence of the pharmacological effects of CCA-1.1 in BC.

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