KIFC1: A Reliable Prognostic Biomarker in Rb-positive Triple-negative Breast Cancer Patients Treated With Doxorubicin in Combination With Abemaciclib

BRETT FLEISHER[1], CAROLIN WERKMAN[1], BREHANNA JACOBS[1],
JUSTIN VARKEY[1], KAREEM TAHA[1] and SIHEM AIT-OUDHIA[2]

[1]*Center for Pharmacometrics and Systems Pharmacology,*
Department of Pharmaceutics, University of Florida, College of Pharmacy, Orlando, FL, U.S.A.;
[2]*Quantitative Pharmacology and Pharmacometrics (QP2), Merck & Co., Inc, Kenilworth, NJ, U.S.A.*

Abstract. *Background/Aim: Triple-negative breast cancer (TNBC) prevalence and risk of relapse are greatest in African American (AA) patients. Doxorubicin (DOX) and abemaciclib (ABE) synergism in Rb-positive TNBC cells (MDA-MB-231), and antagonism in Rb-negative TNBC cells (MDA-MB-468) have been previously shown. Here, we assessed Kinesin-like protein 1 (KIFC1) as an ethnic-specific prognostic biomarker of the DOX+ABE combination for the Rb-status in TNBC. Materials and Methods: Literature search for TNBC prognostic biomarkers in the AA population was conducted. MDA-MB-231 and MDA-MB-468 cells were exposed over 72 h to four treatment arms: 1) control (medium without drug), 2) DOX at 50% inhibitory concentration in MDA-MB-231 (0.565 μM) and MDA-MB-468 (0.121 μM), 3) ABE alone (2 μM), and 4) DOX+ABE combination at their corresponding concentrations in each cell-line. KIFC1 protein expression and temporal changes were quantified in MDA-MB-231 cells using western blot. Results: KIFC1, Kaiso, and Annexin A2 are literature-identified AA-specific TNBC prognostic biomarkers. KIFC1 was found to be uncorrelated to other proposed biomarkers, suggesting it may predict risk independently of other TNBC biomarkers. In both cell lines, DOX alone did not significantly change KIFC1 expression relative to control. Conversely, ABE reduced KIFC1 expression in MDA-MB-231 but not in MDA-MB-468 cells. The combination DOX+ABE resulted in a greatest reduction in KIFC1 in MDA-MB-231 cells with a more rapid time-to-full inhibition of KIFC1 compared to ABE alone. Conclusion: Change in KIFC1 expression is primarily driven by ABE in Rb-positive TNBC cells. DOX increases ABE speed to achieve a full inhibition of KIFC1 in Rb-positive, yet, without influencing its expression in Rb-negative TNBC cells.*

Correspondence to: Sihem Ait-Oudhia, MS, PharmD, Ph.D., Quantitative Pharmacology and Pharmacometrics (QP2), Merck & Co., Inc, 2000 Galloping Hill Rd, Kenilworth, NJ 07033, U.S.A. Tel: +1 7162509828, e-mail: sb.manuscript.submission@gmail.com

Triple-negative breast cancer (TNBC) is a breast cancer (BC) subtype characterized by the lack of the three hallmark receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TNBC occurs in about 10-20% of diagnosed BC patient population and is more aggressive compared to other BC subtypes (1). Diagnoses of TNBC occur at a younger age in African Americans (AA) (average age <50 years) and with more aggressive phenotypes as compared to Caucasian Americans (CA) (1, 2). The American Society of Clinical Oncology (ASCO) recommends TNBC patients be treated with a cytotoxic chemotherapeutic regimen based on anthracyclines and taxanes (3). Despite an initial response to chemotherapy, the risk of TNBC is high (4, 5), especially in AA patients (6), prompting the necessity for enhanced therapeutic alternatives in AA patients with TNBC.

We recently reported a clinical translation of *in vitro* data using mathematical modeling of the interaction of an anthracycline, doxorubicin (DOX), and the CDK4/6 inhibitor, abemaciclib (ABE) (7). The mechanism of action of DOX and ABE involves separate intracellular molecular circuitries. DOX induces cytotoxicity by forming a covalent bond between topoisomerase II and DNA in addition to arresting

TNBC cells in the G2/M phase of the cell cycle (8, 9). ABE inhibits CDK4/6, thereby, halting the G1 to S phase transition and preventing CDK4/6 signaling-dependent cell replication (10). The nature of the drug-drug interaction (synergism, additivity, antagonism) in *in vitro* combinations was dependent on the Rb status of the TNBC (7). The combination produced synergism in the MDA-MB-231 cells, an Rb-expressing and highly metastatic TNBC cell line. Conversely, the combination produced antagonism in the MDA-MB-468 cells, an Rb-negative TNBC cell line (9, 11-13).

Historically, clinical monitoring of disease-associated biomarkers improves patient survival by serving as reliable targets for efficacious treatment (predictive biomarkers) or prognostic biomarkers (14). While Rb may be a predictive biomarker supporting the mechanistic rationale of utilizing CDK4/6 inhibitors in TNBC subtypes, a prognostic biomarker can help preemptively estimate TNBC progression in AA patients following treatment with novel therapeutic options.

In the present work, we first collected from literature reports an up-to-date list of candidate prognostic TNBC biomarkers that were proven specific to AA patients. Then, we analyzed the reported correlations between the biomarker of interest and AA ethnicity as well as TNBC aggressiveness. Our results show that while each biomarker of interest from the TNBC specimens in each study correlates positively with the AA ethnicity within the TNBC samples, the biomarker expression correlates negatively with the survival in donors. Hence, of all the examined biomarkers, we report the time-course change in the negative prognostic biomarker, Kinesin-like protein 1 (KIFC1).

Materials and Methods

Literature search of African American-specific prognostic biomarkers in TNBC. PubMed Central (PMC) and Google Scholar search engines were used to conduct an intense literature search to screen studies examining the role of biomarkers specific for AA TNBC. First, studies were identified using keywords such as "triple-negative breast cancer" and "biomarker" and "African American." Second, only studies with measured biomarker expression using immunohistochemistry techniques in TNBC tissue microarrays from AA and CA donors were selected for the analysis. Lastly, a biomarker was considered as an AA-specific negative prognostic biomarker if the results in the published study (A) report a comparison of the frequency of expression of the biomarker of interest in TNBC samples collected in both AA and CA TNBC donors, (B) report that the biomarker of interest correlates positively with an AA ethnicity, and (C) report that the biomarker of interest correlates negatively with the patients' overall survival.

Cell culture and reagents. The two TNBC cells lines MDA-MB-231 and MDA-MB-468 were obtained from American Type Culture Collection (Manassas, VA, USA). Both were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% sterile filtered fetal bovine serum (Sigma-Aldrich, Saint Louis, MO, USA) and 1% penicillin/streptomycin. The cells were incubated at 37°C in an incubator supplied with 5% CO_2. DOX and ABE were purchased from Selleck Chemicals (Houston, TX, USA). The stock solutions of DOX (30 mM) and ABE (10 mM) were prepared in Millipore water, aliquoted at 20 μl and then stored at −80°C.

Sample collection. The cells were seeded in 6-well plates at density of cells 3×10^5 cells/well. After allowing for an overnight cell adherence, drugs or fresh media alone were added to the corresponding wells and left for incubation at varying time intervals spanning from 0 to 72 h. At the end of each incubation time, all cells (*i.e.*, floating in medium and adherent) were collected *via* trypsinization, pooled, centrifuged at $180 \times g$ for four min, and washed with PBS. Then, cells were re-suspended in 75 μl ice-cold Milliplex® MAP Lysis Buffer supplemented with protease/phosphatase inhibitor. After that the tube containing the cells was shaken for 10 min at 4°C, incubated on ice for 10 min, and centrifuged for 1 min at $10,000 \times g$. The supernatant was then collected, aliquoted, and stored at −80°C. The total cell protein was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Measurement of KIFC1. The western blot technique was used to quantify the change in the expression of the KIFC1 protein. In brief, rabbit primary antibodies against KIFC1 (ab72452; Abcam, Cambridge, UK) and the GAPDH housekeeping protein (2118S Cell Signaling, Danvers, MA, USA) were used. The 12% MP TGX Stain-Free Gel was loaded with 30 μg total proteins per lane, then transferred onto trans-Blot Turbo Mini PVDF (Bio-Rad, Hercules, CA, USA). A 5% non-fat dry milk in 1x phosphate-buffered saline with tween (PBST; 9809S; Cell Signaling Technologies, Danvers, MA, USA) was used for 60 min at room temperature to block (*i.e.*, saturate) the membrane. The latter was then incubated overnight with gentle agitation at 4°C with either rabbit KIFC1 at 1:2,000 dilution or GAPDH at 1:1,000 dilution. Following the incubation, the membrane was thoroughly washed with PBST, and then incubated for 1 h at room temperature with a secondary antibody. The bands were developed using electrochemiluminescence (ECL; Bio-Rad, Hercules, CA, USA). Using ImageLab, a densitometric analysis was carried out to measure the levels of total KIFC1, which were normalized to the total GAPDH.

The drug concentrations producing 50% of maximum inhibitory effects (IC_{50}) for DOX in the TNBC cells lines MDA-MB-231 and MDA-MB-468 were previously reported (7). In order to reproduce the same synergistic interaction of DOX with ABE (7), MDA-MB-231 and MDA-MB-468 cells were exposed to either the medium alone (control), the IC_{50} concentration of DOX that is specific to each cell line, which is 0.565 μM for MDA-MB-231 and 0.121 μM for MDA-MB-468, the ABE alone at 2 μM, or the combination of DOX and ABE over different exposure times spanning the interval of 0-72 h. The time-course analysis of KIFC1 normalized to GAPDH (KIFC1/GAPDH) over 72 h was conducted in MDA-MB-231 cells only. The KIFC1/GAPDH in treated cells was further normalized to KIFC1/GAPDH of control at the respective times. The GraphPad Prism Version 5 for windows (GraphPad Software Inc., San Diego, CA, USA) was used for plotting the results.

Statistical analysis. The statistical differences between the mean values of the measurements obtained in the different treatment arms *versus* controls were calculated using the GraphPad Prism Version 5 software. The one-way analysis of variance test was used, followed by the Tukey test. A *p*-value of <0.05 was used as a threshold to consider the difference between groups as statistically significant.
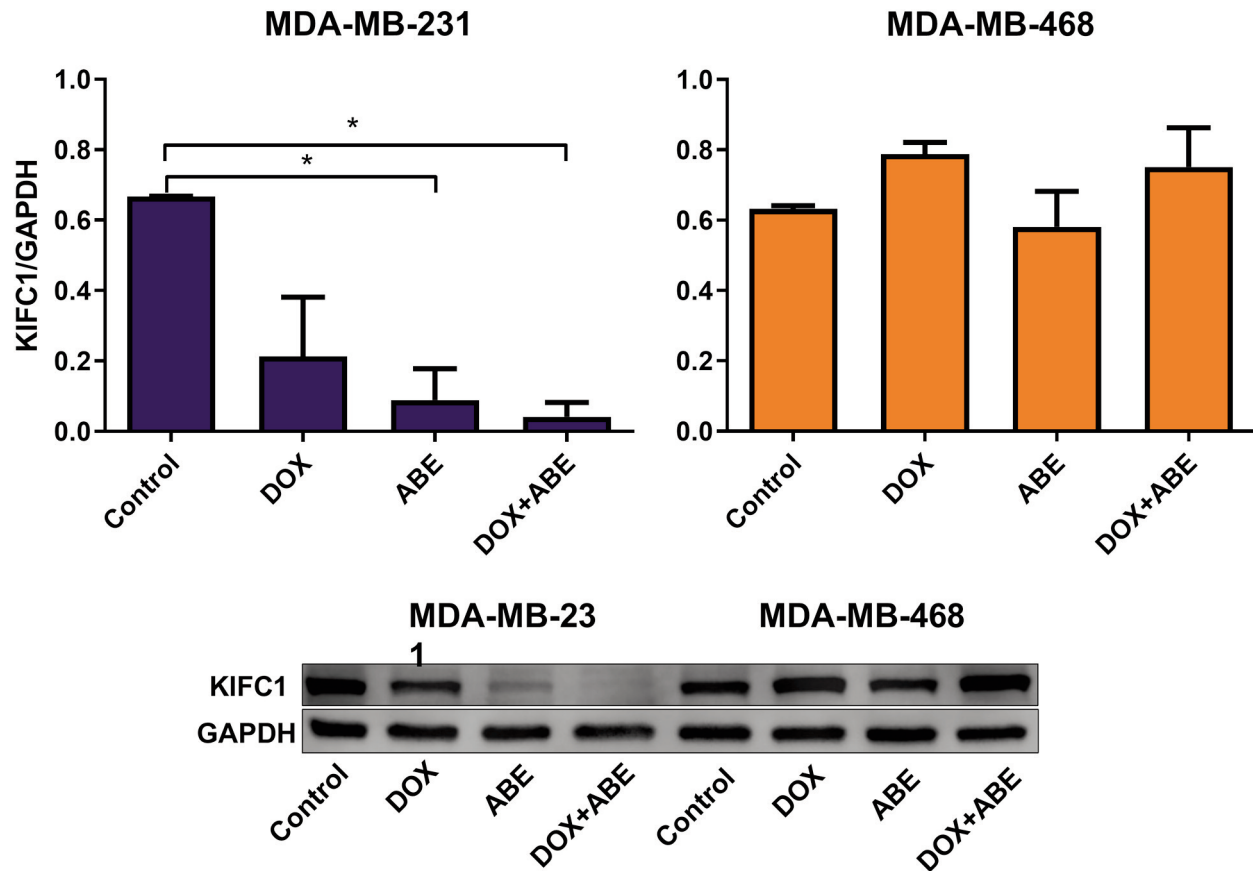
Figure 1. *KIFC1 expression normalized to GAPDH in MDA-MB-231 and MDA-MB-468 cells. MDA-MB-231 and MDA-MB-468 cells were exposed to media alone (control), cell-specific IC50 doxorubicin (DOX; 0.565 and 0.121 μM, respectively), 2 μM abemaciclib (ABE), or the combination DOX+ABE over 72 h. KIFC1 and GAPDH were measured assayed using western blot. Each bar represents a mean from duplicate data values±SEM. *p<0.05.*

Results

AA-specific biomarkers. The identified specific TNBC prognostic biomarkers specific to AA patients included KIFC1 (15) (also known as HSET), Kaiso (16), and Annexin A2 (ANXA2) (17).

ABE inhibits KIFC1 in MDA-MB-231 but not MDA-MB-468 cells. The expression of KIFC1, normalized to GAPDH, was measured in in MDA-MB-231 and MDA-MB-468 cells following treatment with single agents, DOX and ABE, as well as their combination. In the control treatment arm, the baseline level of KIFC1/GAPDH was identical in MDA-MB-231 and MDA-MB-468 (Figure 1). The levels of KIFC1 decreased by 0.6-fold relative to control in MDA-MB-231 cells treated with DOX alone, despite the non-statistical significance of this decrease. The KIFC1 decreased by 0.8-fold compared to control in MDA-MB-231 cells treated with ABE alone, and decreased by 0.9-fold compared to control

when the same cell line was treated with the combination of DOX+ABE. In both groups (ABE single agent and combination DOX+ABE), the difference from control was statistically significant. In MDA-MB-468 cells exposed to either DOX alone or the combination DOX+ABE, KIFC1 increased by 0.3-fold compared to control. However, this difference was not statistically significant. Similarly, MDA-MB-468 cells exposed to ABE alone did not impact KIFC1 expression levels.

The combination of DOX with ABE inhibits KIFC1 faster than single agent ABE. The analysis of the time trajectory of KIFC1/GAPDH fold change from baseline in MDA-MB-231 showed that ABE decreased KIFC1/GAPDH to 0.5-fold that of control at 12 h after administration and resulted in about complete inhibition of KIFC1/GAPDH at 48 h post-exposure (Figure 2). The single agent DOX induced nearly 1.5-fold increase in KIFC1/GAPDH compared to control. The combination DOX+ABE exhibited the same pattern in
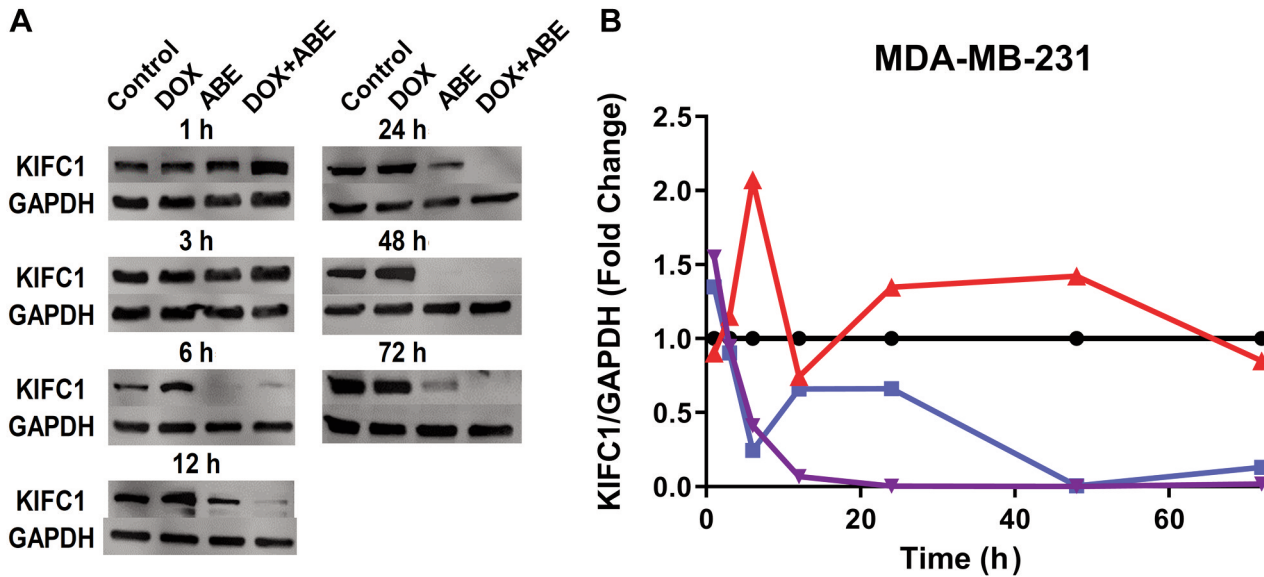
Figure 2. *Fold change from control in the expression of KIFC1 in MDA-MB-231 cells. MDA-MB-231 cells were exposed to media alone, 0.565 μM doxorubicin (DOX), 2 μM abemaciclib (ABE), or the combination DOX+ABE over 72 h. (A) KIFC1 and GAPDH were measured using western blot. (B) Time course data of KIFC1/GAPDH in MDA-MB-231 exposed to DOX (red), ABE (blue), ABE+DOX (purple), and no drug (black) were normalized to time-specific data of samples treated with media alone.*

KIFC1/GAPDH reduction as ABE single agent during the first 6 h, followed by a decay to non-quantifiable levels at 24 h post-administration.

Discussion

The aggressive progression of TNBC is reported to be more frequent in AA women than in any other ethnic subgroup, despite considerations of patients' age, stage of the disease or even the socioeconomic status of the patients (6, 18). One of the major factors involved in this inequal risk between populations is the inherent heterogeneity in tumor biology between different ethnicities, hence warranting the search for diagnostic tools that are specific to each ethnicity. Ethnic-specific prognostic biomarkers are biological entities associated with patients' outcome such as patient-free or overall survival allowing oncologists to anticipate the therapeutic risk in cancer patients (19). Following an intense literature search, three published prognostic biomarkers met our selection criteria of negative patient's clinical outcome in AA population Kaiso (16), ANXA2 (17), and KIFC1 (15).

Kaiso and ANXA2 appear to confer patient mortality *via* the Epidermal Growth Factor Receptor (EGFR) pathway. EGFR activity, a well-studied negative prognostic biomarker in TNBC patients (20-23), induces Kaiso sublocalization to the nucleus (24), where Kaiso, a bi-modal transcription factor (25), silences E-cadherin. The net result is the loss of adherent junctions between cancer cells (16, 24, 26, 27). EGFR also mediates Src-induced phosphorylation of ANXA2, a calcium-dependent phospholipid protein, which increases cell-surface localization and activity of ANXA2 (28, 29). Phosphorylated ANXA2 then mediates the plasmin-induced modification of extracellular matrix (ECM) and the release of angiogenic growth factor (30-32). All three biomarkers induce epithelial-mesenchymal transition (EMT) of the tumor, a process in which modifying cell-cell adhesion or the surrounding ECM attributes to cancer cells a highly metastatic potential (21).

Unlike the other biomarkers, KIFC1 may not be correlated to other commonly examined TNBC markers, including EGFR (14, 15). The KIFC1 protein acts by directing the spindle assembly of chromosomes with supernumerary centrosomes such as the ones present in aggressive TNBC cells (33-37). It cross-links the microtubule minus-end of spindles (33), which ultimately will converge at the acentrosomal microtubule-organizing centers (38). The latter mechanism allows cancer cells to continuously divide. Ogden *et al.* (15) demonstrated that the nuclear KIFC1 is a reliable predictive biomarker of patients' survival in AA women with TNBC. Additional studies have associated the expression of KIFC1 with the increased risk of brain metastases in other types of neoplasms (39). This finding suggests a causal link between the KIFC1 protein and the aggressive progression of cancers. Hence, altogether, these results indicate that KIFC1 protein may be a promising prognostic biomarker candidate that may be very useful in predicting the risk of TNBC aggressiveness in AA independently of EGFR.

We previously published the effects of the combination of DOX+ABE in Rb-positive TNBC cells (MDA-MB-231) as synergistic and in Rb-negative TNBC cells (MDA-MB-468) as antagonistic (7). Here, we sought to further understand whether a change in KIFC1 levels following treatment with single agents DOX or ABE, and both agents in combination DOX+ABE reflects the nature of DOX and ABE interaction (synergism or antagonism) in both cell lines. The baseline levels of KIFC1 protein expression were similar in MDA-MB-231 and MDA-MB-468 cells (Figure 1). This result is in alignment with prior observations (40). The IC_{50} of DOX after 72 h exposure was 0.565 µM in MDA-MB-231 and 0.121 µM in MDA-MB-468 cells (7). These concentrations of DOX altered the expression of KIFC1 protein in both cell lines, albeit neither change was statistically significant compared to control. Next, the KFC1 protein expression was significantly reduced under ABE exposure in MDA-MB-231 cells but remained unchanged in MDA-MB-468cells. The addition of DOX to ABE resulted in a higher decrease in KIFC1 protein expression in MDA-MB-231 cells, although the latter was not statistically significant compared to the effects of ABE as a single agent. The time course profile of the KIFC1 protein expression following treatment with DOX+ABE also revealed that the time to achieve a full inhibition of KIFC1 expression is reduced compared to single agent ABE (Figure 2). Altogether, these findings suggest that the proposed combination therapy of ABE+DOX will result in a more rapid reduction of the prognostic biomarker KIFC1 in Rb-positive TNBC, but will not have an impact on Rb-negative TNBC.

TNBC cells with supernumerary centrosomes, including MDA-MB-231 and MDA-MB-468 cells (41), divide to produce DOX-resistant progeny through centrosomal clustering. Anthracycline-induced DNA damage increases centrosome clustering *via* up-regulation of the KIFC1 pathway (42). DOX exposure increased KIFC1 in both cell lines, whereas the addition of ABE decreased KIFC1 in MDA-MB-231 cells alone (Figure 1 and Figure 2). One hypothesis for the observed reduction in the expression of KIFC1 protein in Rb-positive TNBC is that it may result from a cooperative effect of ABE and DOX on the inhibition of the cell cycle during the replication phase (9, 10). Indeed, the kinesin-14 family is primarily synthesized during the G2 phase of the cell cycle, then is rapidly degraded post-replication (43). An increase in cellular cytostasis following ABE+DOX combination treatment may inhibit the synthesis of the KIFC1 protein without affecting the basal KIFC1 degradation. This hypothesis is in support with the unanticipated increased expression of KIFC1 protein in MDA-MB-468 following exposure to ABE, since ABE exerts its activity on Rb-negative TNBC cells *via* mechanisms that are off-target rather than Rb-mediated cytostasis (44, 45).

It is important to point out that our work presents a few limitations. First, the experiments presented here were conducted using one Rb-positive TNBC cell line, which is MDA-MB-231. In order to examine the hypothesis that the reduction of KIFC1 protein expression is specific to RB-positive TNBC, we may characterize the concentration effect of ABE on KIFC1 protein expression using a second Rb-positive mesenchymal-like TNBC cell line such as HS-578T or MDA-MB-157 (46). Second, it remains unclear whether the metastatic risk attributed to the nuclear KIFC1 activity occurs only during the mitosis phase or also occurs during the interphase (47, 48). One of the suggested roles for the nuclear KIFC1 protein during interphase is keeping the integrity and organization of the Golgi apparatus (48) and aiding in vesicle transport and processing (49, 50). Future examination of the KIFC1 protein degradation rates and KIFC1 interphase activity in TNBC are needed to elucidate the role of KIFC1 as a surrogate for mitosis activity or whether the KIFC1 protein expression is directly influenced by the Rb-CDK4/6 pathway.

Conclusion

In this work, we examined published negative prognostic biomarkers specific to the AA TNBC population. Biomarkers that met our criteria included Kaiso (16), ANXA2 (17), and KIFC1 (15). The combination of ABE+DOX produced the greatest mean reduction in KIFC1. Further, this combination resulted in a more rapid decay in the expression of KIFC1 protein than the corresponding single agents in Rb-positive TNBC, without affecting KIFC1 expression in Rb-negative TNBC. In conclusion, KIFC1 appears to have potential as a robust prognostic biomarker for TNBC in AA patients considered for treatment with the combination therapy of ABE+DOX.

Conflicts of Interest

Authors' Contributions

BF designed the study, conducted the experiments, and wrote the manuscript. CW, BJ, JV, KT helped conducting the experiments, SAO designed the study, provided scientific mentoring to all co-authors, and edited the manuscript.

Acknowledgements

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